RESEARCH PAPER

Insilico, Invitro and Spectroscopic study for Assessment of Antimicrobial and Antioxidant activity of the secondary metabolites containing the leaf extract of *Colocasia esculenta L.*

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ABSTRACT
One of the main sources for developing new, efficient medications is thought to be medicinal plants. In this context, the study's objectives were to determine whether polyphenolic and flavonoid fractions were present using hyphenated techniques in methanol extracts of ***Colocasia esculenta L*** leaf and to assess the antibacterial and antioxidant properties of these fractions. Additionally, *in silico* analysis was carried out using the phenolic, terpenoids, and flavonoids—present in the plant extract as it was confirmed by LC-MS/MS results of the extract obtained during the study. The principle behind this analysis is molecular docking, which uses PyRx software to predict activity and Discovery Studio visualizer were used to validate the receptors or protein structures and for visualizations that were taken into consideration in the docking investigation accompanied with the Network Pharmacology concept for the identification of genes and pathways study. For the in vitro antibacterial investigation, the disc diffusion assay was used against two strains of bacteria that were non-pathogenic in nature. The investigation revealed somewhat strong antibacterial activity, and MIC calculations were also showed promising results. Total phenolic, flavonoid and terpenoid content were quantitatively estimated from the obtained extract. Antioxidant activity of the plant extract also had been performed by using different methods. At the end an electrochemistry method was used to find out the metal ligand complex of a pure flavonoid compounds which can be used in future in the isolated form from the next for more potent antibacterial activity.

Key Words: Electrochemistry, PyRx, Phenolic content, discovery studio visualizer, *Colocasia esculenta L*
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INTRODUCTION

Since ancient times, the tribes have included the use of herbal heritage into their general medical practices. Modern synthetic medications, which produced impressive short-term results in the therapeutic field, came with a number of long-term side effects. Conventional medicines, primarily derived from plants, have been essential to maintain a disease-free human population on Earth [1, 2]. Medicinal plants are widely used in traditional medicine to treat a wide range of diseases [3]. However, a major barrier that has impeded the development of alternative medicine use in developed nations is the lack of documentation and strict quality control measures. Records of all the research done on traditional medicines must be kept in the form of documentation. Due to this disadvantage, it is critical to ensure that the plant and plant parts intended for medicinal use are standardized. We can employ a variety of approaches and methodology, such as phytochemical and pharmacognostic studies, to phase our way towards standardization [4]. World health organization (WHO) has also created awareness towards validation of plant-based drug to maintain the quality, safety and efficacy [5].

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Colocasia esculenta known as Taro, elephant ear & keladi, is an herbaceous plant that belongs to Araceae family [6]. The plant possesses various biological activities and has been widely used as therapeutics. This plant has been cultivated widely from ancient times in the tropical and subtropical latitudinal band around the world. The herb has been known since ancient times for its curative properties and has been utilized for treatment of various ailments such as asthma, arthritis, diarrhoea, neurological disorders, and skin disorders [7]. Colocasia esculenta leaves are widely used in Asian countries, such as, India, Philippines, Sri Lanka, Indonesia and in S. Africa counties as Egypt, Nigeria, as food and feed material [8]. The leaves are often traditionally preserved to be eaten in dry season. Leaf juice is stimulant, expectorant, astringent, appetizer, and otalgia. Looking at the medicinal uses of the plant leaves [9].

Among varieties of herbal plants that grow around us, *Colocasia esculenta* is one of those plants having various ethnomedicinal and pharmacological uses and therefore its antimicrobial, anticoagulant as well as antioxidant activity are performed and explained. To accelerate drug research and to improve success rates, research costs should be decreased. Computer-aided drug design (CADD) has become an important means of designing new drugs [10].

Antimicrobial activity

The ability of a substance to stop the growth of or eradicate microorganisms like bacteria, viruses, fungi, or protozoa is called antimicrobial activity. The hunt for potent antimicrobial agents has become critical when infectious diseases represent serious risks to world health. Following the "golden era" revolution, which saw the discovery of nearly all major antibiotic groups (tetracyclines, cephalosporins, aminoglycosides, and macrolides) and the resolution of chemotherapy's primary issues in the 1960s, history is being repeated today, with these novel compounds facing the threat of losing their effectiveness due to the rise in microbial resistance [11]. At the moment, it has a significant impact on treatment failures linked to bacteria that are resistant to multiple drugs, and public health is concerned about it worldwide [12,13]. The creation of novel antibiotics is therefore a singularly significant goal. Even now, one of the main sources of novel drug molecules is still found in natural products. The majority of the antimicrobial compounds found thus far are derived from microorganisms and plants [14].

Antioxidant activity

Antioxidant activity refers to the ability of certain substances to neutralize harmful molecules called free radicals in the body. Free radicals are unstable molecules that can cause damage to cells, proteins, and DNA, leading to various health problems. Antioxidants work by donating electrons to free radicals, stabilizing them and preventing them from causing damage. They essentially act as scavengers, neutralizing free radicals before they can harm cells. Numerous types of antioxidants are found in plants' secondary metabolites [15]. Plants generate a diverse range of secondary metabolites, such as flavonoids and polyphenols, which have antioxidant qualities among other uses. Polyphenols, for instance, are known for their antioxidant activity, which helps plants combat oxidative stress caused by environmental factors such as sunlight, pollutants, or pathogens. Flavonoids, a subgroup of polyphenols, are particularly abundant in fruits, vegetables, tea, and wine. They contribute to the vibrant colours of many plants and are also associated with health benefits in humans due to their antioxidant and anti-inflammatory properties [16].

Numerous factors, such as the structure of the antioxidants, their concentration, temperature, kind of oxidation substrate, physical state of the system, and the presence of pro-oxidants and synergists, generally affect how effective they are. Over the past few decades, there has been a significant advancement in the techniques and instruments used to measure antioxidant activity [17].

In-silico study

Computational methodologies have become a crucial component of many drug discovery programmes, from hit identification to lead optimization and beyond, and approaches as ligand or structure based virtual screening techniques are widely used in many discovery efforts. One key methodology — docking of small molecules to protein binding sites — was pioneered during the early 1980s, and remains a highly active area of research.

Molecular modeling, docking, and simulation strategies relied on a rigid view of the receptor–ligand interaction mechanism with the help of computational resources. The concept of the molecular docking approach is to how two or more molecular structures (protein or enzyme, nucleic acid, and small lead/drug) fit together. Protein–ligand (small molecule), protein–nucleic acid, and protein–protein docking plays an essential role in predicting the ligand's orientation and binding affinity in the active site of the target protein. The molecular docking studies revealed the intramolecular interaction of small molecules at a binding site of target protein (receptor).

The prediction of binding pocket deduces the functionality and active site domain of the protein, which can obtain the crucial interaction information for computer-aided drug designing. The properties of binding affinity characterize the binding strength of a ligand with a target molecule. Protein–protein docking has been applied to predict the complex structure from known structures of the individual proteins. The results of molecular docking depend on binding energies, the number of hydrogen bonds, and potential hits found in the protein–ligand complex structure. However, docking is unable to capture the conformational changes and flexibility this remains a major challenge for structure-based drug designing [18].

Network pharmacology is an interdisciplinary topic that studies the interdependence of biological systems and identifies important signal nodes for designing pharmacological molecules. It combines the fundamental concepts and research methods of bioinformatics, network science, mathematics, and computer science [19, 20]. As a comprehensive and

interdisciplinary field, it examines the interactions between drugs and their targets using systems-based approaches. This offers a new way of thinking about drug development that may lead to significant advancements [21.22].

The experimental work in this study has been concentrated on the antioxidation, antibacterial, and *in-silico* antibacterial examination of the methanol extract of ***Colocasia esculenta L***. plant constituents due to the dearth of scientific evidence. To complement the in vitro results, a network pharmacological analysis was carried out using bioactive compounds identified through LC–MS analysis, which provides a more comprehensive metabolite profile.

MATERIALS AND METHODS

Materials

The extraction procedure made use of methanol (Merck, India), ethanol (Lobachem, India), and chloroform (Lobachem, India). The phenolic content of the extract was estimated using gallic acid (Lobachem, India), the Folin-Ciocalteu Reagent (Lobachem, India). Quercetin (Merck, India) was used to determine flavonoid content as Ursolic acid (sigmaaldrich, India) was used to determine total terpenoid content. Hydrogen peroxide (Merck, India), was employed for the peroxide method of antioxidant research. Resulting methanolic extract were tested against a panel of two non-pathogenic bacterial strains including *Staphylococcus Aureus* MTCC NO. 87 and *Pseudomonas aerugenosa* MTCC NO. 424 that were purchased from Institute of Microbial Technology, Sector 39, Chandigarh, India.

Preparation of extract

The plant sample, which consisted of leaves, was gathered and it was then air dried in the shade at room temperature, grounded into a fine powder with an electric grinder, and then stored in an airtight container for future use. For extraction, the powdered sample was combined with solvents in a 4:1 methanol to water ratio. The substance was then filtered using Whatman No. 1 filter paper, and the filtrate was combined with (2–3) drops of 2M HCl before being combined with an equivalent volume of chloroform. The dried residue was obtained by taking the lower organic layer after it had formed, separated, and then the solvent was evaporated. The organic layer by mechanism is supposed to have flavonoids/phenolic/terpenoids fraction of the extract which then was mixed with distilled water and 1% w/v of Tween 80 solution for the further antibacterial and antioxidant activity [23].

Preliminary phytochemical screening

A qualitative phytochemical screening was performed on Colocasia esculenta L to examine the presence of various secondary metabolites, including flavonoids, tannins, glycosides, alkaloids, saponins, terpenoids, steroids, and Polyphenols [24].

Estimation of the Total Phenolic Content

A series of gallic acid standard solutions of 10, 20, 30, 40, 50 µg/ml were prepared in test tubes and 1 mL of Folin-Ciocalteu reagent was added to each tube and mixed well. After exactly 5 minutes, 1 mL of 7% sodium carbonate solution was added to each tube or and mixed thoroughly. The solutions were allowed to stand for 2 hours at room temperature, protecting them from light. The absorbance of each solution was measured at 765 nm using UV spectroscopy. A standard curve was prepared by plotting the concentration of gallic acid (µg/mL) on the x-axis and the corresponding absorbance values on the y-axis. 100 mg sample was weighed and transferred into a test tube. A suitable solvent ethanol or water was added to the test tube to extract the phenolic compounds. The contents were thoroughly mixed and allowed the extraction to proceed for 30 minutes with occasional shaking. The sample extract was centrifuged at a suitable speed and duration to remove any insoluble particles. The supernatant extract was transferred to a new test tube, which was used for analysis. The absorbance of this solution was measured by UV spectroscopy. Calculate the total percentage phenolic content of the sample using the equation obtained from the standard curve. The value obtained of the total flavonoid content were expressed as milligrams of gallic acid equivalent per 100 mg of dry mass [25].

Estimation of the Total Flavonoid Content

Aluminium chloride colorimetric assay was used to measure the total flavonoid content of the extract. A 10 ml volumetric flask keeping 4 ml of distilled deionized water was filled with a portion (1 ml) of extracts or a standard solution of (+)-quercetin (20, 40, 60, 80, 100 µg/ml). 0.3 ml of 5% $NaNO_2$ was put in to the flask. 0.3 millilitre of 10% $AlCl_3$ was added after 5 minutes. Following addition of 2 millilitres of 1 M NaOH at the sixth minute, the volume was raised to 10 millilitres using deionized water. The solution was thoroughly mixed, and a Shimadzu UV-Visible spectrophotometer was used to measure the absorbance against a prepared reagent blank at 510 nm with a UV-Visible spectrophotometer. The value obtained of the total flavonoid content were expressed as milligrams of (±) quercetin equivalent (QE) per 100 mg of dry mass [26].

Determination of total terpenoids content (TTC)

200 µl of extract solutions in methanol (0.1 mg/ml) was first mixed with 1 ml of perchloric acid and 300 µl vanillin/glacial acetic acid (5% w/v) solution. 5 ml of glacial acetic acid was then added to it and the absorbance was measured at 548 nm with a Shimadzu UV-Visible spectrophotometer. Ursolic acid at concentrations (20, 40, 60, 80, 100 µg/ml) were used to generate the standard calibration curve [27].

LC–MS analysis of Extract

The phytochemicals of Colocasia esculenta L were investigated by LC– MS (Shimadzu Ultra-Fast LC–MS/MS-8045MS). One gram of the Colocasia esculenta L was completely dissolved in ten mL of methanol, and the resulting solution was then subjected to centrifugation at 10,000 revolutions per minute (rpm) for 15 min to facilitate the effective separation of any suspended particles. Following centrifugation, the

supernatant was carefully filtered through a 0.22 µm membrane filter to remove any remaining impurities before proceeding with the analytical evaluation. Mobile phase A consisted of a formic acid (0.3%) aqueous solution, whereas mobile phase B contained acetonitrile and formic acid in a 99.97:0.3 ratio and detection was performed at a wavelength of 270 nm. [28, 29]

Evaluation of antibacterial activity

Antibacterial assessment Broth dilution method

The minimum inhibitory concentration (MIC) was determined using the broth dilution method as described by a specific method [30]. Specific concentration of extract dilutions were prepared using tubes containing 4 ml of double strength broth and with 0.5ml of inoculums. In all test tubes, test antimicrobial compound is added in the amount of 0.5ml except uninoculated (negative control) and control (positive) tube. The positive control tube is to check for the suitability of the test microorganism and the viability of the inoculums. The final volume was adjusted in all tubes by using sterile water. The tubes were inoculated with the suspension of standardized inocula (0.5 McFarland standard) and incubated at 37°C for 24 h. MIC was recorded as the lowest concentration of extract showing no visible bacterial growth.

Disc diffusion method

The disc diffusion assay will be used to screen for antibacterial activity as described by scientists [31]. The standard inoculum will be introduced onto the surface of the sterile agar plates and a sterile glass spreader was used for even distribution over the media. Blank sterile paper discs (6 mm) will be placed on the inoculated Mueller-Hinton agar surface and impregnated with 50 µl of the different extracts. A concentration of 10 µg/disc of Streptomycin (Sigma Aldrich, India), will be used as a standard. The procedure shall be repeated for all the selected bacterial species used. The plates shall be incubated at 37°C for 24 h. All tests will be performed in triplicate and the antibacterial activity will be expressed as the mean diameter of inhibition zones (mm) produced by the extracts.

Evaluation of Anti-oxidant activity

Peroxide method

The antioxidant activities of the extract was determined at concentrations ranging from 25 to 400 µg/mL using four different *in vitro* assay methods. A fixed volume of 2.5 ml of 1 mM hydrogen peroxide was added and to another test tube a fixed volume of sample dilution 0.5 ml containing the hydrogen peroxide solution. A control tube was included with only the hydrogen peroxide solution without the sample to measure the background reaction. All the reaction mixtures were incubated at a specific temperature for 30 minutes to allow the reaction to proceed. After the incubation period, a suitable stop solution was added to terminate the reaction. Commonly used stop solutions include sulphuric acid, sodium hydroxide, or catalase enzyme. The absorbance of each reaction mixture was measured using a spectrophotometer at 230 nm that corresponds to the absorption peak of hydrogen peroxide. Subtracted the absorbance of the control from the absorbance of each sample to obtain the net absorbance. Ascorbic acid was used as standard compound [32].

Ascorbic acid also was employed as a positive standard for the DPPH radical scavenging activity of the extract was assessed using DPPH as the substrate. The optical density was recorded at 517 nm, and the scavenging capability was expressed as the percentage of inhibition. For the ABTS assay, a solution of 7 mM ABTS was reacted with 2.45 mM potassium persulfate and incubated in the dark for 12 hours to generate the $ABTS^{+}$ radical cation. The resulting solution was then treated with different concentrations of MEAM, and the optical density was recorded at 734 nm. Lipid peroxidation inhibitory activity was established through a modified thiobarbituric acid reactive substances (TBARS) method. Goat liver homogenate was used as a lipid-rich medium, and lipid peroxidation was induced with ferric chloride. Colocasia esculenta L at concentrations ranging from 25 to 400 µg/mL was mixed and incubated for 15 minutes, after which the reaction was terminated using a TCA-TBA-HCl reagent. The mixture was then heated, cooled, centrifuged, and the absorbance of the supernatant was measured at 532 nm. The antioxidant activity was quantified as the percentage inhibition of DPPH, ABTS, and lipid peroxidation radicals. The IC_{50} values were estimated to represent the concentration required to neutralise 50% of the free radicals [33-35].

Ferricyanide ion reduction assay

The reducing power of the plant extract (25 to 400 µg/mL) was determined by mixing it with phosphate buffer and potassium ferricyanide, then incubating at 60 °C for 25 min. Further, TCA (5%) and ferric chloride (0.5%) were introduced into the mixture. The absorbance was recorded at 700 nm, where elevated readings corresponded to enhanced reducing activity [36].

In Silico study for assessment of antibacterial activity [37, 38].

***In-silico* study**

Molecular docking method has been employed here to find out the binding affinity (docking score) between the reported active phytoconstituents (Flavonoids/Phenolic/terpenoids) of the plant extract that served the purpose as a ligand and the microbial protein that was designated as the receptor site or active binding site to have the successfully inhibition of the microbes for the purpose of possible antimicrobial activity.

Protein preparation

In the present study, as per the reported mechanism of action of the selected phytoconstituents (ligand), Receptors were selected as bacterial ***(S. aureus, Pseudomonas aeruginosa)*** target protein as it plays important role in the life cycle of bacteria and it was obtained from the RCSB PDB database for the docking study. The protein was then processed by eliminating water molecules, internal ligands, removing superfluous

chains or heteroatoms, introducing polar hydrogen charges in Discovery Studio Visualizer. After that it was open in PyRx (Algorithm is same as Auto Dock Vina) and was converted to PDBqt format. Ultimately, the ligand was then placed in the centre of the grid box and the docking process was performed in the presence of previously prepared ligand molecule.

Ligand preparation

The bioactive ligand molecules were selected on the basis of it's docking score with the specific receptors and downloaded from the PubChem directory as 3D Standard Data Format (3D SDF) format. OPEN BABEL interface was used to translate the ligand from 3D SDF files to Protein Data Bank (PDB) format. These ligand molecules were independently uploaded into the AutoDock Tools (PyRx) during ligand preparation. It was prepared by minimizing the energy of the structure and also by converting it to PDBqt format for the evaluation of the binding affinity (docking score) of the receptor-ligand complex.

Visualization of the Structure

After the completion of the docking process in the PyRx software best fit of the ligand structure to the receptor surface was identified having the docking score of less than -7 and RMSD value of 0. It was then placed in the prepared protein structure placed on Discovery Studio Visualizer and interaction parameters (Hydrogen Bond formation with residues, distance, Donor-acceptor properties etc.) were examined and all the non-bond parameters were recorded. The 2-dimensional structures of the complex was noted.

In-silico study will be carried out with the reported flavonoid/polyphenolic part (ligand) of the plant extracts against the microorganism (Protein/receptor or specific enzyme) used in the in-vitro study with the help of BIOVIA DISCOVERY STUDIO and AUTODOCK VINA (PyRx) software for the evaluation of specific parameters which will be needed for the completion of the study.

Table 1: Details of *in-silico* study

Plant name	Reported isolated compound (Ligand)	Activity	Microorganism	Protein/Receptor name	Protein/Receptor specification
Colocasia esculenta L	Gallic acid	Antibacterial	*S. aureus*	Integrase	3NKH
	Catechin	Antibacterial	*S. aureus*	DNA gyrase	5CDN
			Pseudomonas aeruginosa	DNA gyrase	7PTG
	Quercetin	Antibacterial	*S. aureus*	ILE TRNA synthetase.	1FFY
				Glycosyltransferase	3VMR
				DNA gyrase	5CDN
			Pseudomonas aeruginosa	3-oxyacyl-[acyl carrier protein] reductase	4BO3
				Enoyl-acyl carrier protein reductase.	4NR0
	β-sitosterol	Antibacterial	*S. aureus*	Sortase B	1QWZ
			Pseudomonas aeruginosa	Virulence factor regulator.	2OZ6
	Kaempferol	Antibacterial	*S. aureus*	Active helicase	5DGK
				DNA helicase sapria.	5XGT
		Antibacterial	*Pseudomonas aeruginosa*	3-oxyacyl-[acyl carrier protein] reductase.	4BO3
				Enoyl-acyl carrier protein reductase.	4NRO

D.S- Docking Score (PyRx), RMSD- Interaction Energy (PyRx), DIAGRAM (DISCOVERY STUDIO)

Network pharmacology analysis

Network pharmacology analysis was performed as described previously [39-41]. The phytoconstituents were identified through LC–MS analysis. Their SMILES were obtained from PubChem database [https://pubchem.ncbi.nlm.nih.gov/].

ADME (absorption, distribution, metabolism and, excretion) analysis

After that, the SMILES of twelve compounds were uploaded to the SwissADME web tool [http://www.swissadme.ch/]. This tool evaluated their pharmacokinetics properties.

Finding potential targets of selected compounds and Screening for target genes related to hepatoprotection

Potential targets of active compounds were retrieved by using online bioinformatics database, Swiss Target Predication [http://www.swisstargetprediction.ch/] next identification of potential target genes related to hepatoprotection by searching the Gene Cards [https://www.genecards.org/] and OMIM [https://www.omim.org/] databases with the keyword

"liver toxicity." We considered targets with scores above the median value to be significant.

Target identification and Venny diagram analysis

However, a Venny diagram [https://bioinformatics.psb.ugent.be/webtools/Venn/] was used to find the overlapping targets between the active compounds in *Argemone mexicana* L. extract and the genes related to liver toxicity. These overlapping targets were seen as potential therapeutic targets of *Argemone mexicana* L. extract for addressing liver toxicity.

Active ingredient–target network construction and enrichment analysis

After that the identified target proteins were analysed using the STRING database [https://string-db.org/] to explore protein–protein interaction (PPI) networks. The enriched pathways were found using the KEGG database as well as, Gene Ontology (GO) enrichment analysis was done to predict the associated cellular components, molecular functions, and biological processes using the software ShinyGO 0.85.1 [https://bioinformatics.sdstate.edu/go/]. Lastly, the visualization of network was done that integrated phytoconstituents, target proteins, and signalling pathways using Cytoscape (version 3.10. 3) [https://cytoscape.org/] and interpreted the connections based on edge count scores.

Statistical analysis

Statistical evaluation was performed utilising SPSS Statistics software, version 23.0 (SPSS Inc., Chicago, IL, USA). Each experimental procedure was independently repeated three times. The obtained data are shown as mean values accompanied by standard deviation (mean±SD). For comparison among multiple groups, one-way analysis of variance (ANOVA) was carried out, and then Duncan's multiple range tests were used to identify statistically significant differences. Statistical significance was defined as a p-value less than 0.05.

Evaluation of ligand-metal complex

Monovariation method (Mole ratio method/Yoe Jones method) (Theoretical validation/ Standardization)

This method was introduced by Yoe and Jones [42]. A series of solutions are prepared in which the total concentration of the metal is kept constant and the concentration of the ligand is varied under similar conditions. A plot was prepared of absorbance values as a function of the ratio of moles of ligand to moles of the metal. The corresponding point where the end point was visualized as the occurrence of sudden decrease or increase (lowest or highest) in absorbance value gave the idea about molar metal: ligand complexation process.

Modified Job's method (Method of Continuous Variations) [43]

The modification of Job's [44] continuous variation method performed by Vesburgh and Cooper [45] was applied to find the stoichiometric and formation constant of the complex. The case of co-ordination may be described by:

mM + nL = M mLn

Equimolar (0.1M) solutions of ligand and metal solutions were prepared in which the sum of total concentration of M and L is kept constant but their proportions are continuously varied. The conductance values of the series are plotted against the mole fraction of the ligand. The ratio of the stoichiometric coefficients is determined from the mole fraction at the point of decrease or increase (lowest or highest) in absorbance value. That value corresponding to the mole metal: ligand ratio will be the endpoint of the titration [46]. Graphs were plotted between absorbance values and mole metal-ligand ratio. The composition and stability constant can be determined from the equivalence point in the graph [47, 48].

RESULT & DISCUSSION

Phytochemical Screening

Experimental Plant- *Colocasia esculenta L.*

Table 2: Phytochemical screening of *Colocasia esculenta L* extract

Terpenoids	***Alkaloids Test***		***Glycoside***	***Flavonoids***		***Steroid***	***Phenol***	***Tannin***
Salkowski test	Dragendorff	Wagner	Fehling test	Shinoda test	Alkaline reagent	Salkowski test	10% Ferric Chloride test	5% Ferric Chloride test
-	+	+	+	+	+	-	+	-

Positive Control- Tulsi (*Ocimum sanctum)*

Table 3: Phytochemical screening of *Ocimum sanctum* extract

Terpenoids	***Alkaloids Test***		***Glycoside***	***Flavonoids***		***Steroid***	***Phenol***	***Tannin***
Salkowski test	Dragendorff	Wagner	Fehling test	Shinoda test	Alkaline reagent	Salkowski test	10% Ferric Chloride test	5% Ferric Chloride test
+	+	+	+	+	+	+	+	+

Ocimum sanctum was chosen as the positive control plant for the validation of the chemicals was used in phytochemical screening. Table 2 and Table 3 results indicates that the final extract residue contains primarily the flavonoids/phenolic group, which was one of the experimental objectives which was screened through LC-MS/MS study of the plant extract.

LC-MS analysis of MEAM

Table 4: Compounds identified in Methanol extract of plant entity by positive mode of analysis

Name	Retention time (min.)	Molecular formula	Molecular mass (g/mol)	Classification	Area%
Quercetin	**18.828**	**$C_{27}H_{30}O_{15}$**	**594.15**	**Flavonoid**	**9.067**
Catechin	14.911	$C_{15}H_{10}O_6$	286.24	Flavonoid	**4.033**
Benzenepropanoic acid	10.389	$C_{10}H_{13}N_5O_4$	267.09	Phenolic	1.119
Flavone	18.918	$C_{15}H_{10}O_2$	222.29	Phenolic	0.255
Trimethoxy flavone	8.689	$C_{18}H_{16}O_5$	312.31	Flavonoid aglycone	3.139
Phytol	11.380	$C_{20}H_{40}O$	296.53	Terpenoids	1.967
Ferulic acid	11.875	$C_{10}H_{10}O_4$	194.18	A phenolic antioxidant	1.422
Quercetin trimethyl ether	10.534	$C_{18}H_{16}O_7$	344.32	flavonoid glycosides	2.060
Kaempferol	**23.685**	**$C_{15}H_{10}O_6$**	**286.23**	**Flavonoid compound or Methoxylated flavone**	**4.895**
Gallic acid	23.471	$C_6H_2(OH)_3CO_2H$	170.12	Phenolic acid	2.232
Chlorogenic acid	28.292	$C_{16}H_8O_9$	354.311	Caffeic acid group	4.321
Luteolin 7-rutinoside	26.321	$C_{27}H_{30}O_{15}$	594.5	Flavonoid	2.212
Rutin	29.209	$C_{27}H_{30}O_{16}$	610.5	flavonol glycoside	1.658
Vitexin	30.230	$C_{21}H_{20}O_{10}$	432.4	flavone	1.212
β-Sitosterol	28.123	$C_{29}H_{50}O$	414.7	Terpenoids	3.654

According to Table 4, quercetin and kaempferol are the most abundant compound in the Colocasia esculenta L extract as identified by the LC–MS technique.

Network pharmacology

Identification and screening of bio actives

Molecular weight and SMILES of the compounds based on % area observed in the LC-MS study were retrieved from PubChem database (Table 5) and drug likeliness, bioavailability score, GI absorption and blood brain barrier permeability data was retrieved from Swiss ADME, where every compound possess high GI absorption (Table 5). Ligands were analysed based on drug likeliness based on Lipinski, Ghose, Veber, Egan, and Muegge rules, as well as their gastrointestinal absorption characteristics to determine their potential as drug candidates.

Table 5: Identification and Screening of Phytoconstituents

Sl.no.	Phytochemical name	SMILES	DRUG LIKELINESS	BIOAVAILABILITY SCORE	MOLECULAR WEIGHT	GI absorption	BBB permeation
1	Kaempferol	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O	0	0.55	286.23	High	Yes
2	Quercetin	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O	0	0.55	302.236	High	Yes

3	Catechin	C1[C@@H]([C@H](OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)O	0	0.55	290.27	High	Yes

Screening of potential targets

Using 3 candidate compounds, a total of 219 potential targets were predicted using the Swiss Target Prediction database. Selected bacterial genes were screened from Gene Cards and OMIM databases, after duplicate removal, 2708 non-redundant targets were found. Furthermore, the intersection analysis was carried out on the Venny 2.1.0. software, that demonstrated 10 targets (EGFR, ADORA1, SRC, MMP13, ABCC1, MMP9, MMP2, CA4, ABCB1 and BACE1) that overlaps representing potential targets for antibacterial activity (Figure 1).

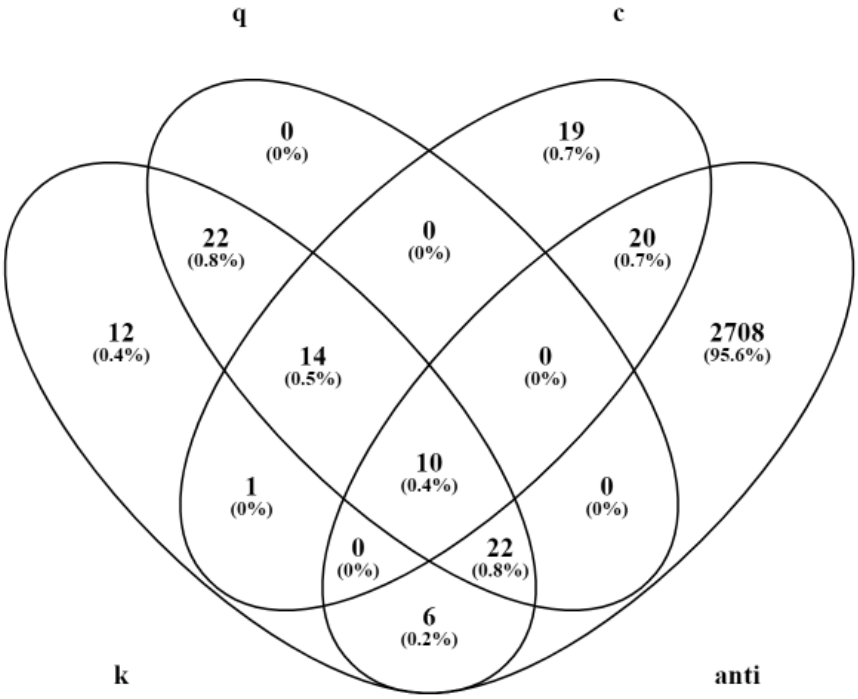


Figure 1: Venny diagram of the intersection points between the phytoconstituents of *Colocasia esculenta L* and Bacterial infection.

Active Ingredient–Target Network Construction:

The potential 10 target interventions were analysed using STRING database to construct a protein- protein interaction network. Parameters were submitted as follows species limitation to Homo sapiens, network type selection as "full STRING network", edge definition restricted to experimentally validated interactions, activation of all available interaction sources. The resulting PPI network contains 10 nodes connected by 17 edges of interaction where nodes denote proteins and edges represent interaction strength (Figure 2).

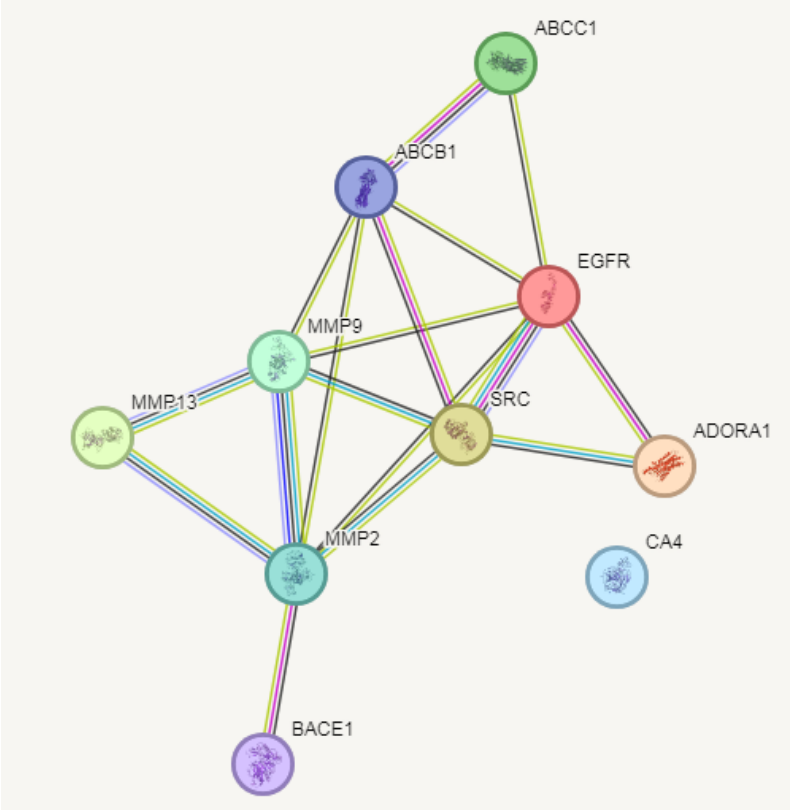


Figure 2: Protein-protein interaction image

Further, subsequent topological optimization was carried out using Cytoscape software and their key node characteristics were visually highlighted, with node colour intensity, where darker shades represented higher network centrality. After progress filtering, 9 hub targets have been identified such as EGFR, ADORA1, SRC, MMP13, ABCC1, MMP9, MMP2, ABCB1 and BACE1 (Figure 3).

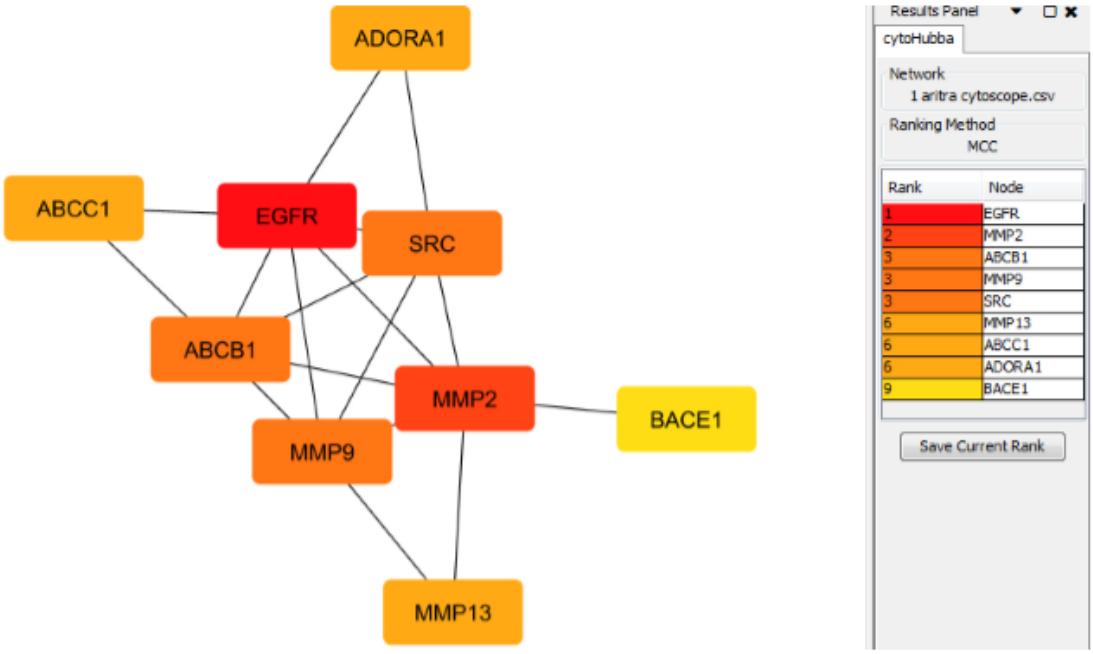
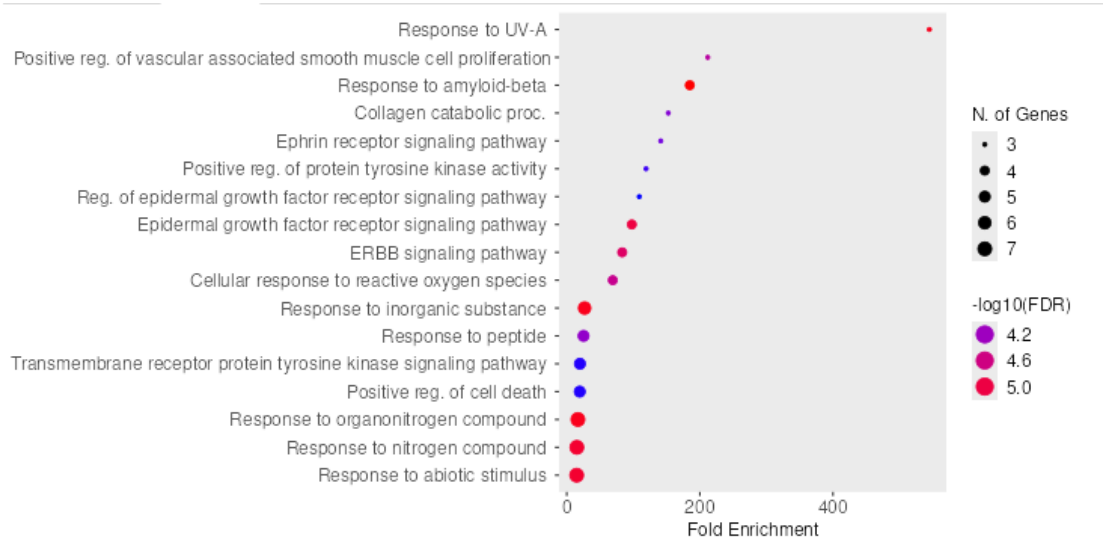


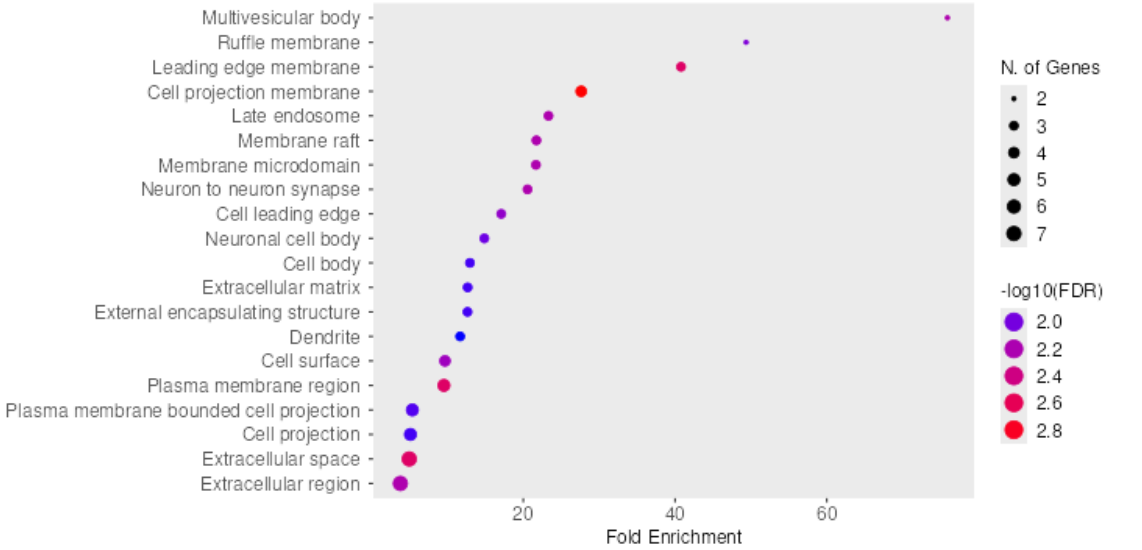
Figure 3: Hub targets after screening

Enrichment Analysis:

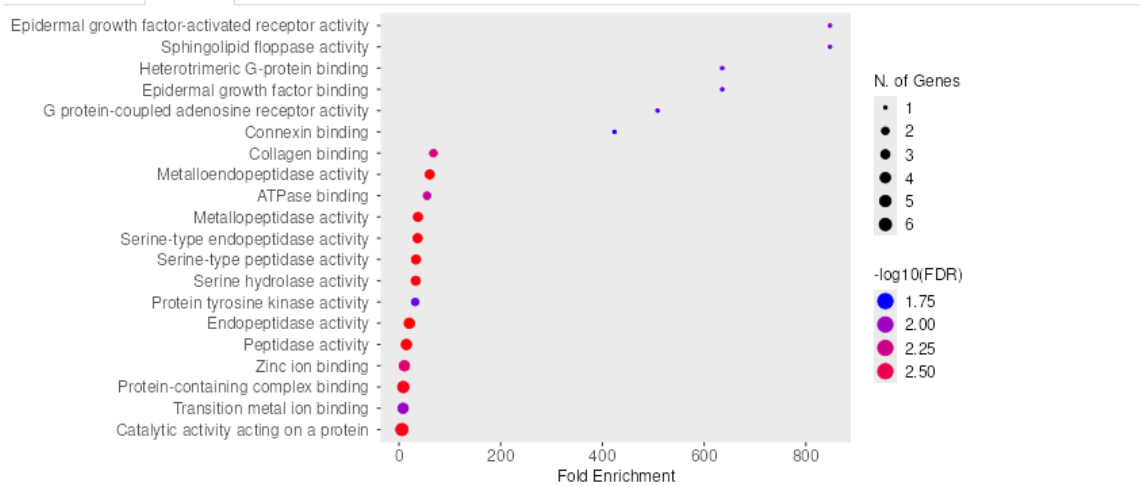
Pathway enrichment and Gene Ontology (GO) analyses of the overlapping proteins were performed using ShinyGO platform to determine their biological characteristics. Figure 4. Illustrates the top 20 enriched KEGG pathways along with the 20 GO terms categorized under biological processes (BP), cellular component (CC) and molecular function (MF). The gene set shows significant enrichment in cellular response to oxidative species, particularly involving protein tyrosine kinase activity can have a effect on antioxidant and antibacterial activity. However, KEGG enrichment analysis indicates that the gene set is highly linked to GnRH signalling pathway for antioxidant and antibacterial activity. Receptor or protein set that was selected for docking study essentially based on the above interpretation mentioned in figure 4.



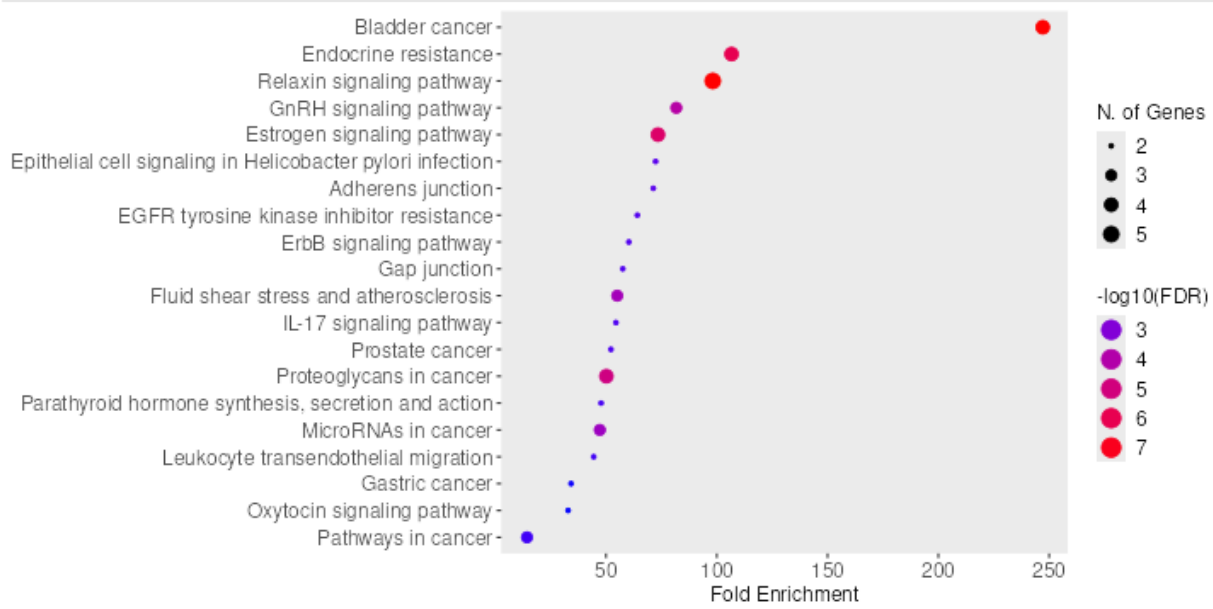
BIOLOGICAL PROCESS



CELLULAR COMPONENT



MOLECULAR FUNCTION



KEGG PATHWAY

Figure 4: GO enrichment analysis (A) Biological process, (B) Cellular components, (C) Molecular function and (D) KEGG pathway Analysis

Assessment of TPC, TFC and TTC

Table 6 expressed the TPC, TFC and TTC of the extract were determined to be 32.5 ± 2.15 mg GAE/g and 25.23 ± 2.03 mg QE/g and 26.23±2.10 Ursolic acid/g respectively. The phenolic-to-flavonoid ratio suggests that flavonoids may play a key role in the biological activities of the plant extract. The findings of this study indicate a significant presence of both total phenolic and flavonoid content. Phenolic compounds are recognised for their antioxidant, hepatoprotective, and Reactive Oxygen Species (ROS) scavenging properties.

Evaluation of Antioxidant activity

Table 6: *In Vitro* Assessment of Antioxidant Properties of extract

Sample	**IC_{50} values (µg/mL)**			
	DPPH	**ABTs**	**Lipid peroxidation**	**Peroxide method**
Methanolic Plant extract	62.5 ± 2.3	55.7 ± 3.8	38.2 ± 2.2	42.2± 2.3
Ascorbic acid	12 ± 1.3	24 ± 2.3	28 ± 0.4	31±2.5

Results are expressed as the mean ± standard deviation (SD) (n=3).

Antibacterial Assessment

Table 7: Assessment of Antibacterial activity of the plant extract

Microorganisms	Diameter of inhibition zone (mm)		MIC (mg/ml)
	Methanol extract	Streptomycin	Methanol extract
S. aureus	**8.1 ± 0.31**	**6.8 ± 0.12**	**6**
P. aerugenosa	7.1 ± 0.35	6.5 ± 0.25	5

Note: The control disc used for solvent had no zone of inhibition, so there data was omitted from the above data. Inhibition zones including the diameter of the paper disc (6 mm). Results are expressed as the mean ± SEM of triplicate measurements.

According to Table 7, MIC of methanol extract of ***Colocasia esculenta*** were 6 mg/ml and 5 mg/ml against *S. aureus* and *P. aerogenosa*. The MIC determination was performed in triplicate for each organism.

The methanolic extract exhibited (Table 8) potent anti-bacterial activity against *S. aureus* (8.1 ± 0.31 mm) and *P. aerugenosa* (7.1 ± 0.12 mm). The same for the standard drug was found to be (6.8 ± 0.12 mm) and (6.5 ± 0.25 mm) against *S. aureus* and *P. aerugenosa* respectively.

***IN-SILICO* STUDY**

5CDN

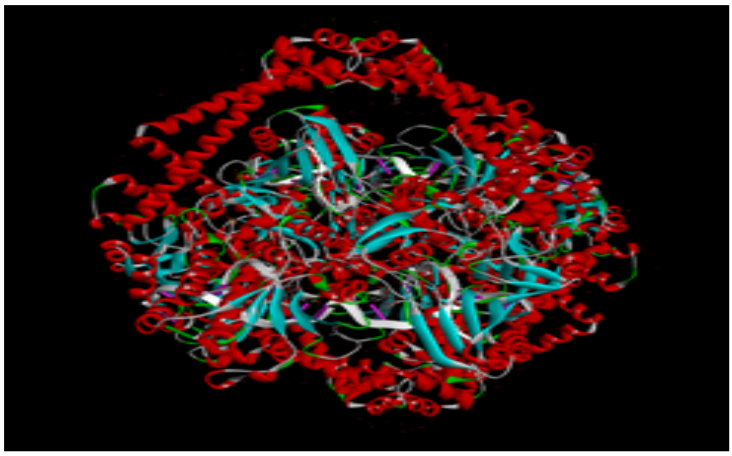
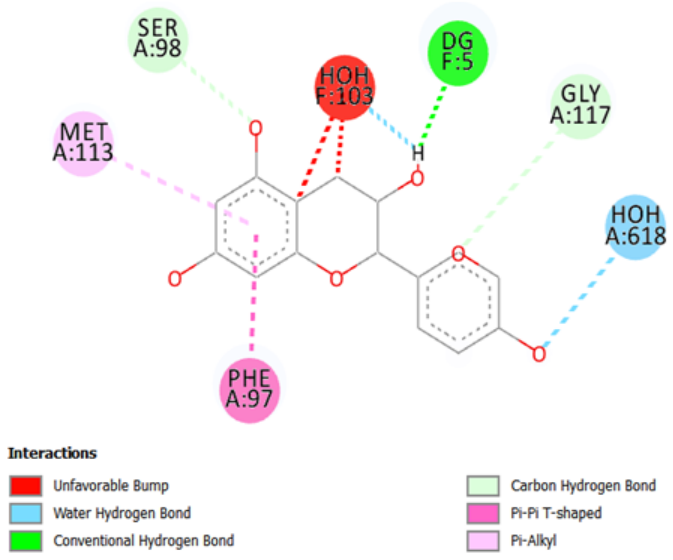


Figure 5: Protein structure of binding site data base code (5CDN)

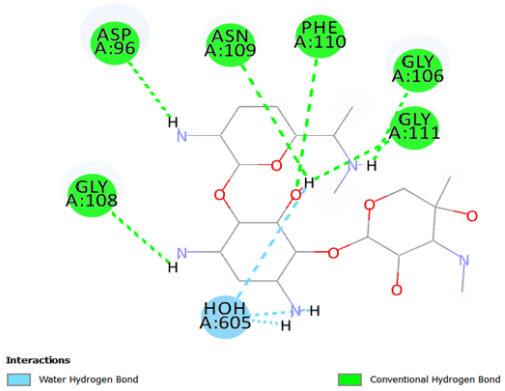
Best fit docking score: -8.1



Hydrogen-bond interacting active binding site residues: **DGF 5**.

Figure 6: 2D diagram of Quercetin - 5CDN complex best fit interaction

Gentamycin (Standard drug)
Best fit docking score: -6.3



Hydrogen-bond interacting active binding site residues: **GLY108, ASN109, GLY111, PHE110, GLY 106, ASP96.**
Figure 7: 2D and 3D diagram of Gentamycin- 5CDN complex best fit interaction
4NRO

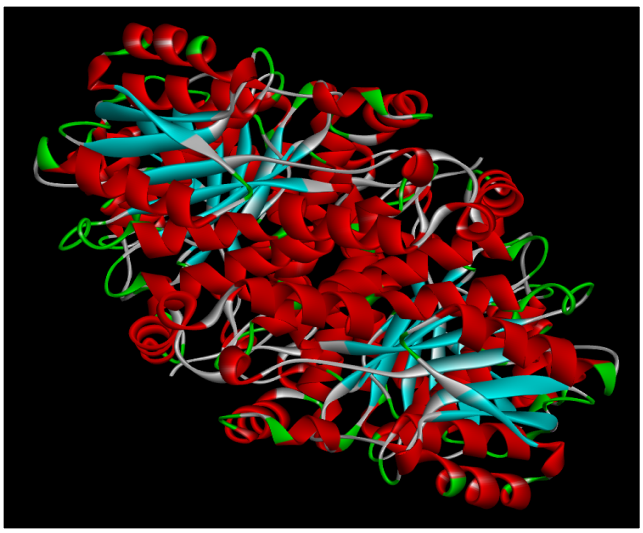
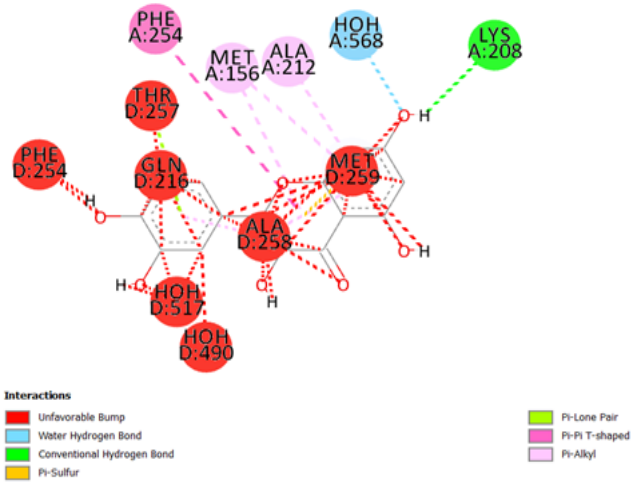


Figure 8: Protein structure of binding site data base code (4NR0)
Best fit docking score: -7.5

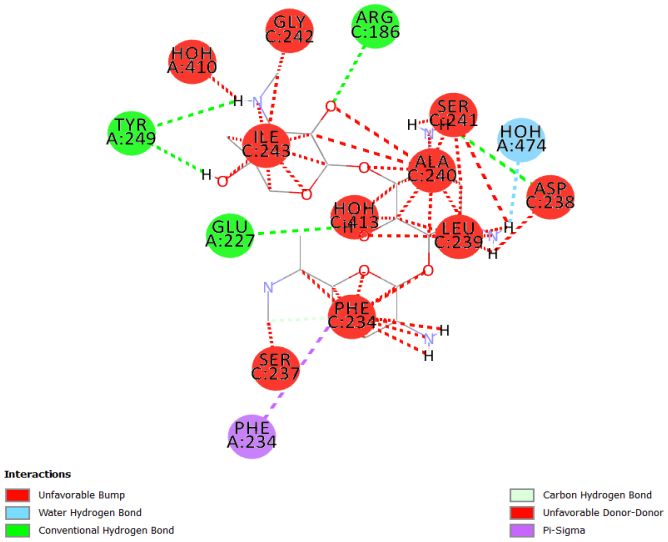


Hydrogen-bond interacting active binding site residues: **LYS 208**

Figure 9: 2D and 3D diagram of Quercetin- 4NR0 complex best fit interaction

Gentamycin (Standard drug)

Best fit docking score: -8.2



Hydrogen-bond interacting active binding site residues: **ARG 186, TRY 249, GLU 227**

Figure 10: Docking parameters of Gentamycin- 4NR0 best fit interaction

5DGK

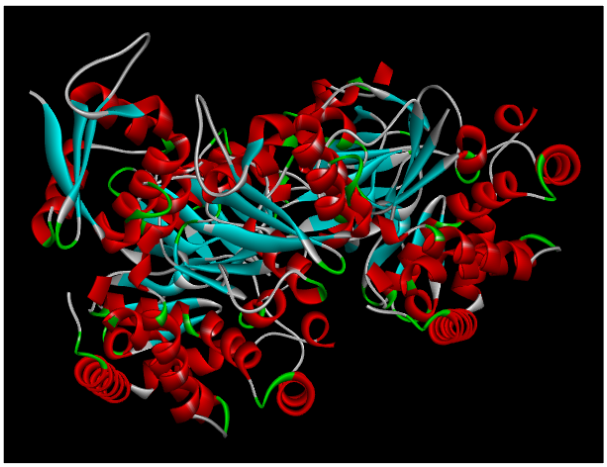
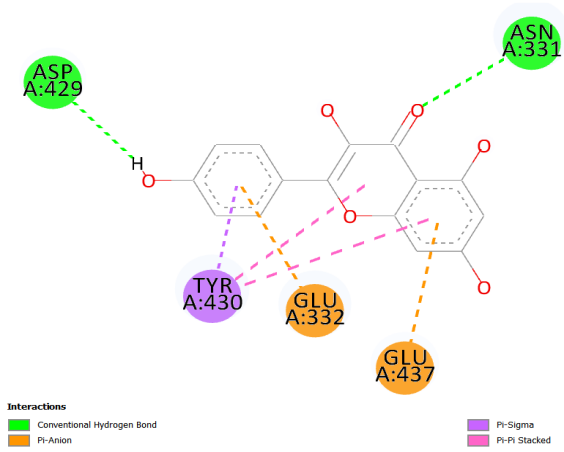
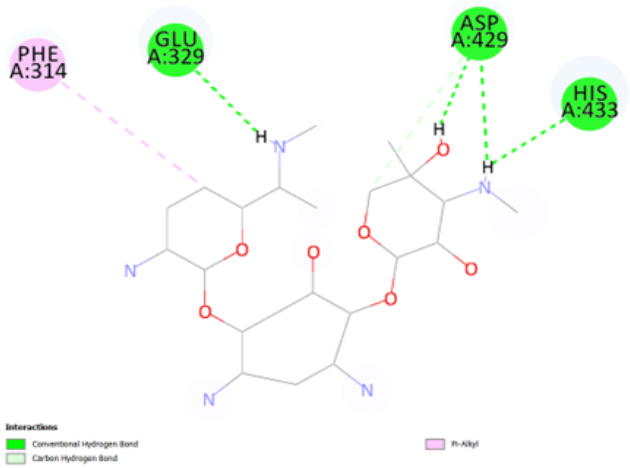


Figure 11: Protein structure of binding site data base code (5DGK)
Best fit docking score: -8.6



Hydrogen-bond interacting active binding site residues**: ASP 429, ASN 331**
Figure 12: 2D and 3D diagram of Kaempferol- 5DGK complex best fit interaction
Gentamycin (Standard drug)
Best fit docking score: -7.8



Hydrogen-bond interacting active binding site residues**: GLU 329, HIS 433, ASP 429.**
Figure 13: 2D and 3D diagram of Gentamycin- 5DGK complex best fit interaction
4BO3

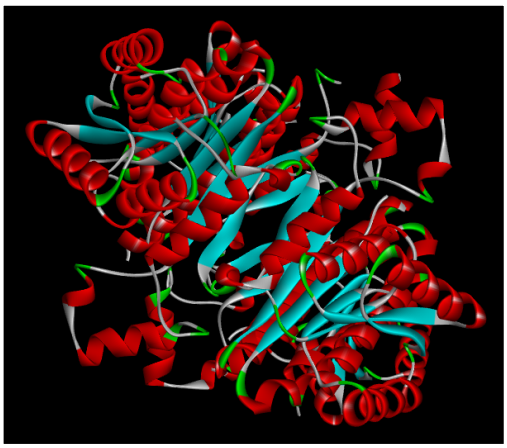
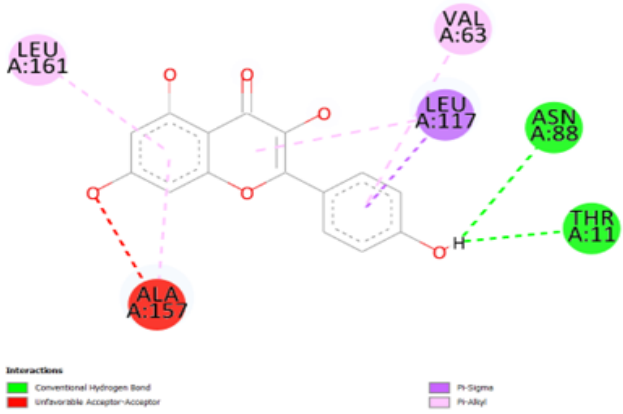
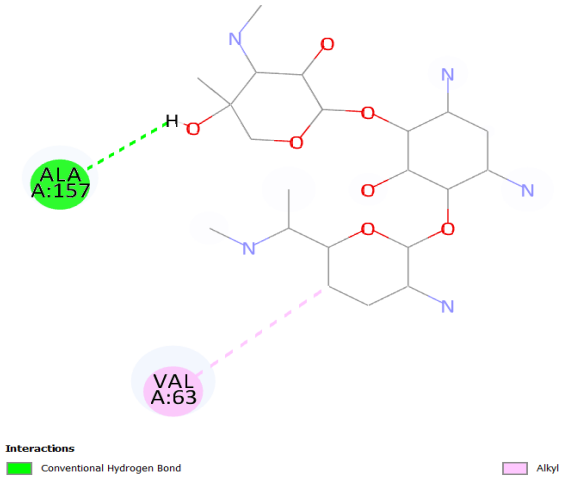


Figure 14: Protein structure of binding site data base code (4BO3)
Best fit docking score: -8.2



Hydrogen-bond interacting active binding site residues: **ASP 88, THR11**
Figure 15: 2D and 3D diagram of Kaempferol- 4BO3 complex best fit interaction
Gentamycin (Standard drug)
Best fit docking score: -7.5



Hydrogen-bond interacting active binding site residues: **ALA 157**
Figure 16: 2D and 3D diagram of Gentamycin- 4BO3 complex best fit interaction

Table 8: Summary of Docking Study

LIGAND	RECEPTOR	MICROORGANISM	DOCKING SCORE (Best fit)	RMSD VALUE

Reported Test Compound (**Quercetin**)	5CDN	*S. aureus*	**-8.1**	0
Standard Compound (Gentamycin)			**-6.3**	0
Reported Test Compound (**Quercetin**)	4NRO	*P. aeruginosa*	-7.5	0
Standard Compound (Gentamycin)			-8.2	0
Reported Test Compound **(Kaempferol)**	5DGK	*S. aureus*	**-8.6**	0
Standard Compound (Gentamycin)			**-7.8**	0
Reported Test Compound **(Kaempferol)**	$4BO_3$	*P. aeruginosa*	-8.2	0
Standard Compound (Gentamycin)			-7.5	0

According to Table 8, the plant extract claimed phytoconstituents like **Quercetin** and **Kaempferol** demonstrated more *in silico* antibacterial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* than the standard drug Gentamycin and out of the both ligands ***Kaempferol*** complex with receptor shows best docking score of the studies against *Staphylococcus aureus*.

Estimation of ligand-metal complex

Metal- 0.005M Ferric Chloride

Ligand- 0.005M Gallic Acid

Determination of stoichiometric ratio, *n* (ML_n) 0.005M (Complexation)

Table 9: Determination of stoichiometric ratio

Mol fraction of Ligand in the mixture L/(L+M) Mol fraction = (VLXML)/ (VLXML) + (VMX MM) VL= Volume of ligand solution at each interval (values) ML = Molarity of ligand solution VM = Volume of Metal solution at each interval (values) MM = Molarity of Metal solution	(L:M) (Ligand volume: Metal Volume)	L/M (Ligand volume/Metal Volume)	(A_{264}) required A_{264} = Absorbance of mixture (Ferric chloride+ Gallic acid) 264= Absorption Maxima of Gallic acid in water A0= Absorbance of ligand (Gallic acid) without metal & with distilled water
0.0	0.0:5.0	0.00	0.0862
0.1	0.5:4.5	0.11	0.1824
0.2	1.0:4.0	0.25	0.3659
0.3	1.5:3.5	0.43	0.4781
0.4	2.0:3.0	0.67	0.6690
0.5	**2.5:2.5**	**1**	**0.5483**
0.6	3.0:2.0	1.5	0.9040
0.7	3.5:1.5	2.33	0.9206
0.8	4.0:1.0	4	1.1785
0.9	4.5:0.5	9	1.4248
1.0	5.0:0.0	-	0.8059

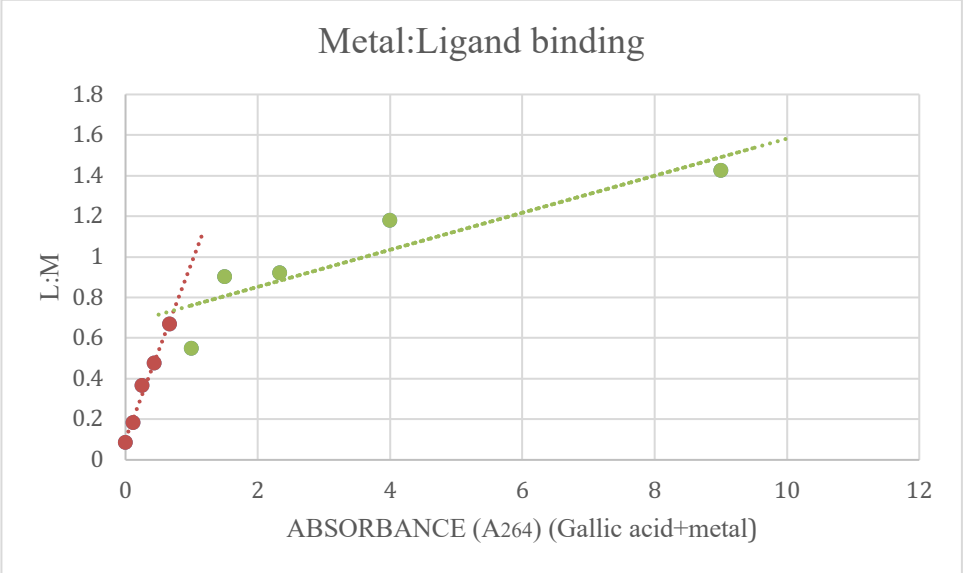


Figure 17: Determination of stoichiometric ratio, *n* (ML_n) 0.005M (Complexation)

Table 9 and Figure 17 represented the results which confirms that Gallic acid makes complexation with ferric ion in the ratio of 1:1 which could later serves as a indicator while performing the study after isolation study of the extract.

CONCLUSION

Currently, a number of conventional and folkloric medical systems employ medications that originate from the leaves of ***Colocasia esculenta*** for treatment of different ailments in a random manner. These results could be used to distinguish this species from its other species diversity, identify the species using its pharmacognostic features, and assist in the creation of an herbal monograph for the species. Moreover, the findings of this study indicated that the phytochemical screening and LC-MS/MS study of the Methanol extract of leaves of ***Colocasia esculenta*** confirmed the presence of Phenolic/Flavonoid/terpenoids fraction mainly due to which quercetin and kaempferol were selected for the *in-silico* study based on the reported phytoconstituents present on the plant leaves. In addition, same extract has a considerable amount of antibacterial activity against different non- pathogenic species when compared with the standard drug. Additionally, the plant extract exhibited spectrophotometrically determined antioxidant activity, which may result in the scavenging of free radical forms inside the biological systems. Also, the quantitative estimation of total phenolic content gave satisfactory result that may affect the antioxidant and antibacterial properties. An *in-silico* investigation has been conducted for all ligands against specific receptor types present in two different bacterial species which showed good binding affinity by forming hydrogen bonds with the interacting residues. SWISS ADME portal confirmed the drug likeness property of the reported ligand molecules along with the other satisfactory results during the *in-silico* study specially network Pharmacology gave an idea about the pathways and genes involved in the antibacterial activity which confirmed after docking study by using the receptors representing the same pathways and gene set. Gallic acid- ferric ion binding study gave an idea about the future studies if possible can be done after isolation from the plant extract for the biological activities.

Future research can use the GC-MS method to separate and identify other fractions of plant extract, with the quantification of the extract by HPLC technique. The *in-vitro* MIC values of separated phytoconstituents can be compared with the *in-silico* docking score against the same set of bacterial strains that may be compared against the same standard ligand molecule.

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ABBREVIATIONS

ABS: Absorbance, GC-MS: Gas chromatography-Mass spectrometry, BP: biological processes, CC: cellular component, MF: and molecular function.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ETHICAL STATEMENT

The manuscript assures originality, proper attribution, and adherence to research standards, confirming the work is unpublished, plagiarism-free, and all authors contributed significantly; while disclosing conflicts of interest and providing proof of ethical approval (IRB/ethics committee) and informed consent for studies involving humans or animals (No animal or human sample were used during study).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.