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Appendices

APPENDIX I

DETERMINATION OF PHYSICOCHEMICAL PARAMETERS

(Gupta *et al*., 2006)

Moisture content

Weigh about 1.5 gm of powdered sample in to a weighed flat and thin porcelain dish. Dry in oven at 100-105˚C. Cool in a desiccator and watch. The loss in weight is usually recorded as moisture.

Ash value

The determination of ash values is meant for detecting low grade products, exhausted drugs and sandy or earthy matters. Ash contains inorganic radicals like phosphates, carbonates and silicates of sodium, potassium, magnesium and calcium. Sometimes inorganic variables like calcium oxalate, silica, and carbonate content of the crude drug affect the total ash value. Such variable ash value can be determined by different methods which measure total ash, acid insoluble ash and water soluble ash.

Total ash

The total ash method is designed to measure the total amount of material remaining after ignition. This includes both the “physiological ash” which is derived from the plant tissue itself, and “non physiological ash”, which is the residue of the extraneous matter such as sand and soil adhering to the plant surface. About 2 g of the crude powder was accurately weighed in a tarred silica crucible and weighed. The powder was scattered into fine even layer on the bottom of the crucible. It was ignited by gradually increasing the heat up to 500˚C until free from carbon. Cooled in desiccator and weighed. Percentage of ash with reference to the air dried plant sample was calculated.

Acid insoluble ash

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. The total ash obtained in previous experiment is boiled for 5 minutes with 25 ml of dilute hydrochloric acid. The insoluble

matter is collected on an ash less filter paper and washed with hot water. Then it was ignited and weighed, after cooling in desiccator. The percentage of acid insoluble ash was calculated with reference to the air dried plant sample.

Water soluble ash

- Water soluble ash is the difference in weight between the total ash and the residue after the treatment of the total ash with water.
- The total ash obtained previously is boiled with 25 ml of water for 5 minutes. The insoluble matter is collected on an ash less filter paper and washed with hot water.
- Then it was ignited in a crucible for 15 minutes at a temperature not exceeding 450˚C.
- The weight of this residue is subtracted from the weight of the total ash and the content of water soluble ash in mg per g of air dried material is calculated.

Extractive value

- Extractive value is useful for the evaluation of a crude plant sample.
- Give idea about the nature of the chemical constituents present in a crude drug.
- Useful for the estimation of specific constituents, soluble in that particular solvent used for extraction.

Determination of alcohol soluble extractives

Procedure

- Weigh 5 gm of powder in a weighing bottle and transfer it into 250 ml conical flask.
- Fill a 100 ml flask to the delivery mark with methanol as solvent. Wash out the weighing bottle and pour the washings together with the remainder of the solvent into the conical flask.
- Cork the flask and set aside for 24 hrs with frequent shaking.
- Filter into 50 ml cylinder. Collect 25 ml from that filtrate and pour it into porcelain
- dish, as used for the determination of alcohol soluble extracts.

- Evaporate to dry on water bath and complete the drying in an oven at 100˚C.
- Cool in desiccator and weigh.
- Calculate the percentage w/w of extractive with reference to the air dried plant sample.

Determination of water soluble extractives

Procedure

- Weigh 5 gm of powder in weighing bottle and transfer it into 250 ml conical flask.
- Fill a 100 ml flask to the delivery mark with 90% alcohol. Wash out the weighing bottle and pour the washings together with the remainder of the solvent into the conical flask.
- Cork the flask and set aside for 24 hrs with frequent shaking.
- Filter into 50 ml cylinder. Collect 25 ml from that filtrate and pour it into porcelain dish, as used for the determination of alcohol soluble extracts.
- Evaporate to dry on water bath and complete the drying in an oven at 100˚C.
- Cool in desiccator and weigh
- Calculate the percentage w/w of extractive with reference to the air dried plant sample

APPENDIX II

PHYTOCHEMICAL ANALYSIS (Kokate *et al*., 2008)

Test for alkaloids

Mayer's test: A small quantity of extract was treated with Mayer's reagent (mercuric chloride and potassium iodide) and observed for yellowish buff color precipitate.

Dragendroff's test: A small quantity of extract was treated with Dragendroff's reagent (sodium iodide, basic bismuth carbonate, glacial acetic acid and ethyl acetate) and observed for the presence of orange brown precipitate.

Wagner's test: A small quantity of extract was treated with Wagner's reagent and observed for reddish brown precipitate.

Hager's test: A small quantity of extract was treated with Hager's reagent and observed for the presence of reddish brown precipitate.

Test for flavonoids

Ferric chloride test: A small quantity of the extract was added to a few drops of neutral ferric chloride solution and noted for the development of intense green colour.

Alkaline reagent test: To the test solution, a few drops of sodium hydroxide solution were added, formation of an intense yellow colour which turns to colourless by the addition of a few drops of dilute acetic acid indicated the presence of flavonoids.

Test for sterols and triterpenoids

Libermann-burchard test: 5 ml of test solution was boiled with two drops of acetic anhydride boiled and cooled then concentrated sulphuric acid was added along the side of the test tube. Appearance of brown ring at the junction of two layers is taken as reference. If the upper layer turns green, sterols are present whereas formation of deep red colour indicates the presence of triterpenoids.

Salkowski's test: Test solution was treated with a few drops of concentrated sulphuric acid and shaken well and the solution was allowed to stand for some time. Appearance of red color in the lower layer indicates the presence of sterols whereas formation of yellow color in the lower layer indicates the presence of triterpenoids.

Test for carbohydrates

Molish's test: To small quantities of extract, a few drops of 1% -naphthol in ethanol were added. Concentrated sulphuric acid wasthen added to the sides of the test tube. A brown purple ring formed at the junction of the two liquids indicates the presence of sugars.

Benedict's test: A few drops of test solution were boiled with equal volume of Benedict's reagent. Formation of brick red precipitate confirmed the presence of sugars.

Fehling's test: A few drops of test solution were boiled with equal volume of Fehling's solution. Formation of brick red precipitate confirmed the presence of reducing sugars.

Test for tannins

Lead acetate test: To 5 ml test solution, a few drops of 10% lead acetate were added. Appearance of yellow colour precipitate indicates presence of tannins.

Ferric chloride test: To 5 ml test solution, a few drops of 5% ferric chloride solution was added. Appearance of intense green or blue colour indicates presence of tannins.

Test for proteins

Biuret test: The extract was treated with equal volume of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution. Pink or purple colour indicated the presence of proteins.

Warming test: Test solution was boiled in a boiling water bath. Appearance of coagulation indicated the presence of proteins.

Hydrolysis test: The extract was hydrolyzed with conc.H_2SO_4 or HCl followed by ninhydrin test.

Test for amino acids

Ninhydrin test: A small quantity of test solution was boiled with 5% solution of Ninhydrin. Appearance of violet color indicated the presence of free amino acids.

Million's test: To 2 ml of test solution, equal volume of Million's reagent was added. Appearance of white precipitate which turns red upon gentle heating indicated the presence of amino acids.

Test for saponins

Froth test: To the extract, 20ml of distilled water was added and agitated on a graduated cylinder for 15 min. Persistence of characteristic honey comb froth at least 1cm in height for 30 minutes indicated the presence of saponins.

Test for phenols

Ferric chloride test: 2ml of the extract was treated with 2ml of 5% ferric chloride solution and the formation of deep blue or black colour indicated the presence of phenols.

Libermann's test: 1ml of the extract was heated with a pinch of sodium nitrite. To this solution 0.5ml of dilute H_2SO_4 was added with 1ml of dilute NaOH. The formation of deep red or green or blue colour indicated the presence of phenols.

Test for glycosides

Borntrager's test (Anthraquinone glycosides): 0.5g of the plant extract was shaken with benzene and organic layer got separated. One part of 10% ammonia solution was added to 2 parts of organic layer. A pinkish red or violet colouration in the ammonical phase indicated the presence of anthraquinone glycosides.

Keller Killiani test (Cardiac glycosides): To 0.5g of plant extract, 0.4ml of glacial acetic acid containing trace amount of ferric chloride was added. Contents were transferred to small test tube and 0.5ml of conc.H_2SO_4 acid was added along the sides of the test tube. Appearance of blue color in the acetic layer indicated the presence of cardiac glycosides.

APPENDIX III

PREPARATION OF LIPOSOMES

(Fathy *et al*., 2019)

The EEHA (Ethanol extract of *H. auriculata*) and betulin encapsulated liposomes were prepared using thin-film hydration method coupled with sonication. Cholesterol and phosphatidylcholine (Lecithin) were used at a molar ratio of 2:1. The mixture of cholesterol and lecithin was dissolved in 10 ml of chloroform until the formation of a clear solution. Using a rotary evaporator, the chloroform was evaporated at 40ºC and then the flask was kept in vacuum overnight for the removal of the organic solvent completely which results in the thin lipid film formation. This thin film was then hydrated with the 5ml of the sample dissolved in DMSO by placing it in a rotary evaporator for 5minutes. Thus obtained EEHA (Ethanol extract of *H. auriculata*) and betulin loaded liposomes were subjected to sonication to reduce the size of the liposomes. Then the non-loaded samples present in the supernatant were removed by centrifugation

APPENDIX IV

ENCAPSULATION EFFICIENCY OF THE EEHA (ETHANOL EXTRACT OF *H. AURICULATA*) AND BETULIN LOADED LIPOSOMES (Nayyer *et al*., 2019)

The encapsulation efficiency was determined using the indirect spectrophotometric method. To determine the amount of the EEHA and betulin encapsulated, the liposomes were treated with chloroform separately and shaken well. This process releases the EEHA and betulin encapsulated in the liposomes which can be measured spectrophotometrically at 450 nm. The encapsulation efficiency can be calculated using the following formula:

Encapsulation Efficiency = Amount of Encapsulated Nanoparticle/Amount of Encapsulated + Free Nanoparticle × 100

APPENDIX V

Plant authentication certificate

സിദ്ധ പ്രാദേശിക ഗവേഷണ സ്ഥാപനം
(സി.സി.ആർ.എസ്സ്., ചെന്നൈ, ആയുഷ് മന്ത്രാലയം, ഭാരത സർക്കാർ)
പൂജപ്പുര, തിരുവനന്തപുരം695012, കേരളം

सिद्ध क्षेत्रीय अनुसन्धान संस्थान
(सी.सी.आर.एस., चेन्नई, आयुष मंत्रालय, भारत सरकार के अंतर्गत)
पूजप्पुरा, तिरुवनंतपुरम - 695012, केरल

SIDDHA REGIONAL RESEARCH INSTITUTE
(Under C.C.R.S., Chennai, Ministry of AYUSH, Govt. of India)
Poojappura, Thiruvananthapuram - 695 012, Kerala
E-mail: srritvm10@gmail.com Phone: 0471-2341832

दिनांक Date:30.09.2021

AUTHENTICATION CERTIFICATE FOR 001-300921001

Certified that the Herbarium specimen submitted by E.Deepika, Research Scholar, Dept. of Zoology, Avinashilingam Institute, Coimbatore, Tamilnadu was identified as:

S.No	Botanical Name	Family	Code
01	***Hygrophila auriculata* (Schmach.)Heine.**	**Acanthaceae**	**300921001**

The image of the plant is provided below.

***Hygrophila auriculata* (Schmach.)Heine**

S. Ghanthikumar 30/09/2021
Dr. S. Ghanthikumar
Research Officer (Botany)
SRRI, Thiruvananthapuram

डॉ. एस. गंथी कुमार Dr.S.Ghanthi Kumar
अनुसंधान अधिकारी (वनस्पति विज्ञान) Research Officer (Botany)
सिद्ध क्षेत्रीय अनुसंधान संस्थान Siddha Regional Research Institute
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30/9/21
Dr.A.Kanagarajan
Assistant Director (Siddha)S-IV & I/c,
SRRI, Thiruvananthapuram

डॉ. ए. कनगराजन / Dr. A. Kanagarajan
प्रभारी सहायक निदेशक (सिद्ध) / Assistant Director(S) In-charge
सिद्ध क्षेत्रीय अनुसंधान संस्थान / Siddha Regional Research Institute
पूजप्पुरा, तिरुवनंतपुरम-695 012 / Poojappura, Thiruvananthapuram-695 012

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APPENDIX VI

IAEC Certificate

Avinashilingam Institute for Home Science and Higher Education for Women
(Deemed to be University under Category 'A' by MHRD, Estd. u/s 3 of UGC Act, 1956)
Re-accredited with 'A+' grade by NAAC, Recognised by UGC under Section 12 B
Coimbatore – 641 043, Tamil Nadu, India
(Reg. No. 623/PO/ReBi/S/02/CPCSEA)

Certificate

This is to certify that the project proposal no.**AIW:IAEC.2021:ZOO:01** entitled **"Validation of the Antidiabetic and Wound Healing potential of *Hygrophila auriculata* leaves and roots"** submitted by **Ms. Deepika E** has been approved by the IAEC of **Avinashilingam Institute for Home Science and Higher Education for Women** in its meeting held on **01.10.2021** and **62 Swiss Albino Mice** have been sanctioned under this proposal for a duration of **120-490** days.

Authorized by	Name	Signature	Date
Chairman:	Dr. Smitha Subash		01.10.2021
Member Secretary:	R. NIRMALADEVI		01.10.2021
Main Nominee of CPCSEA:	Dr. C. GUNASEKARAN		01.10.2021

\

Avinashilingam Institute for HomeScience and Higher Education for Women
(Deemed to be University Estd. u/s 3 of UGC Act 1956, Category 'A' by MHRD
Re-accredited with A++ Grade by NAAC. CGPA 3.65/4, Category I by UGC
Coimbatore - 641 043, Tamil Nadu, India

Appendix L2

(Item No 5 of Check List) Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	PHYTO CONSTITUENTS BASED ANTIRADICAL DEFENSE RESPONSE OF HYGROPHILA AURICULATA ROOT	RESEARCH JOURNAL OF AGRICULTURAL SCIENCES	13-VOL ISSUE: 06 YEAR: 2022 Pg.no: 1906-1911	UGC-CARE
2	UNRAVELLING THE MODE OF ACTION OF WOUND HEALING EFFICENCY WITH THE COMPOUNDS SCREENED FROM HYGROPHILA AURICULATA BY MOLECULAR DOCKING	THE JOURNAL OF PLANT SCIENCE RESEARCH	VOL: 39 ISSUE: 2 YEAR: 2023	UGC-CARE

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : E. Deepika

Supervisor : Sandhya K.S. 29.5.2023

K. Ravikumar 29/5/23

Checked By:

HoD/Dean of Respective School

The scholar miss. Deepika, E has published her article in "Research Journal of Agricultural Sciences" which is active and indexed in UGC Care List Group I and got acceptance from the journal "The journa

of Plant Science Research" indexed and active in Ukc care Group I from September 2019 to present.

29.05.23.

Phytoconstituents based Antiradical Defense Response of Hygrophila auriculata Root

Deepika E and Santhy K. S.

Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 06

Res. Jr. of Agril. Sci. (2022) 13: 1906–1911

CARAS

Res. Jr. of Agril. Sci. (Nov-Dec 2022)
13(6): 1906–1911

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

Phytoconstituents based Antiradical Defense Response of *Hygrophila auriculata* Root

Deepika E[1] and Santhy K. S.*[2]

Received: 05 Oct 2022 | Revised accepted: 05 Dec 2022 | Published online: 27 Dec 2022

A B S T R A C T

The accumulation of free radicals in the body leads to many diseases due to oxidative stress. Numerous studies are currently being conducted to prevent oxidative stress and neutralize the effects of free radicals by antioxidants primarily of plant origin rather than synthetic ones. *Hygrophila auriculata* belonging to the family Acanthaceae was used in this study to assess the phytochemical constituents, free radical scavenging ability, and the amount of enzymatic and non-enzymatic antioxidants. Four solvents, namely ethanol, ethyl acetate, chloroform, and water, were taken to extract *Hygrophila auriculata* root. Preliminary phytochemical analysis was carried out by adopting the standard protocol. Further, the free radical scavenging activity was assessed against two radicals, DPPH and FRAP, along with the reducing power ability. The enzymatic antioxidants include superoxide dismutase, catalase, peroxidase, polyphenol oxidase, glutathione-S-transferase, and non-enzymatic antioxidants such as ascorbic acid, α-tocopherol, and total phenols were assessed. The phytochemical screening revealed the presence of compounds such as alkaloids, flavonoids, sterols, phenols, saponins, tannins, proteins, carbohydrates, cardiac glycosides, and terpenoids firmly in the ethanol extract compared to all other solvents. The free radical scavenging activity showed a maximum scavenging power in the ethanol extract. The scavenging efficacy of DPPH and ABTS was 89.53 and 85.71%, respectively, at 100 µg/ml. Similarly, the reducing power was highest at the dose of 100 µg/ml. The ethanol extract possesses enzymatic antioxidants such as superoxide dismutase (111.3 ± 3.21 Units/mg), peroxidase (0.34 ± 2.11 Units/mg), catalase (4.53 ± 0.9 Units/mg), polyphenol oxidase (0.4 ± 0.01 µg/g), glutathione-S-transferase (2.3 ± 0.5 µg/g) and non-enzymatic antioxidants such as flavonoids (243.40 ± 1.23 mg/g), α – tocopherol (32.3 ± 0.18 µg/g), vitamin – C (2.93 ± 0.02). Thus, our findings suggest that roots of *Hygrophila auriculata* have the potential to scavenge the free radicals and prevent oxidative stress-related diseases, which pave the way for the plant to serve as a good phytotherapeutic agent against many diseases and disorders.

***Key words*:** *Hygrophila auriculata*, Antioxidants, Free radicals, Phytochemicals, DPPH

Traditionally used medicinal plants play a prominent role in human health as therapeutic remedies. Natural products have been discovered to be a repository of diverse biomolecular structures far beyond human knowledge [1]. Phytochemicals are bioactive plant compounds that alleviate many human physiological disorders and suppress synthetic antibiotics' consumption. Normal cellular metabolic reactions of the human body in a more exposed environment and higher levels of ingested xenobiotics lead to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In distinct pathophysiological situations, ROS and RNS are responsible for causing oxidative stress. This further produces unstable molecules known as free radicals, enhancing many chronic and degenerative ailments [2]. Oxidative stress and free radical suppression could be achieved by efficiently neutralizing cellular responses in antioxidants [3].

The defense response against the reactive oxygen species could be activated by two antioxidants: enzymatic and non-enzymatic. The body safeguards itself from ROS by employing enzymatic antioxidant processes, which include enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase and glutathione-S-transferase (GST). These enzymes are essential in preventing the cells from lipid peroxidation and supporting cell membranes' stability and proper functioning. Non-enzymatic antioxidants disrupt the free radical chain reaction. Consequently, both antioxidants protect the body from DNA damage, tumor growth, cardiovascular diseases, neuroprotective diseases, etc. [4].

Phytoconstituents can scavenge free radicals by donating electrons or ions to those unpaired electrons. Several researchers have been exploring potent antioxidants to be

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extracted from medicinal plants because they are financially sustainable and have excellent antioxidant properties without adverse effects. *Hygrophila auriculata* (Buch. -Ham) (*H.auriculata*) is a thorny sub-shrub of the family Acanthaceae that grows widely throughout the moist places of India. The leaves and roots of this plant are medicinally utilized for treating many disorders, such as inflammation, jaundice, diabetes, etc. [5]. With the above context, the present investigation aimed to evaluate *H. auriculata* for its potential use as a natural source of phytochemicals and antioxidants.

MATERIALS AND METHODS

Plant collection and sample preparation

The roots of *H. auriculata* were collected from the areas of Coimbatore. The roots were washed entirely and let dry for 5-7 days at room temperature. The dried-out leaves were ground to powder and stored in screw-cap bottles until further analysis. Preparation of the extract A 50 g of sample was dissolved in 500 ml of various solvents (ethanol, ethyl acetate, water, and chloroform). It was then filtered and further concentrated by evaporation.

Phytochemical analysis

The extracts were subjected to preliminary phytochemical evaluation, which was done using standard color test methods [6].

Free radical scavenging activity

The radical scavenging activities of the extracts were determined *in vitro* against a battery of radicals, namely DPPH and ABTS, and FRAP.

DPPH radical scavenging activity [7]

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. About 3 ml of graded concentration (25 - 100µg/ml) of extracts were taken in different test tubes, and 1 ml of 0.3mM DPPH methanol solution was added to these test tubes and shaken vigorously. Methanol served as the blank, and DPPH in methanol, without the rhizome extracts, served as the positive control. After 30 min incubation of samples at 25 °C in the dark, the absorption was measured at 517 nm. The inhibition percentage of DPPH was calculated as follows:

Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control) × 100

Abs (control)- absorbance of DPPH radical with methanol

Abs (sample)- absorbance of DPPH radical with sample extract

ABTS radical scavenging activity [8]

ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 hours before use. Aliquots (5µl) of the different extracts were added to 0.3ml of ABTS solution, and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer, and the percent scavenging was calculated using the formula:

Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control) × 100

FRAP (Ferric reducing power assay) [9]

Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%), and varying concentrations of extracts (25 - 100µg/ml). After the reaction, mixtures were incubated at 50°C in a water bath for 30 min, allowed to cool at room temperature (28 °C), and 2.5 ml of 10% TCA (Trichloroacetic acid) was mixed into each reaction mixture, followed by the centrifugation at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in the test tube, added with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1.0%), and allowed to react for 10 min and absorbed at 700 nm.

Antioxidant activity

The antioxidant status of the roots of *H. auriculata* was estimated by analyzing various enzymic and non-enzymic parameters.

Estimation of catalase activity [10]

H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of enzyme extract (0.01 - 0.04), and mixed thoroughly. The time taken for a decrease in absorbance for 0.5 units is noted. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with a more concentrated sample solution.

Estimation of peroxidase activity [11]

Three milliliters of 0.05M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract were taken in a test tube. 0.5 ml of 1% hydrogen peroxide was added to the test cuvette. The spectrophotometer was adjusted to read '0' at 400nm. Changes in absorbance were recorded every 30 seconds up to 3 minutes.

Estimation of superoxide dismutase activity [12]

The incubation medium contained a 300µl of each reagent (50mM potassium phosphate buffer (pH 7.8), 45mM Methionine, 5.3mM Riboflavin, 84mM Nitro Blue Tetrazolium (NBT), and 20 mM potassium cyanide. 300µl of the sample was added to this mixture, and the final volume was made up to 3ml with water. The tubes were placed in an aluminum foil lined box maintained at 25 °C and equipped with 15W fluorescent lamps. The NBT reduction was measured at 600nm after 10 minutes of exposure to light. The maximum reduction was evaluated in the absence of an enzyme giving 50% inhibition of the reduction of NBT.

Estimation of polyphenol oxidase activity [13]

2.5 ml of 0.2M phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01 M) were taken into the cuvette and added the enzyme extract (0.2 ml). The spectrophotometer was set at 495nm and recorded the change in absorbance every 30 seconds up to 5 minutes.

Estimation of glutathione-s-transferase [14]

A total of 1.0 ml of buffer, 1.7 ml of water, and 0.1 ml of CDNB were added to the 0.1 ml of sample and incubated for 5 minutes at 37 °C. This was followed by the addition of 0.1 ml of glutathione s transferase was added. At 340 nm, the enzyme's optical density was calculated compared to a blank.

Non-enzymic antioxidants

Estimation of vitamin C or ascorbic acid [15]

The assay volumes were made up of 2.0ml with 4% TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate, respectively, were pipetted out into a clean, dry test tube, the volume of which was also made up to 2.0ml with 4% TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by two drops of 10% thiourea solution. The sample was incubated at 37 °C for 3 hours. The osazones formed were dissolved in 2.5ml of 85% sulphuric acid in cold, drop by drop, with no appreciable rise in temperature. The

DNPH reagent and thiourea were added to the blank alone after adding H_2SO_4. The tubes were incubated for 30 min at room temperature, and the absorbance was read spectrophotometrically at 540nm. The ascorbic acid content in the sample was calculated using the standard graph.

Estimation of α-tocopherol [16]

Into three stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5ml of extract, 1.5ml of standard (10mg of α-tocopherol was dissolved in 10ml of absolute alcohol), and 1.5ml of water, respectively. To the test and blank, 1.5 ml of ethanol was added, and to the standard, 1.5 ml of water was added. Added 1.5ml xylene to all the test tubes, stoppered, mixed well, and centrifuged. From this, 1.0ml of the xylene layer was transferred into another stoppered tube. Added 1.0ml of 2, 2'- dipyridyl reagent to each tube, stoppered, and mixed well. 1.5ml of this mixture was pipetted into colorimeter cuvettes, and the test's extinction and standard against the blank were noted at 460nm. 0.33 ml of ferric chloride solution was added to all the test tubes, including the blank. The amount of vitamin E can be calculated using the formula:

Amount of tocopherols in µg = reading at 520 nm- reading at 460 nm/reading of standard at 520 nm × 0.24 × 15

Estimation of flavonoids [17]

An aliquot of the extract was pipetted out and evaporated to dryness. Different volumes of standard catechin (0.2 to 1.0ml) were taken and made up to 1.0ml with distilled water. An aliquot of 4.0ml of vanillin reagent was added, and the tubes were heated for 15 minutes in a boiling water bath and cooled. The optical density of the solution was read at 340 nm. The standard curve was constructed in an electronic calculator set to the linear regression mode, and the concentration of flavonoids was calculated. The values are expressed as mg flavonoids/g tissue.

RESULTS AND DISCUSSION

The preliminary phytochemical screening was carried out on various solvents and revealed the presence of a wide range of phytoconstituents, including alkaloids, flavonoids, sterols, phenols, saponins, tannins, proteins, carbohydrates, cardiac glycosides and terpenoids which showed better result on ethanolic extract among the other three solvents (Table 1). This indicates that ethanol is highly capable of extracting secondary metabolites of *H. auriculata* compared with all other solvents since the high polarity of the solvent accounts for the extraction of a wide range of compounds.

Table 1 Qualitative phytochemical analysis of the extracts of *Hygrophila auriculata*

Constituents	Solvents			
	Chloroform	Ethanol	Ethyl acetate	Aqueous
Alkaloids	+	+	+	-
Flavonoids	+	+	+	+
Sterols	-	-	-	+
Phenols	-	+	+	-
Saponins	-	-	+	-
Tannins	+	+	+	-
Quinones	-	-	-	-
Proteins	+	+	-	+
Carbohydrates	-	+	-	+
Cardiac glycosides	-	+	-	+
Terpenoids	-	+	-	+

+ Present; - Absent

The capacity of the plant extracts to scavenge the free radicals such as DPPH (2,2- di (4-test-octyl phenyl) -1-picrylhydrazyl radical) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation followed by the ability to reduce ferric (III) iron to ferrous (II) iron were assessed by performing the *in vitro* antioxidant assays. Comparative analysis was done using various solvents on the antioxidant activity of *H. auriculata* roots (Table 2). In the present study, the results revealed that ethanol extract of *H. auriculata* has the highest antioxidant capacity against both DPPH (89.53%) and ABTS radicals (85.71%) and also exhibited the potential reducing power (86.24%). This was then followed by ethyl acetate (81.73%), aqueous (77.41%), and chloroform (59.45%). The solvent polarity strongly impacted the presence of secondary metabolites and their antioxidant potential [18]. The radical scavenging ability ranged from 38.76% to 89.53%, which was nearer to the standard (89.89%).

In the DPPH assay, the antioxidant was able to reduce and scavenge the violet-colored radical DPPH to the yellow-colored 1, 1-diphenyl-1, 2-picryl hydrazine stable compound [1]. The antioxidant in the *H. auriculata* root donates an H-atom to DPPH radical making it to DPPH-H. Consequently, as DPPH loses its reactivity, this reaction is distinguished by a decrease in absorbance [19]. Similar interactions occur in the ABTS assay, where the antioxidants act with the generated ABTS radical and decolorize its blue color. Our results showed that the order of ABTS radical scavenging activity of all the extracts was similar to that observed for DPPH. The FRAP assay is generally associated with the presence of reductones which have been shown to exert antioxidant action by donating a hydrogen atom and breaking the free radical chain. During the reducing power assay, reductants (antioxidants) in the *H. auriculata* root would reduce the Fe_3^+/ ferricyanide complex to the ferrous form (Fe_2^+). The amount of Fe_2^+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Here occurs the color change of yellow to multiple shades of green and blue, which is proportional to its antioxidant ability.

Bioactive compounds such as natural phenols and flavonoids quench the reactive oxygen species, which could defend against oxidative stress and inhibits lipid peroxidation [20-21]. In this study, phyto compounds such as flavonoids, phenolic acids, and phenolic diterpenes in the *H. auriculata* root extract naturally elevated the radical scavenging activities. These phenolic components possess many hydroxyl groups, including the o-dihydroxy group, which have a powerful radical scavenging effect and antioxidant power. Hydroxyl groups play a vital role in hydrogen bond donation that aids the scavenging of free radicals, reducing metal ions, and interacting with biomolecules [22]. A previous study regarding the free radical scavenging ability of *H. auriculata* root reported that petroleum ether extract indicated 93.91±6.57% DPPH radical scavenging ability at 120 µg/mL concentration, which was followed by 62.07±4.34 % of ferric reducing power at 120 (µg/mL) [23].

Toxic free radicals are effectively squelched by the cellular antioxidant defence mechanism endogenously, which shields the biomolecules from oxidative alteration. The endogenous compounds in cells are classified into enzymatic and non-enzymatic antioxidants. In addition to cellular antioxidants, exogenous antioxidants from herbal plants are proved to improve the body's natural defences against disorders and stress. The phytocompounds from the plant accelerate the scavenging and interruption of free radical chain in the body by generating enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase, glutathione-S-transferase (GST) and also the exogenous non-enzymatic antioxidants such as flavonoids, α – tocopherol and

Vitamin – C. These enzymes activate the plant's antioxidant system, which together alleviates the toxic effects of oxidative damage [24].

Table 2 Free radical scavenging activity of *Hygrophila auriculata*

Drug	Concentration	Scavenging ability (%)		
		DPPH	ABTS	FRAP
Chloroform	25	38.76	40.22	33.22
	50	41.09	43.21	47.32
	100	49.80	51.20	59.45
Ethanol	25	77.87	73.33	61.05
	50	81.22	80.07	73.37
	100	89.53	85.71	86.24
Ethyl acetate	25	73.23	74.88	70.33
	50	78.66	77.63	75.88
	100	81.73	79.55	79.38
Aqueous	25	59.21	60.22	61.88
	50	64.35	64.33	67.29
	100	68.20	67.30	77.41
	25	67.89	72.14	67.44
Standard	50	75.23	78.09	71.21
	100	89.89	84.77	87.37

In the present study, the activities of enzymatic and non-enzymatic antioxidants were assessed using ethanol extract of *H. auriculata* due to its high potential free radical scavenging ability. The accumulation of enzymatic antioxidants such as superoxide dismutase (111.3 ± 3.21 Units/mg), peroxidase (0.34 ± 2.11 Units/mg), catalase (4.53 ± 0.9 Units/mg), polyphenol oxidase (0.4 ± 0.01 µg/g), glutathione-S-transferase (2.3 ± 0.5 µg/g) and non-enzymatic antioxidants such as flavonoids (243.40 ± 1.23 mg/g), α – tocopherol (32.3 ± 0.18 µg/g), vitamin – C (2.93 ± 0.02) in the ethanolic extracts of *H. auriculata* showed significant results (Table 3). All the enzymatic antioxidants which involve in the act of initial defense mechanisms converts reactive oxygen species (ROS) and superoxide anion to lipid hydrogen peroxide (H_2O_2) and hydroperoxide. Further, the enzyme catalase facilitates the degradation of hydrogen peroxide (H_2O_2) and hydroperoxide as water and oxygen. The above process is accomplished in the presence of zinc (Zn), copper (Cu), and manganese (mn) metal ions. Thus, SOD, POD, and CAT are the most potent antioxidants that prevent the body from lipid peroxidation, toxification of the cells, and excessive oxygen radicals and maintain the cell structure and growth [4].

Table 3 Quantitative estimation of enzymatic and non-enzymatic antioxidants

Enzymatic antioxidants	Level
SOD (Units/mg)	111.3 ± 3.21
POD (Units/mg)	0.34 2.11
CAT (Units/mg)	4.53 ± 0.9
Polyphenol oxidase (µg/g)	0.4 ± 0.01
GST (µg/g)	2.3 ± 0.5
Non-enzymatic antioxidants	
Flavonoids (mg/g)	243.40 ± 1.23
α – tocopherol (µg/g)	32.3 ± 0.18
Vitamin – C (µg/g)	2.93 ± 0.02

Similarly, the reduction of glutathione in the body promotes defense against oxidants and neutralizes the hydrogen peroxide in the cell. Glutathione S-transferases (GSTs) is a detoxification enzyme that helps reduce and conjugates glutathione with various electrophilic compounds, making the macromolecule more soluble [25-26].

Polyphenol oxidase is a vital copper enzyme known as catechol oxidase, tyrosinase, etc. [27]. Polyphenol oxidase is a potent antioxidant that scavenges H_2O_2 thereby neutralizing oxidative stress and regulating the other oxidases in the body [28-29]. This is because the electrons in the phenolic hydroxyl structure have a conjugation effect, which weakens the binding ability of hydrogen ions and raises their risk of dissociation. As a result, the active hydrogen ion suppresses the reactive oxygen species and other oxidants, stabilizing themselves [30].

Following this, the second line defense mechanism inhibits the production of damaged cell species and the progressing of harmless free radicals, thereby reducing the impact of oxidative reaction. This was worked by some of the non-enzymatic antioxidants such as flavonoids, α-tocopherol, and Vitamin – C [31].

The presence of flavonoids in the qualitative phytochemical analysis of ethanol extract of *H. auriculata* root is comparable to the accumulation of 243.40 ± 1.23 mg/g of flavonoids in the quantitative analysis. In the phenolics group, flavonoids constitute polyphenols, highly associated with antioxidant potential [32]. Flavonoids play a vital role in protecting DNA from damage caused by hydroxyl radicals. The chelation reaction involving the metal ions like copper and iron elucidates the preventive effect of flavonoids on DNA damage. The flavonoids suppress the production of free radicals by complexing with the chelating metal ions, thereby neutralizing it [33-34].

Subsequently, vitamin E is a lipid-soluble vitamin with high antioxidant potency. α -tocopherol, a stereoisomer of vitamin E, is the most bioactive form in humans. As fat-soluble, α -tocopherol safeguards cell membranes from damage by free radicals. Its direct antioxidant action is to prevent lipid peroxidation. It interrupts lipid peroxyl radicals (LOOc) and stops the lipid peroxidation reactions. Tocopheroxyl radical is generated when α -tocopherol reacts with lipid peroxyl radicals, transferring the phenolic hydrogen ion. Although relatively stable, this tocopheroxyl radical can neither trigger nor initiate additional lipid peroxidation, which is a crucial characteristic of a powerful antioxidant [35-37].

The antioxidant Vitamin C, otherwise known as ascorbic acid, retains the α-tocopherol radical to its original form by reducing the generated vitamin E radicals. Therefore, vitamin C can function as an antioxidant by contributing electrons to various enzymatic and non-enzymatic activities [38-39]. The protection of the macromolecules against biological oxidation is achieved through the reduction of transition metal ions of numerous biosynthetic enzymes by vitamin C. This also aids in the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) by behaving as a substrate for the enzyme ascorbate peroxidase [40].

Like vitamin E, vitamin C also inhibits the lipid peroxidation chain reaction by contributing an electron to lipid radical and changing itself to an ascorbate radical. The rapid interaction between ascorbate radicals leads to the generation of one molecule of ascorbate and one molecule of dehydroascorbate, where the dehydroascorbate doesn't have the antioxidant ability. The addition of two electrons to the radical converts it into ascorbate, which has been proposed to carry out by oxidoreductase [4]. Hence the synergistic activity of vitamin C and E suppress the formation of hydroperoxide and other radicals. Thus, the aforementioned function of all enzymatic and non-enzymatic antioxidants demonstrates their potent role

in the antiradical defense mechanism. Hence the observed elevation in SOD, CAT, POD, polyphenol oxidase, and GST indicates that *H. auriculata* can inhibit the effect of oxidative stress.

CONCLUSION

According to the findings of the current study, it was observed that the root of *H. auriculata* contains certain active phytochemical constituents that improve the antioxidant status. It was clearly demonstrated that in each investigation such as free radical scavenging assay and quantitative estimation of the antioxidants significant percentage of scavenging capacity was determined with potent antioxidants. This experimental evidence suggests the candidate plant's use to treat human pathologies in which free radicals play a significant role. However, further investigation is required on the isolation and characterization of the antioxidant constituents.

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Unravelling the Mode of Action of Wound Healing Efficiency with the Compounds Screened from *Hygrophila auriculata* by Molecular Docking Studies

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Hygrophila auriculata **is one of the traditional herbal plants utilized by many rural folks to heal wounds. According to the traditional knowledge, the roots of the plant would be highly effective for wound healing. The plant-based secondary metabolites remain an essential aspect in developing novel drug candidates. The early screening of bioactive compounds for a therapeutic property would be strongly evidenced to carry out the *in vitro* and *in vivo* studies. The current study focuses on screening bioactive compounds involved in wound healing using *in silico* molecular docking studies. The LCMS analysis of *H. auriculata* yielded about 15 compounds in positive and negative modes with the greatest medicinal properties. The drug-likeness, physicochemical properties, and bioactivity scores were evaluated for 15 compounds using SWISS ADME online tool. The compounds that indicate drug like properties and adhere to the permissible parameters are epiafzelechin, betulin, caffeic acid 3-glucoside, quercetin, palmitic acid, linoleic acid, chlorogenic acid, myristic acid, and 5, P coumaroyl quinic acid implying to be a cell-permeable and orally active drug. The bioactivity scores indicated that the compounds to be highly active. The binding affinities were checked with the wound pathogenic proteins such as Elastase, Glycogen synthase kinase-3β, gelatinase, and collagenase. The compounds that fall within the drug-likeness limit were further evaluated for ADMET properties and predicted that majority compounds are nontoxic and easily absorbed. The docking score ranged from -1.56 kcal/mol to -9.65 kcal/mol which exhibited the strongest binding affinity. The effective binding of compounds like epiafzelechin, betulin, kaempferol-7-O-Glucoside, linoleic acid, chlorogenic acid, and quercetin with these proteins provides evidence for further research to elucidate the underlying mechanism that promotes wound healing of *Hygrophila auriculata* root.**

Keywords: Docking, Wound healing, *Hygrophila auriculata,* Drug-likeness, ADMET.

INTRODUCTION

Skin is the primary barrier that shields the human body from the hostile atmosphere. Any physical harm to the skin's surface causes it to lose its integrity, exposing the subcutaneous layer known as the wound. Upon an injury, it is critical for the wound to close promptly and regenerate the injured skin. When the integrity of the skin is disrupted due to acute or chronic lesions, a sequence of dynamic cellular reactions known as wound healing is instigated to regain healthy skin. Wound healing comprises four phases: inflammation, proliferation, maturation, and remodelling. There is a massive influx of proinflammatory cytokines at the inflammatory phase, followed by the upsurge of Wnt/β-catenin. Wnt proteins belong to glycoproteins which modulate cell division and cell fate. The Wnt/β-catenin pathway stabilizes the cytoplasm by signalling, and β-catenin accumulates in the nucleus, promoting cell division and proliferation.

GSK-3β also plays a vital role in the canonical Wnt pathway, dependent on β-catenin. During the proliferation phase, mesenchymal cells generate more β-catenin. Generally, GSK-3 protein phosphorylates β-catenin on serine and threonine residues targeting it for breakdown. The Wnt/β-catenin pathway has been proven to enhance wound healing by blocking the glycogen synthase kinase-3 (GSK-3) protein. The elevated Wnt reaction raises the β-catenin deposition in the cytoplasm. Also, increased β-catenin causes the transcription of some genes like the

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matrix metalloproteinases, leading to extracellular matrix accretion, angiogenesis, and propagation of various cell structures. The last maturation phase involves remodeling the extracellular matrix. Due to oxidative stress in the wound site, extracellular matrix (ECM) deterioration of collagen, gelatin, and elastin occur. These are catalyzed by the improper regulation of matrix metalloproteinases (MMPs) such as Collagenase (MMP1), Gelatinase (MMP2), and elastase (MMP12) enzymes, respectively, which delays the healing process.

Naturopathic remedies with medicinal plants have sparked interest in treating numerous medical conditions. *Hygrophila auriculata* (K. Schum) Heine (*H. auriculata*) is an erect semi-woody plant belonging to Acanthaceae. It is reported that the rural people of Assam revealed that the roots of *H. auriculata* is effectively used as traditional wound healing agent. Scientific validation of such conventional practices is more profound and adds value to such methods. The advancement of modern technology impacts the pharmacology and mechanisms of action of many therapeutic herbs. Structure-based drug design using bioactive constituents can reduce uncertainty and speed up the process. However, research on the molecular interaction of phytoconstituents in *H. auriculata* with wound healing mediators is scanty. Impelling to screen the potential bioactive drug candidates, the present study focuses on *in silico* molecular docking of selected phytocompounds from *H. auriculata* against the proteins responsible for the pathogenesis of wounds. Therefore, the effective bioactive chemicals can be determined earlier, before *in vitro* and *in vivo* approaches, by evaluating their binding affinities and molecular interactions.

MATERIALS AND METHODOLOGY

LCMS

LCMS analysis was carried out using XEVO-TQS micro #QEA0592. At 40°C, a reversed-phase C18 analytical column (HSS T3, C18 Column from Waters, USA) with 2.1×100 mm and 2.5 m particle size were employed. In the mobile phase, a binary gradient of water and acetonitrile was utilized at a constant flow rate of 0.4 mL/min. The following was the gradient elution programming: 5% B (0 seconds), 10% B (60 seconds), 23% B (90 seconds), 28% B (3.8 minutes), 35% B (6.5 minutes), 43% B (8 minutes), 45% B (9.5 minutes), 95% B (12 minutes), 5% B (20min). Empower3 software was used for gathering data and processing.

Retrieval of ligands and proteins

The chemical structures of the selected ligands were obtained from the pub chem database. The X-ray crystal structures of the receptors Elastase (1HNE), Glycogen synthase kinase-3β (GSK-3β;1Q5K), Gelatinase (1QIB), Collagenase (2Y6I) were retrieved from the Protein Data Bank.

Drug likeliness and Physicochemical properties

The SWISS ADME online server was utilized to calculate the physicochemical properties and drug likeliness parameters based on Lipinski, Ghose, Veber, Egan, and Muegge rules. The SMILES notations of each compound were obtained from the Pubchem database to calculate both properties.

Bioactivity score

SMILES notations of the selected compounds were fed in the online Molinspiration software version 2011.06 (www.molinspiration.com) to predict bioactivity score (GPCR ligands, kinase inhibitors, ion channel modulators, enzymes, and nuclear receptors).

ADMET properties

ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction provides valuable facts about the compound that could be evidenced for drug design. The computational pkCSM tool (http://biosig.unimelb.edu.au/pkcsm/prediction) was used to conduct ADMET studies. The molecules were fed in the canonical SMILE format for calculating the ADMET properties.

Ligand protein docking

Docking simulation was performed using Autodock4 (version 4.2.8). A scoring function based on energy is used to rank the receptor-ligand poses obtained during docking calculations. The best-docked confirmation was then visualized using the Discovery studio visualizer (BIOVIA), and the docking site, binding interaction, and bond length were identified.

RESULTS AND DISCUSSION

The present study is of great significance as it is the first to report *in silico* findings of *H. auriculata* compounds with the target proteins.

LCMS

The bioactive compounds of the *Hygrophila auriculata* root were qualitatively characterized by LCMS analysis in both positive (+) and negative (-) modes. In the present study, the protonated [M+H] + and deprotonated [M−H] − molecules of various phytoconstituents were revealed in the positive and negative ion modes (Figure 1). The phyto compounds are listed in Table 1. The identified compounds belong to secondary metabolites like terpenoids, alkaloids, aliphatic compounds, and phenolics.

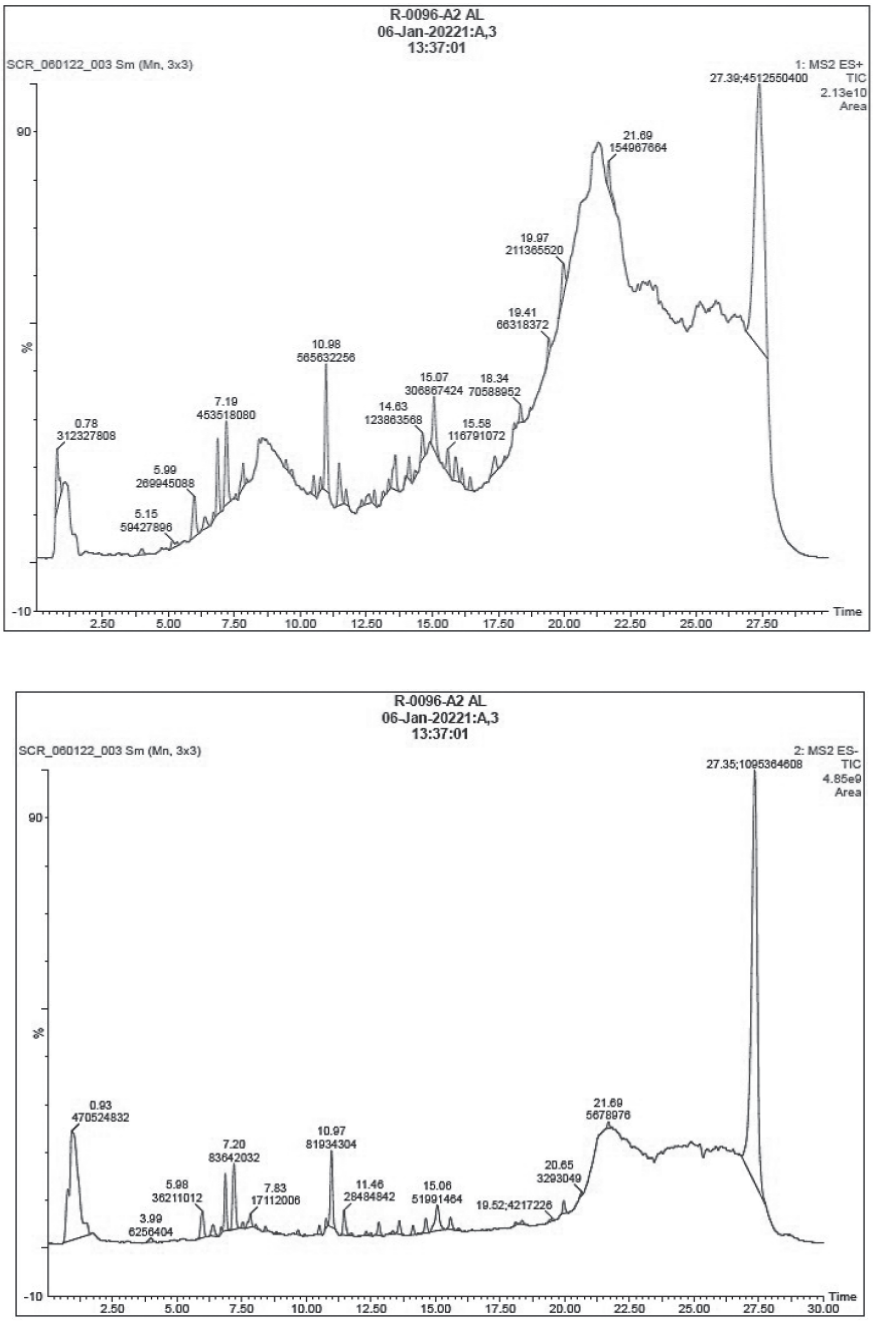


Fig. 1: LCMS Chromatogram of *Hygrophila auriculata* in both positive and negative mode

Table 1: The significant compounds of *Hygrophila auriculata* analyzed using LCMS

Compound name	Molecular formula	Molecular weight	m/z	Mode (+/-)
Caffeic anhydride	$C_{15}H_{18}O_9$	342	341.12	-
Quercetin	$C_{15}H_{10}O_7$	302.24	301.03	-
Myristic acid	$C_{14}H_{28}O_2$	228.37	227.37	-
Palmitic acid	$C_{16}H_{32}O_2$	256.42	255.70	-
1,3-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.4	515.05	-
Coumaroyl quinic acid	$C_{16}H_{18}O_8$	338.31	337.12; 337.52	-
Epiafzelechin	$C_{15}H_{14}O_5$	274.27	273.78	-
Kaempferol-7-O-Glucoside	$C_{21}H_{20}O_{11}$	447.4	446.76	-
Betulin	$C_{30}H_{50}O_2$	442.72	443.45	+
Linoleic acid	$C_{18}H_{32}O_2$	280.45	281.13	+
Luteolin 7-O-rutinoside	$C_{27}H_{30}O_{16}$	610.52	611.36	+
4-Feruloyl-5- caffeoylquinic acid	$C_{26}H_{26}O_{12}$	530.5	531.99	+
Chlorogenic acid	$C_{16}H_{18}O_9$	354.3	355.67	+
Myricetin-3-O-hexoside	$C_{21}H_{20}O_{13}$	480.4	481.24	+
Apigenin-6-C-glucoside 8-C-arabinoside	$C_{26}H_{28}O_{14}$	564.49	563.11	-

Drug likeliness and Physicochemical properties

The physicochemical property of the compounds influences their biological effects through pharmacokinetic and pharmacodynamic features. The drug likeness properties of the 15 compounds were assessed by using Swiss ADME. Out of 15 molecules, only 10 molecules were found to satisfy drug-like properties based on Lipinski's rule of five. Lipinski's rule of five defines a molecule as drug like only if the molar weight (MW) is less than 500 Daltons (Da); the logarithm of the octanol/water partition coefficient (Q PlogPo/w) is less than 5, the number of hydrogen bond acceptors (HBA) less than10 and the number of hydrogen bond donors (HBD) less than 5. The distributions of the compound MW, log P, HBA and HBD were calculated and used to assess the likely drug like nature of the compounds derived from *H. auriculata* root (Table 2). In the present study except the compounds such as Luteolin 7-O-rutinoside, 4 Feruloyl-5-caffeoylquinic acid, Myricetin-3-O-hexoside, Apigenin-6-C-glucoside 8C-arabinoside and 1,3-Dicaffeoylquinic acid all other compounds expressed to be highly drug-like.

Table 2: Drug likeliness and physicochemical properties of the ligands chosen for docking

S. No	Molecule	Molecular formula	MW g/mol	HBA	HBD	LOG P	Lipinski violation
1.	Caffeic anhydride	$C_{15}H_{18}O_9$	342	7	4	2.04	0
2.	Quercetin	$C_{15}H_{10}O_7$	302.23	7	5	1.23	0
3.	Myristic acid	$C_{14}H_{28}O_2$	228.37	2	1	4.45	0
4.	Palmitic acid	$C_{16}H_{32}O_2$	256.42	2	1	4.20	0
5.	Coumaroyl quinic acid	$C_{16}H_{18}O_8$	338.31	8	5	0.05	0
6.	Epiafzelechin	$C_{15}H_{14}O_5$	274.27	5	4	1.20	0
7.	Kaempferol-7-O- Glucoside	$C_{21}H_{20}O_{11}$	447.4	11	5	0.13	0
8.	Betulin	$C_{30}H_{50}O_2$	442.72	2	2	6.39	0
9.	Linoleic acid	$C_{18}H_{32}O_2$	280.45	2	1	4.8	0
10.	Chlorogenic acid	$C_{16}H_{18}O_9$	354.3	9	5	-0.39	0
11.	Luteolin 7-O- rutinoside	$C_{27}H_{30}O_{16}$	610.52	10	3	2.39	1
12.	4 Feruloyl-5- caffeoylquinic acid	$C_{26}H_{26}O_{12}$	530.5	12	6	1.32	3
13.	Myricetin-3-O- hexoside	$C_{21}H_{20}O_{13}$	480.4	13	9	-0.96	2
14.	Apigenin-6-C- glucoside 8C-arabinoside	$C_{26}H_{28}O_{14}$	564.49	14	10	-1.54	2
15.	1,3-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.4	12	7	0.83	3

Lipophilicity comprises parameters such as XLOGP3, WLOGP, and MLOGP. The partition coefficient P is considered the essential physical property evaluated as one of the standards in nearly all drug-likeness indices. The LOGP of a given molecule between n-octanol and water system is a quantitative descriptor of Lipophilicity. N-octanol is a good mimic of phospholipid membrane properties due to its amphiphilic nature. In the lipophilicity analysis, the compounds that fall within the acceptable range are epiafzelechin, betulin, caffeic acid 3-glucoside, quercetin, palmitic acid, linoleic acid, chlorogenic acid, myristic acid, and 5-p-coumaroyl quinic acid implying to be a cell-permeable and orally active drug.

ADMET properties

These selected compounds were further evaluated for their drug-like behaviour through analysis of pharmacokinetic parameters required for absorption, distribution, metabolism, excretion and toxicity (ADMET) by using PKCSM (Table 3).

The predicted values of the active compounds of H. auriculata root were compared with the normal range of predicted parameters of ADMET. Overall, all the compounds showed a significant ADMET properties that enable every compound to be defined as drug-like and non-toxic (Table 3).

Table. 3 Prediction ADMET profile of the selected compounds

Pharmacokinetic properties		C 1	C 2	C 3	C 4	C 5	C 6	C7	C8	C9	C10	Unit
Absorption	Water solubility	-3.1	-2.95	-4.95	-5.56	-2.96	-2.96	-3.25	-2.55	-5.44	-5.86	log mol/L
	Human intestinal Absorption (HIA)	67.34	77.20	92.69	92.01	30.30	27.57	91.48	32.93	94.53	92.32	% Absorbed
	Skin permeability	-2.73	-2.73	-2.70	2.711	-2.73	-2.73	-2.73	-2.73	-2.7	-2.7	Log Kp
	P-glycoprotein Substrate	N	N	N	N	N	N	N	N	N	N	Yes/No
	BBB permeability	0.11	1.09	-0.02	-0.11	-1.98	-1.34	-0.81	-1.35	-0.29	-0.14	log BB
	CNS permeability	-1.11	-1.06	1.92	-1.81	-0.84	-1.62	-1.74	1.90	-1.03	-1.6	log PS
Metabolism	CYP2D6 substrate	Yes	Yes	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes/No
	CYP3A4 substrate	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes/No
	CYP2D6 inhibitor	No	No	No	No	No	Yes	Yes	No	No	No	Yes/No
	CYP3A4 inhibitor	No	No	No	Yes	Yes	Yes	Yes	No	No	No	Yes/No
Excretion	Total clearance	0.11	0.40	1.69	1.76	-0.05	0.44	0.25	0.56	0.23	1.933	log ml/ min/kg
	Renal OCT2 substrate	Yes	Yes	No	No	No	No	Yes	No	Yes	No	Yes/No

Toxicity	AMES toxicity	No	No	No	No	No	No	No	No	No	No	Yes/No
	Maximum tolerated dose (Human)	-0.2	0.49	0.55	0.70	0.56	0.48	0.13	0.51	0.79	-0.8	log mg/kg/ day
	hERG inhibitor	No	No	No	No	No	No	No	No	No		Yes/No
	Oral rat acute toxicity (LD50)	2.17	2.47	1.47	1.44	2.56	1.75	2.36	2.59	2.69	1.4	mol/kg
	Hepatotoxicity	No	No	No	No	No	No	No	No	No	No	Yes/No
	Skin sensitivity	No	No	No	No	No	No	No	No	No	No	Yes/No

C1-Caffeic anhydride; C2-Quercetin; C3-Myristic acid; C4-Palmitic acid; C5- Chlorogenic acid; C6- Coumaroyl quinic acid; C7-Epiafzelechin; C8-Kaempferol-7-O-Glucoside; C9-Betulin; C10-Linoleic acid

A favorable ADMET profile is necessary for the molecules in new drug discovery. The adsorption properties predict whether the compound is adsorbed efficiently by oral or intestinal administration and its water solubility. Caco-2 cells have been utilized generally as an *in vitro* model to study absorption efficiency through epithelial barriers related to cellular transport in the body due to their resemblance to small intestine epithelial cells. A drug is expected to be permeable when the permeability of Caco-2 cells is greater than 8×10^{-6}. Similarly, the phytocompounds in the present study are highly permeable to Caco-2 cells and had high HIA in the range of 77.207 to 81.13 percent, implying that they all tend to be easily absorbed in the gut. Also, the Log Kp of all the screened phytocompounds were less than -2.5, revealing their high skin permeability. The P-glycoprotein extrudes toxins from the cells. From this analysis, quercetin and Epiafzelechin were found to be substrates of P-glycoprotein. The other compounds were inhibitors of P-glycoprotein. These compounds could inhibit the P-glycoproteins when overexpressed in the cell surfaces and prevent the excessive efflux of drugs.

In the distribution analysis, the blood-brain barrier blocks molecules from entering the central nervous system, allowing only water and lipid-soluble and selective transport molecules like plasma glycoprotein and glucose transporters to pass through. In this study, quercetin was anticipated to cross the blood-brain barrier (BBB) as they had log BB > 0.3 and were permeable to CNS.

In this study, CYP2D6 and CYP3A4, the isoforms of CYP450, were utilized for metabolism evaluation. Cytochrome P450 is a detoxifying enzyme that aids xenobiotics' metabolism by oxidizing and facilitating detoxification. In the metabolism analysis, the compounds were examined to determine whether they were inhibitors or substrates of CYP2D6 and CYP3A4. Except the compounds C3, C5, C6, C7 and C9 were substrates of both CYP2D6 and CYP3A4. Hence, these compounds were predicted to be effectively metabolized in the liver.

Total clearance is an essential pharmacokinetic characteristic affecting a drug's metabolism and excretion since it regulates its half-life, bioavailability, dosage concentrations, and frequency. In the excretion, the organic cation transporter substrate was analyzed. All the reported phytocompounds had significant total clearance values and renal clearance by renal OCT2 substrate.

Further, in the toxicity prediction, the toxic nature of all the ligands using various tests was analyzed. All the drug candidates in the present study were predicted to be non-toxic by the AMES toxicity test, Hepatotoxicity test and not sensitive to skin. Maximum tolerated dose (Human), Oral rat

acute toxicity (LD50), and Oral rat chronic toxicity (LOAEL) doses were given in the ADMET analysis table. The hERG potassium channel (human ether-a-go-go-related gene) tends to be the biomarker responsible for the cardiotoxicity of a broad range of clinical medications. It is actively involved in cardiac activity, which regulates the heartbeat. Blocking the hERG potassium channel in humans leads to QT interval prolongation and major cardiovascular complications, a significant issue in pharmacological trials. All compounds tested for hERG activity act as a non-inhibitor of hERG, lowering the risk of cardiovascular complications. Overall toxicity analysis revealed that all the screened phytocompounds were noncarcinogenic to mice, had less lethality, and were non-hepatotoxic.

The ADMET analysis supports the above-discussed molecular docking results where the compound betulin showed significant binding affinity towards the target proteins, causing wound healing. Further, *in vitro* studies, clinical trials, and various advanced techniques could be adopted to analyze the drug development from the above studied compounds.

Bioactivity score

The bioactivity scores were calculated using a signaling cascade that included GPCR ligands, ion channel modulators, protein kinase inhibitors, nuclear receptor ligands, and protease inhibitor ligands. GPCR ligand-based signaling cascade was used to develop a new functional drug with an increased binding selectivity profile and fewer undesirable effects. Ion channel modulators allow the movement of charged particles across cell membranes and are essential therapeutic targets modulated by various medicinal drugs. For developing selective inhibitors to block or modulate diseased signaling pathways, Kinase inhibitors are considered a promising approach for drug development. Following the physicochemical properties, the bioactive scores were evaluated. The scores fall in the following ranges: active > 0, moderately active −5.0 - 0.0, and inactive < -5.0. The bioactivity analysis revealed that all the compounds were highly bioactive except myristic acid (Table 4).

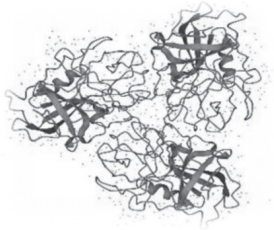
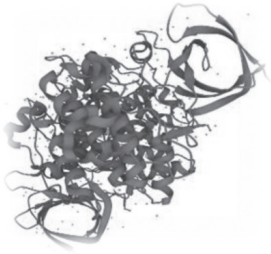
Table 4: Bioactivity score of the selected ligands

S. No	Compounds	GPCR Receptor	Ion channel modulator	Protein Kinase inhibitor	Nuclear receptor	Protease Inhibitor	Enzyme inhibitor
1	Caffeic anhydride	0.17	0.07	0.22	0.17	0.07	0.22
2	Myristic acid	-0.02	-0.07	-0.02	-0.07	-0.02	-0.07
3	Chlorogenic acid	-0.11	0.03	-0.51	-0.06	-0.19	0.13
4	Coumaroyl quinic acid	0.04	0.01	0.10	0.19	0.03	0.16
5	Betulin	0.77	0.26	0.88	0.52	0.94	0.17
6	Quercetin	0.21	0.04	0.41	0.85	0.09	0.51
7	Palmitic acid	0.06	0.19	0.28	0.36	0.25	0.28
8	Epiafzelechin	0.03	0.47	0.08	0.15	0.02	0.14
9	Kaempferol-7-O-Glucoside	0.12	0.08	0.52	0.74	0.02	0.53
10	Linoleic acid	0.14	0.04	0.51	0.73	0.07	0.51

Target proteins

Four target proteins were retrieved from the RCSB PDB database, and their structures and functions are depicted in Table 5.

Table 5: Target wound pathogenic proteins with their characteristics and structure

Name of the protein	PDB code	Role of the protein	3D Structure of the protein
Elastase	1HNE	Degrades elastin which provides strength and recoil to the skin due to oxidative stress in the wound site	
Glycogen synthase kinase-3β (GSK-3β)	1Q5K	GSK-3 phosphorylates -Catenin destabilizes and blocks the Wnt pathway, where the catenin is activated in mesenchymal cells during the proliferative phase of wound healing.	
Gelatinase	1QIB	Reduces the keratinocyte migration in wound	
Collagenase	2Y6I	Modulation of collagenase expression can affect the efficiency of re-epithelialization in circular excisional wounds	

Molecular docking

After the preliminary investigations for druglike properties, all the ligands were subjected to docking with the target proteins. Totally 40 *in silico* docking analyses were performed. The present computational study aims to find an efficient drug candidate from the *H. auriculata* root which can inhibit the target proteins responsible for the pathogenesis of the wound. The binding energy, which reflects the binding affinity of the selected compounds with the target proteins, is shown in Table 6. Among the docked complexes, compounds such as betulin (-9.65 kcal/mol), chlorogenic acid (9.12 kcal/mol), Kaempferol-7-O-Glucoside (-8.19 kcal/mol), Linoleic acid (-7.96 kcal/mol), and Epiafzelechin (6.46 kcal/mol) showed good binding affinity towards the target proteins (Table 6).

Table 6: Binding energy scores of docked complexes

S. No	Compound name	Binding energy (Kcal/mol) against wound-healing proteins			
		1HNE Elastase	2Y6I Collagenase	1Q5K GSK	1QIB Gelatinase
1	Caffeic anhydride	-2.32	-1.55	-3.71	-2.05
2	Epiafzelechin	-6.46	-5.61	-6.62	-5.86
3	Coumaroyl quinic acid	-2.69	-4.75	-5.42	-5.23
4	Palmitic acid	-4.42	-2.43	-4.89	-4.22
5	Betulin	-6.48	-9.65	-8.11	-7.90
6	Quercetin	-3.89	-4.96	-6.12	-5.63
7	Myristic acid	-1.61	-1.56	-2.09	-1.75
8	Kaempferol-7-O-Glucoside	-6.19	-7.48	-8.19	-8.05
9	Linoleic acid	-6.39	-7.32	-6.54	-7.96
10	Chlorogenic acid	-6.45	-7.18	-9.12	-7.90

The binding pattern analysis between the protein and the chosen ligands varies according to the nature of the ligand. In the molecular docking analyses, the highest negative value indicates the strongest binding affinity. A protein-ligand interaction will be effective only when the ligand fits proactively into the receptor pockets. The best docking interaction of elastase was found to be with betulin and epiafzelechin (-6.48 kcal/mol; 1 H-bond and -6.46 kcal/mol; 2 H-bond, respectively). Likewise, compared to other compounds, collagenase was docked well with betulin and kaempferol-7-O-Glucoside. The docking profile of the collagenase complex shows two H-bond interactions with betulin and five H-Bond interactions with kaempferol-7-O-glucoside and had the highest binding energy of -9.65 kcal/mol and -7.48 kcal/mol, respectively. In the docking profile of glycogen synthase, kinase-3β protein, chlorogenic acid, and

kaempferol-7-O-glucoside showed better binding affinity (-9.12 kcal/mol; 5 H-bond and -8.19 kcal/mol; 2 H-bond respectively).

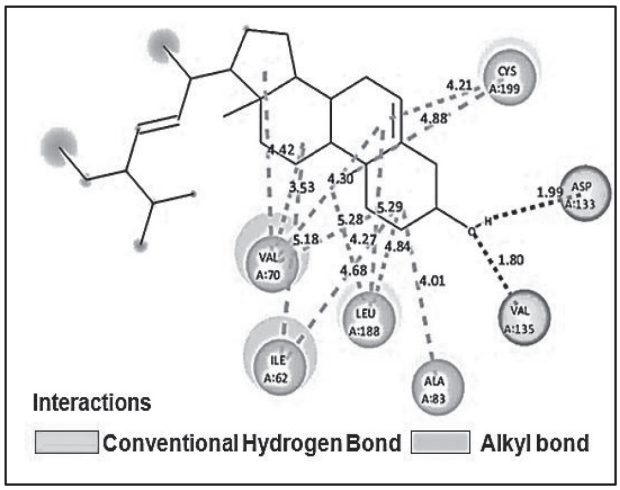
The protein gelatinase also possessed the best docking interactions with kaempferol-7-O-glucoside and linoleic acid, followed by this. These complexes, gelatinase-kaempferol-7-O-glucoside, and gelatinase-linoleic acid, had an excellent binding affinity with a binding energy of -8.05 kcal/mol and -7.96 kcal/mol, where kaempferol-7-O-glucoside interacted with 6 H-bonds and linoleic acid interacted with alkyl and nonbonded interactions. The 2D interactions were depicted using Discovery studio and presented in Figure 2.

During wound formation, the intracellular signaling pathway changes, which leads to the destruction of the extracellular matrix (ECM), collagen degradation, and induction of *matrix metalloproteinases* (MMPs). The elevated MMPs delay wound healing by degrading the newly formed ECM in the wound site. MMP may be triggered by oxidative stress and inflammatory responses generated by wounds. Hence, the present docking work deals with the interaction of the bioactive compounds of *H. auriculata* against MMPs, which catalyses the ECM degradation pathway and delays wound healing.

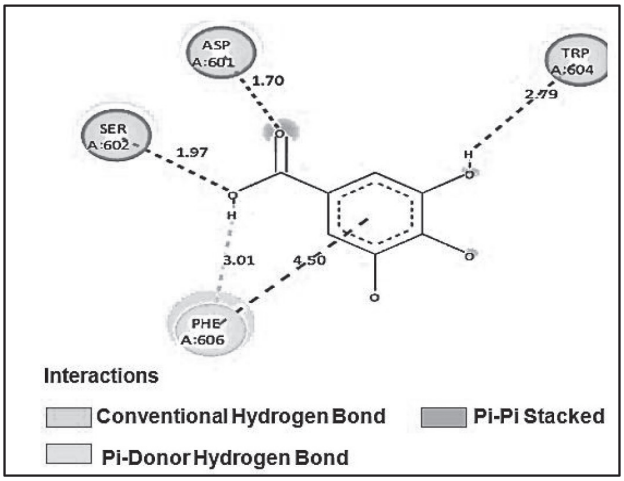
In the present study, the ligands epiafzelechin, coumaroyl quinic acid, quercetin, betulin, palmitic acid, kaempferol-7-O-Glucoside, linoleic acid, and chlorogenic acid exhibited the maximum binding energy of above -4 kcal/mol with collagenase, gelatinase, and elastase. Hence, we speculate that these compounds accelerate the wound healing process by inhibiting the MMPs, thereby increasing the amount of gelatin, collagen, elastin, and fibronectin in the wound site and minimizing platelet aggregation and pro-inflammatory factors. Since the collagenase deactivation at the wound site slows down the function of chemokines that reduces inflammation and enhances the healing process. Following that, the overexpression of mettaloelastase enzymes stimulates the influx of inflammatory cells and affects the wound-healing process. Hence, inhibition of matrix metalloproteinase enzyme is crucial in tissue repair.

Also, it can be evidenced from the molecular docking that the ligands Betulin and Kaempferol-7-O-Glucoside had a better binding affinity with GSK 3β. Hence, these ligands are expected to promote cutaneous wound healing by eliciting the beta-catenin-dependent Wnt pathway by inhibiting the GSK 3β protein.

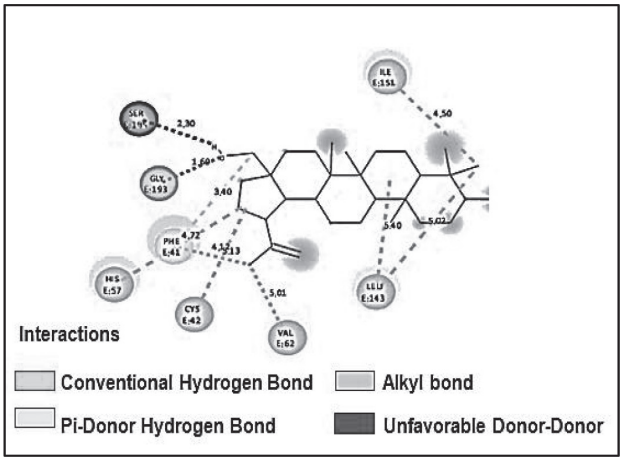
The triterpenoid compound betulin showed a high affinity with all the target proteins among other selected compounds, which may increase cell proliferation, and promote healing. Hence, from the present computational analysis, it could be stated that the strong thermodynamic of the compound betulin can be recommended for further *in vitro* and *in vivo* research for its wound healing activities.



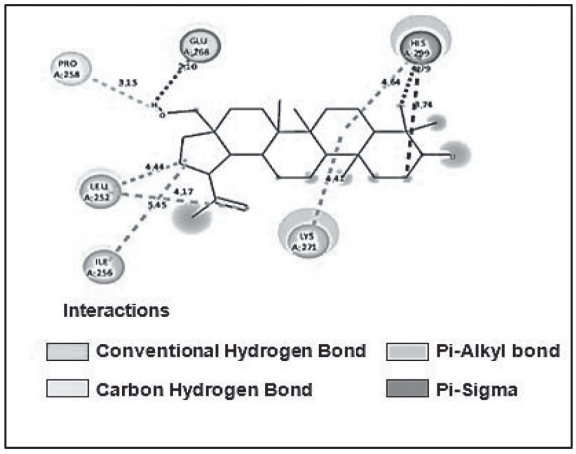
Epiafzelechin with Elastase



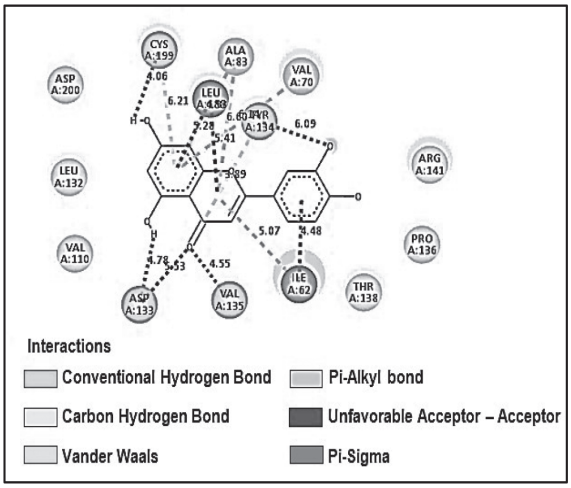
Chlorogenic acid with Glycogen synthase kinase-3



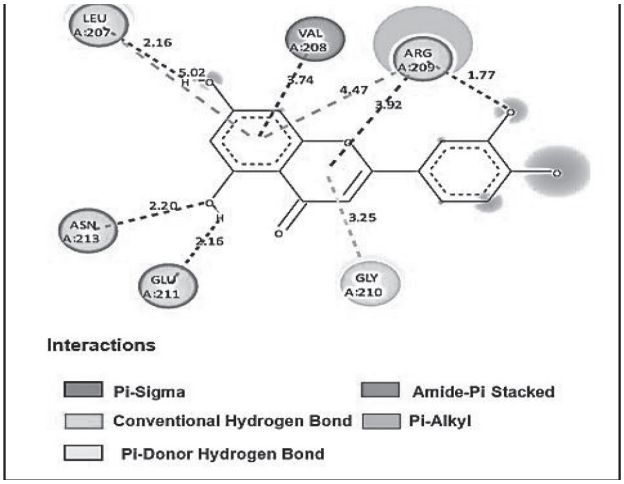
Betulin with Elastase



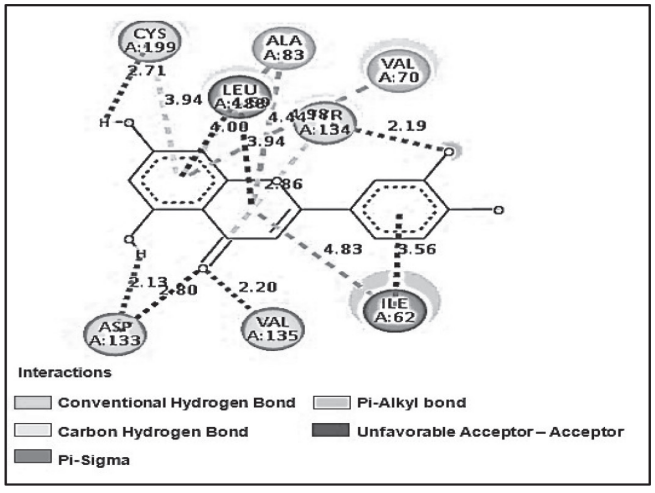
Betulin with Collagenase



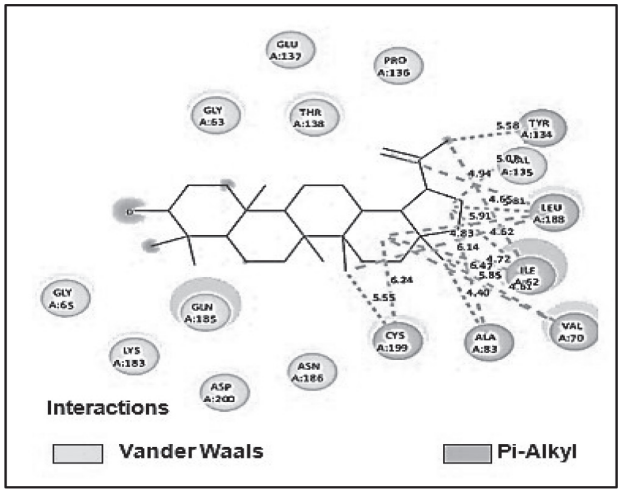
Kaempferol-7-O-Glucoside with Glycogen synthase kinase-3β



Kaempferol-7-O-Glucoside with Collagenase



Kaempferol-7-O-Glucoside with -Gelatinase



Linoleic acid with gelatinase

Fig. 2: 2D interactions of the best-docked complexes

CONCLUSION

The bioactive compounds from *H. auriculata* were utilized in this experiment to analyse the drug development process for wound healing. Several parameters like physicochemical properties, Drug likeliness, bioactivity score, and ADMET properties of the selected compounds revealed significant results supporting its healing ability. The molecular docking study demonstrated that the compound betulin has a high binding affinity toward all three MMPs and glycogen synthase kinase-3β. Hence, the present study reflects that roots of *H. auriculata* possesses significant wound-healing properties against various proteins responsible for the pathogenesis of the wound. The above findings illustrate a valid *in silico* research which would raise those phytochemicals as potential drug candidates in wound healing and pave the way for drug development. Furthermore, this could be explored to divulge the mechanism of action of potential wound-healing drugs.

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Declaration of interest

The authors have no competing interests to declare relevant to this article's content.

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Author's contributions

Santhy KS- Providing research guidance, constructing the study, and revising the manuscript. Deepika E-Performed data analysis, docking, and drafted the manuscript.

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Introduction

Over the course of millennia, the intricate evolution of our skin has yielded a remarkably adaptive and multifunctional organ, serving as a formidable barrier against the daily barrage of challenges posed by chemical, physical, and ultraviolet radiation. Any infringement upon the integrity of living tissue is designated as a wound. Such wounds manifest when the protective epidermal layer of the skin is breached, exposing the underlying dermis to the external environment. Depending on the depth and extent of the skin damage, the exposed tissues may range from blood vessels to bone. Consequently, wounds are broadly categorized into three classifications. A surface-level injury specifically involves damage confined to the outermost layer of the skin, known as the epidermis. In contrast, a wound of partial thickness extends into the deeper layers of the dermis, affecting structures such as blood vessels, sweat glands, and hair follicles. In the case of a full-thickness wound, the underlying subcutaneous fat or deeper tissues become compromised.

The challenging external conditions frequently expose our skin to injuries and therefore, it is anticipated that our skin is endowed with sophisticated reparative mechanisms facilitating swift and efficient healing. The wound healing process unfolds as a meticulously orchestrated cascade of events, each phase seamlessly interacting with the next. These phases include coagulation, immune response and inflammation, proliferation, and remodelling. The healing of cutaneous wounds is a crucial physiological process that requires coordinated actions from different cell types and their substances. The commencement of the restoration process for injuries resulting from local damage occurs in the early stages of inflammation, ultimately progressing towards repair and regeneration.

Repair involves the substitution of specialized structures through collagen deposition, while regeneration entails the proliferation and subsequent differentiation of cells within the tissue and/or stem cells. Disruption of this highly regulated healing process results in the cessation of healing, leading to the development of chronic wounds. Addressing the intricate symptoms that emerge from the metabolic disorder in the wound microenvironment is a significant medical need for effectively treating chronic wounds. This requirement remains substantial but yet un fulfilled in the field of comprehensive wound care.

Comparative Evaluation of Liposome Encapsulated Hygrophila auriculata (Schumach.) Heine Root and Betulin on Wound Healing Activity through In silico, In vitro and In vivo approaches

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