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ISOLATION OF EMBELIN FROM *Embeliaribes* BERRIES FOR THE DEVELOPMENT OF TOPICAL ANTI–INFLAMMATORY PREPARATION

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Abstract

Purpose: Embelin is a natural benzoquinone compound, isolated from *Embeliaribes* berries, known extensively for its anti-inflammatory properties. Due to its hydrophobic nature, it was less explored for topical preparation. Therefore, the purpose of this study was to explore the potential of embelin as an anti-inflammatory agent for topical preparations as well as assess its potential to replace synthetic drugs, which can be resistant to bacterial strains. A topical preparation with embelin would be potentially more effective as well as safer than synthetic drugs. Method: Embelin has been converted into its potassium salt, and a cream was developed, which was then subjected to physiochemical evaluation. The isolated Embelin and Embelin Potassium salts were characterised by various spectral analysis. The in vitro anti-inflammatory activity was determined by the heat-induced hemolysis and albumin denaturation. Docking scores of embelin and diclofenac were compared and the drug content per gram of cream was estimated. Result: The UV spectral absorption peak of Embelin was determined to be at 274 nm, while that of the potassium salt of Embelin was at 515 nm. The IR bands were found at 1328.951cm-1,361.74 cm-1, 1616.35 cm -1,1616.35 cm -1 ,1452.4 cm -1, 748.28 cm -1 and 2947.23 cm -1 respectively due to phenolic OH bending, C=O stretching, aromatic ring bending, methylene bending and methyl stretching . The IR spectra of Embelin potassium salt exhibited similar pattern as that of Embelin but with shifted band due to presence of potassium salt. Heat-induced hemolysis using diclofenac as standard and embelin potassium salt as test showed 98.01% inhibition and 92% inhibition, respectively, at50µg/ml. The binding affinity of diclofenac was determined to be -4.9, while the binding affinity of embelin was -5.7, indicating a higher affinity for embelin. Lastly, the drug content per gram of cream was found to be 95%, indicating the amount of active drug (embelin or embelin potassium salt) present in the cream formulation. Conclusion: Embelin was isolated from *Embeliaribes* berries and formulated into a herbal cream. The in-vitro studies conducted on the cream showed that it has a significant effect on inflammation. However, in–vivo studies are required for the prepared topical cream to confirm its effectiveness.

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Introduction

Medicinal plants also called medicinal herbs, have been used for centuries. *Embeliaribes* Burn, which is widely known as Vidanga, Vaividang, and Vavding, is a potential herb with a wide range of therapeutic applications. These are species found in the Primulaceae family. It was first described by Nicolas Laurens Burman in his 1768 publication, Flora indica. This is widely used in most of the ayurvedic formulations for the treatment of various ailments, and it is available in various ayurvedic formulations such as asava, arista, loha, and thaila. It is listed in the Red Book as a threatened species. The various studies found that the fruits of the plants are used as an anthelminthic, carminative, diuretic, contraceptive, antibacterial, anti-inflammatory, astringent, antioxidant, and anticancer agent, and the seeds have antibiotic1 and anti-tubercular properties [1-10]. Inflammation is an adaptive defence mechanism initiated by the immune system in response to adverse events such as infection, allergens, and

tissue damage. In recent years. Non-steroidal anti-inflammatory drugs (NSAIDS) have been the first choice of drug in the classic treatment of inflammatory diseases [11]. However, NSAIDS are frequently associated with cardiovascular, renal, gastrointestinal and haematological side effects that demand a better and safer alternative [12-16]. Embelin, [Fig1] a bioactive molecule isolated from dried berries of *Embeliaribes berries, has* been selected for the development of anti-inflammatory formulations as there is evidence to support the use of these in treating inflammation, as studies have shown. Embelin has been found to inhibit the activity of two enzymes, 5-lipoxygenase (5-LO) and microsomal prostaglandin E synthase-1 (mPGES-1), which are responsible for the production of pro-inflammatory molecules. Furthermore, Embelin act as an inhibitor of the NF-κB and MAPK pathways, two pathways responsible for regulating the production of cytokines that are involved in inflammation [17-19].The in vivo anti-inflammatory potential of embelin has been demonstrated in a number of animal studies [20-22]. Molecular docking is a powerful tool that allows scientists to predict the interactions between a ligand and a receptor as well as the relative affinity of those interactions. This helps to identify the most promising candidate molecules for use in drug discovery as well as gain insight into the molecular mechanisms of drug action. This method provides a more accurate comparison of the anti-inflammatory effects of two compounds than traditional methods. Additionally,docking technique predicts tentative binding parameters of the ligand receptor[23-24].

Taking into account the considerable low oral bioavailability of embelin[25-26],topical cream formulationis the most effective means of drug delivery because they are non-greasy and absorb quickly into the skin, allowing the active ingredients to penetrate deeper into the skin and produce the desired pharmacological effect. They are also more pleasant to use as they leave the skin feeling softer and smoother compared to ointments and other topical medications. Additionally, creams are thicker than lotions, which helps lock in moisture while allowing the skin to breathe. This makes them more effective at preventing dehydration and restoring the suppleness of the skin [27-29].

The purpose of the research is to formulate an embelin based anti-inflammatory cream. The isolated embelin (Fig1) was first converted into its potassium salt, and then both were characterised by spectral analysis. In vitro Anti-inflammatory activity was determined by heat-induced hemolysis using diclofenac as a standard along with albumin denaturation. The potassium salt of Embelin is water-soluble, which allows it to be incorporated into an herbal cream, followed by its physiochemical evaluation. In addition, molecular docking studies were also carried out using PyRx software on the active site of the oxidoreductase enzyme (PDB-1NR6). Through the study, it was determined that embelin had a higher docking score than standard diclofenac, indicating that it could potentially have a stronger anti-inflammatory effect. Thus, embelin-based preparations can be an alternative to synthetic topical preparations with significant adverse effects.

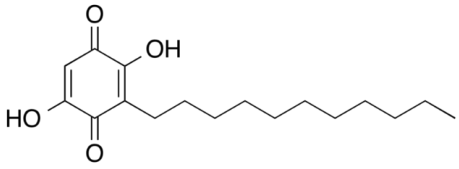


Fig 1:Chemical structure of Embelin[30]

Embelin [CAS: 550–24–3; IUPAC: 2,5–Dihydroxy–3–undecylcyclohexa–2,5–diene–1,4–dione]

Materials and Methods

Materials and Equipments

Embelin procured from YUCCA enterprises with certificate of analysis having the purity of 98%. All the chemicals and reagents were obtained from the store of Nirmala College of Pharmacy and were of analytical grade: n–hexane, chloroform, petroleum ether, dichloromethane, sodium citrate, citric acid,sodium chloride, dextrose, bovine albumin, stearic acid, glycerine, potassium hydroxide, tri-ethanol amine, formic acid, ethyl acetate, acetic acid, carbopol 934.

Methods

Isolation of Embelinand Prelimary Phytochemical Screening

The pure embelin was extracted from *Embeliaribes* berries based on the method described by Suthar et al [31]. The dried Embeliaribes berries were ground into a coarse powder. The dried powder (250g) was subjected to extraction with n-hexane for 6 hours using a Soxhlet apparatus (Fig2). The solvent was distilled out of the mixture, resulting in a residue. The residue was then washed with cold petroleum ether to remove impurities. The purified residue was dissolved in a mixture of methanol and dichloromethane (DCM). The solution was left to crystallize for 24 hours, allowing the formation of embelin crystals. The crystals were filtered from the mother solution and washed with DCM, followed by n-hexane, to further purify them. To confirm the presence of embelin in both the raw material and the obtained extract, a specific test was performed using n-hexane and dilute ammonia. The obtained golden-coloured embelin crystals were further characterized using various methods, including organoleptic properties such as appearance, colour, and consistency, determination of melting point, chemical tests, thin-layer chromatography (TLC), and solubility tests[32]. These analyses provided additional information about the properties and identity of the embelin crystals .An initial phytochemical screening was conducted on the isolated embelin crystals. Standard procedures were used to test for the presence of alkaloids, steroids, glycosides, and proteins [33-34].

Figure–2: Extraction of embelin

Preparation of Embelin Potassium Salt

Accurately measured 0.1 g of embelin. Added 0.37 g of potassium hydroxide pellets to the embelin. The mixture of embelin and potassium hydroxide was heated with constant stirring. The heating process was continued for a few minutes until the formation of grape wine-coloured embelin potassium salt [Fig 3]

Figure–3: Embelin & Prepared Embelin Potassium salt

Physiochemical and Spectral Characterization of Embelin Potassium Salt

Physiochemical and spectral characterization of embelin potassium salt is typically conducted to determine its physical and chemical properties [32]. These characterization techniques help in determining the physicochemical properties, molecular structure, and overall identity of embelin potassium salt

Solubility

The procedure for determining solubility involves adding a known amount of Embelin potassium salt to various solvents and stirring the mixture until the compound is completely dissolved. If the compound dissolves completely, it is considered soluble in that particular solvent. If it does not dissolve or only partially dissolves, it is considered insoluble or sparingly soluble in that solvent.

Melting Point

Melting point determination of a substance, such as embelin potassium salt, was carried out using an open capillary tube on a melting point apparatus. The obtained melting points are typically reported as uncorrected values. The process involves filling a small amount of the substance into an open capillary tube, ensuring it is tightly packed. The capillary tube is then placed in the melting point apparatus, where it is heated gradually until the substance melts. The temperature at which the substance begins to melt and fully liquefies is recorded as the melting point.

Thin Layer Chromatography

1 mg of embelin potassium salt was dissolved in 10 mL of dichloromethane (DCM) to prepare the sample solution. A pre-coated silica gel G aluminium plate was used as the stationary phase. The plate was prepared beforehand according to standard protocols. 10 µL of the prepared embelin potassium salt sample solution was spotted onto the TLC plate. This spot represents the sample. The mobile phase used was a mixture of ethyl acetate, toluene, methanol, and formic acid in a ratio of 0.5:0.4:0.1:0.05, respectively. This solvent system was carefully prepared. The TLC plate, with the spotted sample, was placed in a developing chamber. The chamber was saturated with the mobile phase vapor before inserting the plate. The plate was then allowed to develop, with the solvent moving up the plate by capillary action. After the development was complete, the TLC plate was removed from the chamber and allowed to dry.

The distance travelled by the compound spot (Embelin potassium salt) and the distance travelled by the solvent front were measured. The ratio of these distances is known as the Rf (retention factor) value. The Rf value obtained for the Embelin potassium salt spot on the TLC plate was then compared with the Rf value of a standard compound run under the same conditions. This comparison helps to confirm the identity of the compound**.** Rf value is calculated as

$$R_f \text{ value} = \frac{\text{Distance travelled by solute from the point of origin}}{\text{Distance travelled by solvent from the point of origin}}$$

UV Spectroscopy

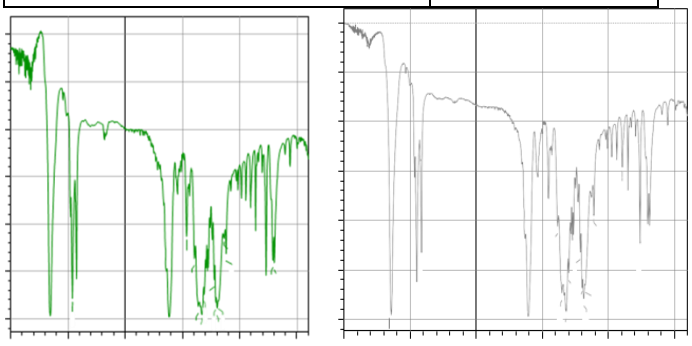
UV spectroscopy is a valuable analytical technique used to identify compounds based on their characteristic absorbance patterns. To identify embelin potassium salt using UV spectroscopy, a series of concentrations of the compound are prepared in distilled water, and the absorbance of each concentration is measured at a specific wavelength, such as 560 nm

IR Spectroscopy

IR spectroscopy is employed to study the functional groups present in embelin potassium salt**.** The technique utilized in this study was the potassium bromide (KBr) disk method, where a small quantity of drug powder was combined with spectroscopic KBr and compressed under vacuum conditions to produce a disk .The Infrared spectrum was then captured by scanning across a range of wave numbers from 400 to 4000 cm-1 using Nicolet Omnic-1 software. Table 1 provides the infrared[IR] range of embelin. The distinctive infrared spectrum and its corresponding peaks were observed and compared to the reference spectrum of embelin.[Fig 4]

Table–1: IR range of Embelin

Groups	Embelin
Phenolic	1410–1310
Ketone	1650–1600
Aromatic ring stretch	1615–1580
Methylene	1485–1445
Methyl	2970–2950



Figure–4: IR Spectra of Standard Embelin & Isolated Embelin

In–Vitro Anti–Inflammatory Activity of Embelin Potassium Salt

In order to assess the in-vitro anti-inflammatory properties of embelin potassium salt, heat-induced hemolysis and albumin denaturation tests were conducted[35,36,37]. Heat-induced hemolysis is employed to quantify the extent of cell membrane damage, while albumin denaturation is used to measure the level of protein denaturation. By evaluating these two

parameters, the in vitro anti-inflammatory action of embelin potassium salt can be determined.

Heat Induced Hemolysis

Preparation of Human Blood Cells (HRBC) Suspension

A fresh sample of whole blood was obtained and combined with an equal volume of sterilized Alsever solution, which consists of 2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% NaCl dissolved in water. The mixture was then subjected to centrifugation at a speed of 3000 rpm for a duration of 10 minutes. After centrifugation, the packed cells were subjected to three cycles of washing using isosaline solution, which has a concentration of 0.85% and a pH of 7.2. The initial volume of the blood sample was measured and subsequently reconstituted as a 10% volume/volume (v/v) suspension using isosaline.

Procedure

The assay mixture was prepared by combining 1 ml of phosphate buffer with a pH of 7.4, 2 ml of hyposaline solution with a concentration of 0.36%, and 0.5 ml of HRBC (Human Red Blood Cell) suspension at a concentration of 10% volume/volume (v/v). Various concentrations of embelin potassium salt were added to the assay mixture to assess its effects on the HRBCs. In the control test tube, all the components of the assay mixture were included except for the embelin potassium salt. The resulting mixture, along with the control, was then incubated at a temperature of 37℃ for a duration of 30 minutes. Following incubation, both the assay mixture and the control were subjected to centrifugation. The absorbance was subsequently estimated using a spectrophotometer set at a wavelength of 560 nm. The same experimental procedure was then repeated using different concentrations of diclofenac sodium as the standard drug. The inhibitory effect of diclofenac sodium on the HRBCs was assessed and compared to that of embelin potassium salt.

The percentage of hemolysis of HRBC membrane can be calculated as follows:

$$\% \text{ hemolysis} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Where Abs Control is the absorbance of control and Abs Sample is the absorbance of test sample.

Inhibition of Albumin Denaturation

The anti-inflammatory activity of embelin was assessed using the inhibition of albumin denaturation technique. The reaction mixture was prepared by combining the equal volume of test extracts with a 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using a small amount of 1N HCl. The sample extracts were then incubated at a temperature of 37℃ for duration of 20 minutes. Subsequently, the samples were heated to 51℃ and maintained at this temperature for an additional 20 minutes. After cooling the samples, the absorbance was measured at a wavelength of 660 nm. To ensure accuracy, the experiment was performed in triplicate.

The % inhibition of protein denaturation was calculated as follows:

$$\% \text{ inhibition} = \frac{(\text{Abs Control} - \text{Abs Sample})}{\text{Abs Control}} \times 10$$

Where Abs Control is the absorbance of control and Abs Sample is the absorbance of test sample.

Preparation of Cream

The Embelin potassium salt is formulated into an herbal cream and the specific quantities and proportions of the ingredients required to formulate 10 grams of the cream is given in Table 2.Melt stearic acid and heat it together with glycerine on a water bath until the temperature reaches around 70℃Dissolve KOH in water, as well as embelin potassium salt and triethanolamine in separate containers. Heat both solutions to 70℃ on a water bath. Slowly add the aqueous phase (KOH solution, embelin potassium salt solution, and triethanolamine solution) to the oil phase (melted stearic acid and glycerine) while stirring continuously until a cream-like consistency is achieved. Once the mixture has cooled down to 50℃, add the desired perfume or fragrance. Transfer the cream mixture to a clean mortar and triturate it until a cream with a pearl lustre is obtained. Finally, transfer the cream to a clean container for storage [38, 39].

Table–2: Formula for 10gm cream

Ingredients	Quantity for 100gm	Quantity for 10gm
Stearic acid	24 g	2.4 g
Glycerine	7.2 mL	0.72 mL
Tri ethanol amine	1.44 mL	0.144 mL
KOH	0.048 g	0.0048 g
Embelin potassium salt	1 g	0.1 g
Water	86.8 mL	8.68 mL
Perfume	q.s	q.s
Preservative	0.24 g	0.024 g

Physical Evaluation Of Cream

After formulating the cream containing embelin potassium salt, it undergoes a series of physical evaluations to assess its quality and performance. The results of these physical evaluations help determine the quality, effectiveness, and user-friendliness of the formulated cream [40,41].

Organoleptic Characters

Colour, texture, homogeneity, and phase separation: The visual appearance of the cream was examined to assess these characteristics. Homogeneity and texture: A small amount of the cream was pressed between the thumb and index finger to evaluate its homogeneity and texture. The consistency of the formulation and the presence of coarse particles were considered. Immediate skin feel: Upon application, the cream was evaluated for attributes such as stiffness, grittiness, and greasiness

Spreadability

The spreading diameter of 1g of the cream was measured between two horizontal glass plates. After 1 minute, a standard weight of 25g was applied to the upper plate. This test was conducted three times to ensure accuracy and consistency.

pH determination

1g of the cream was dispersed in 25ml of deionized water, and the pH of the resulting mixture was measured using a pH meter. This measurement was performed three times to ensure reliability. The pH meter [Fig 5] was calibrated with a standard buffer solution of pH 4 before each use.

Figure–5: pH meter

Viscosity Measurement

To measure the viscosity of the cream, a Brookfield viscometer [Fig 6] equipped with a concentric cylinder spindle 64 was used. The spindle was set to rotate at a speed of 100 revolutions per minute (rpm). The viscosity measurements were conducted in triplicate to ensure accuracy and consistency of the results.

Figure–6: Brookfield viscometer

Drug Content Determination

This procedure helps quantify the drug content in the cream sample by measuring the absorbance of the solution after appropriate dilution and filtering.

Preparation of Standard

Weighed accurately 1g of embelin potassium salt and dissolved in 100ml distilled water. Pipette out 0.8, 1, 1.2, 1.4, 1.6ml and made up to 10ml with distilled water.

Assay of cream

Accurately weighed 1 g of the cream. Dissolved the weighed cream in 100 ml of methanol, ensuring complete dissolution. Transferred the solution to a volumetric flask and allowed it to sit for 2 hours, shaking it well in a shaker to ensure thorough mixing. Passed the solution through filter paper to remove any particulate matter or impurities and filtered solution was collected. The absorbance of the solution was measured spectrophotometrically at a wavelength of 660 nm. Appropriate dilutions of the solution were made, if necessary, using distilled water as a blank. The drug content in the cream was determined based on the absorbance measurements and appropriate calibration curve.

Results and Discussion

Isolation of Embelin and Physiochemical Characterization

Embelia Ribes were procured and subjected to extraction using n–hexane as solvent, washed with petroleum ether and purified using dichloromethane and n-hexane. The percentage yield was found to be 1.1 % w/w [Table 3]. A specific test was carried out using n–hexane and dilute ammonia which gives a blue purple colour confirming the identity of embelin in raw material and in obtained extract. Furthermore, solubility tests were performed on the isolated compound, revealing that embelin is soluble in organic solvents such as chloroform and methanol. However, it was found to be insoluble in water. Chemical analysis of embelin indicated the presence of alkaloids, proteins, glycosides, and steroids. However, tannins and flavonoids were absent in the compound.[Table 4].The melting point of embelin was investigated using a melting point apparatus, and it was determined to be within the range of 142℃–145°C. The compound was also identified through Thin Layer Chromatography (TLC).The optimized mobile phase was ethyl acetate: toluene: methanol: formic acid (0.5:0.4:0.1:0.05). The isolated embelin exhibited an Rf value of 0.75[Fig 9], which closely matched the Rf value of the standard embelin.

Table–3: Percentage yield of isolated embelin

Drug Loaded (g)	%weight obtained ± S.D	Percentage yield: % w/v ± S.D
90	1.01±0.02	1.10±0.03
95	1.05±0.03	1.1±0.01
98	1.2±0.01	1.2±0.04
Average	1.08	1.11

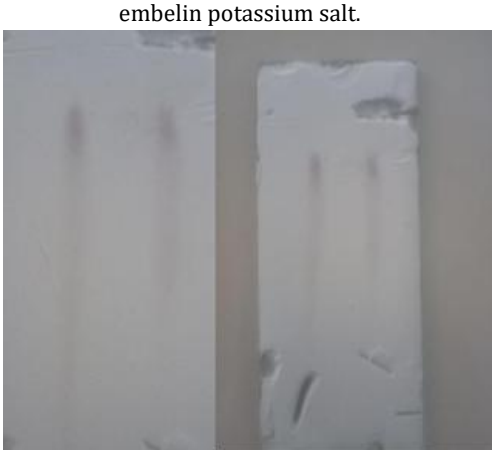
Table–4: Phytochemical screening of petroleum ether extract of *Embeliaribes* seeds

Phytochemical Screening Test	Indication
Alkaloids	+
Tannins	–
Flavonoids	–
Proteins	+
Glycosides	+
Steroids	+

Preparation of Embelin Potassium Salt and Physiochemical Characterization

The Embelin potassium salt was prepared using 1 mole of Embelin and 2 moles of KOH by heating in a water bath which turns to grape wine-coloured crystals (1.28g and the percentage yield was found to 93 % w/w.).The principle behind the formation of salt form may be due to that Embelin possess quinone ring having two ketone groups in 1,4 position having four double bonds with four π electrons forming four π bonds. The alkyl chain at position 3 creates positive inductive effect on quinonoid auxochrome chromatophore which can be melted easily with KOH to form potassium salt with two phenolic groups which are again 1,4 positions of each. Two phenolic groups(1,4 position) and two ketone groups(1,4 positions) are adjacent with each other which produce electron transfer (negative charge) towards ketone after formation of dipotassium salt during dry fusion with Embelin with KOH. 2,5–dihydroxy–3–undecylcyclohexa–2,5–diene–1,4–dione is IUPAC name of Embelin and dipotassium 3,6–dioxo–2–undecylcyclohexa–1,4–diene–1,4–diolate is IUPAC name of dipotassium salt.The melting point of embelin potassium salt was observed to be within the range of 141-145°C. Thin Layer Chromatography (TLC) was performed using the same mobile phase as that of embelin, and the resulting Rf value was found to be 0.75[Fig 7]. This indicates that both embelin and embelin potassium salt share a similar Rf value to the standard

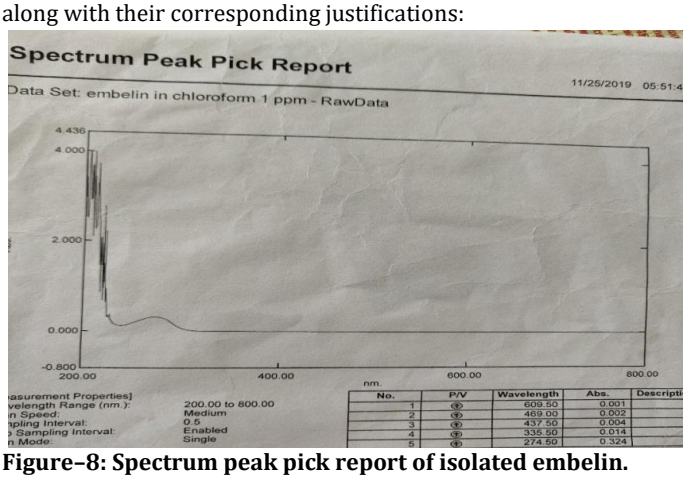
embelin. Table 5 provides the physicochemical evaluation of embelin potassium salt.



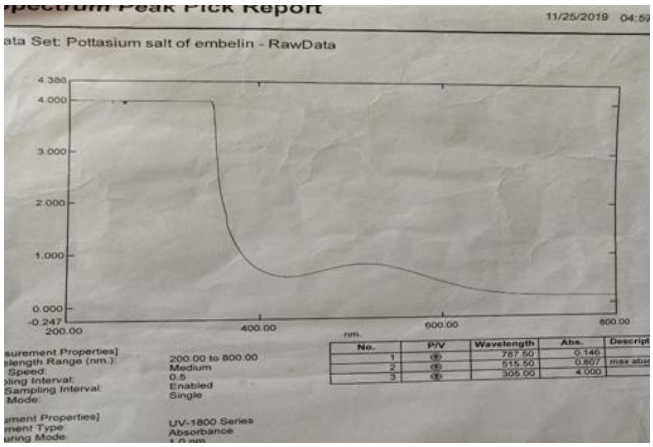
Figure–7: TLC of Embelin & Embelin Potassium salt

Spectral Characterization of Embelin And Embelin Potassium Salt

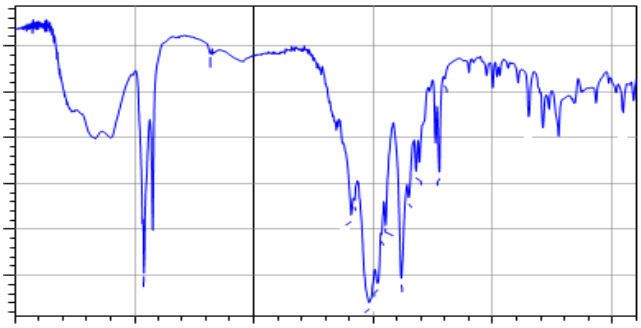
The isolated embelin and Embelin Potassium salt were characterised by various spectral analysis (UV, IR). UV spectral analysis was carried out for the identification of embelin in *Embeliaribes* berries and the absorption peak was found to be at 274nm[Fig8] whereas potassium salt of Embelin was found to be 515 nm[Fig9]. The presence of functional groups were ascertained by IR spectroscopy. The IR spectral analysis of Embelin and Embelin Potassium salt revealed characteristic vibrations associated with specific functional groups. For Embelin, the IR spectrum exhibited bands near 1328.95 cm^{-1} and 1361.74 cm^{-1}, indicating the presence of phenolic OH bending. A band at 1616.35 cm^{-1} suggested C=O stretching. The spectrum also displayed vibrations related to aromatic ring bending (1616.35cm^{-1},), methylene bending at 1452.4 cm^{-1} and 748.28 cm^{-1}, and methyl stretching at 2947.23 cm^{-1}. In the case of Embelin Potassium salt, the IR spectrum showed phenolic OH bending at 1350.17 cm^{-1} and 1382.96 cm^{-1}. C=O stretching vibrations were observed at 1625.99 cm^{-1}, while aromatic ring bending occurred at 1593.2 cm^{-1}. Methylene bending vibrations were detected at 1454.33 cm^{-1} and 750.31 cm^{-1}, and methyl stretching vibrations appeared at 2929.87 cm^{-1}. These IR results provide insights into the presence of specific functional groups, aiding in the identification and characterization of Embelin and Embelin Potassium salt.Table 6 provides IR results for Embelin and Embelin Potassium salt, along with their corresponding justifications:



Figure–8: Spectrum peak pick report of isolated embelin. Maximum absorbance ofembelin was found to be at 274nm



Figure–9: Spectrum peak report of Embelin Potassium Salt. Maximum absorbance of Embelin Potassium Salt was found at 515nm



Figure–10: IR Spectrum of Embelin Potassium Salt

Table–6: IR results for Embelin and Embelin Potassium salt

Groups	Embelin [cm^{-1}]	Embelin Potassium Salt [cm^{-1}]	Justification
Phenolic (Bending)	1328.95, 1361.74	1350.17, 1382.96	Value increases due to formation of **dipotassium 3,6–dioxo–2–undecylcyclohexa–1,4–diene–1,4– diolate** from **2,5–dihydroxy–3–undecylcyclohexa–2,5–diene–1,4–dione**
Ketone (Stretching)	1616.35	1625.99	Value slightly increases due to release of H^+& formation of O^-
Aromatic ring stretch (Bending)	1616.35	1593.2	Value decreases due to shifting of π bond
Methylene (Bending)	1425.4 & 748.38	1454.33 & 750.31	Value remains same as same inductive effect is playing
Methyl (Stretching)	2947.23	2929.87	No such significant change as methyl group plays same inductive effect

N–Vitro Anti–Inflammatory Activity of Embelin Potassium Salt

The heat-induced hemolysis method was used to estimate the in-vitro anti-inflammatory activity of Embelin potassium salt at concentrations of 10, 20, 30 and 40,50 µg/ml. The results showed percentage inhibitions of 19.52%, 44.2%, 77%, 84.7% and 92.1% respectively (Table 8). In comparison, the standard diclofenac sodium, also tested at concentrations of 10, 20, 30, and 40 µg/ml, displayed percentage inhibitions of 86.6%, 89.5%, 91.6% 95.2% and 98.01% respectively(Table 7).Figures 11-14depict regression equation graphs for diclofenac sodium and embelin potassium salt using absorbance at 560 nm, as well as graphs for the percentage inhibition of both substances. All graphs exhibit positive slopes, indicating that as the concentration increases, absorbance and percentage inhibition also increase. The high R-squared values (0.9978 and 0.9698 for absorbance, 0.9982 and 0.9176 for percentage inhibition) demonstrate strong correlations between concentration and the respective variables. These findings suggest a dose-dependent relationship and significant inhibitory effects of both diclofenac sodium and embelin potassium salt. Fig 15 illustrates the comparison of anti-inflammatory activity between the standard and test samples using the heat-induced hemolysis Additionally, the albumin denaturation method was employed to estimate the in-vitro anti-inflammatory activity of Embelin potassium salt. Concentrations ranging from 10µg/ml to 40µg/ml were utilized, resulting in percentage inhibitions of 96.3%, 97.3%, 97.8%,98.6%, and 99.2%, as indicated in Table 9. The regression graphs (Figures 16 and17) for embelin potassium salt, based on absorbance at 660 nm and percentage inhibition, respectively, demonstrate a positive relationship between concentration and the observed effects of embelin potassium salt in terms of absorbance and inhibition.Overall, these findings demonstrate the significant anti-inflammatory activity of Embelin potassium salt in vitro, as determined by both the heat-induced hemolysis and albumin denaturation

Heat Induced Hemolysis

Table–7: % inhibition of standard diclofenac sodium on heat induced hemolysis

Standard Diclofenac Sodium (DMSO+distilled Water)			
Sl.No.	Concentration in µg/mL	Absorbance*: Mean±SD	% inhibition
1	10	0.28 ± 0.005	86.6
2	20	0.22±0.01	89.5
3	30	0.17±0.01	91.6
4	40	0.1±0.015	95.2
5	50	0.04±0.01	98.01

*N=3 (Each conc. determined 3 times)

Table 8: % inhibition for embelin potassium Salt on heat induced hemolysis

Sl.No.	Concentration in µg/mL	Absorbance*: Mean±SD	% Inhibition
1	10	1.69±0.01	19.52
2	20	1.17±0.005	44.2
3	30	0.77±0.005	77
4	40	0.32±0.01	84.7
5	50	0.19±0.01	92.1

*N=3 (Each conc. determined 3 times)

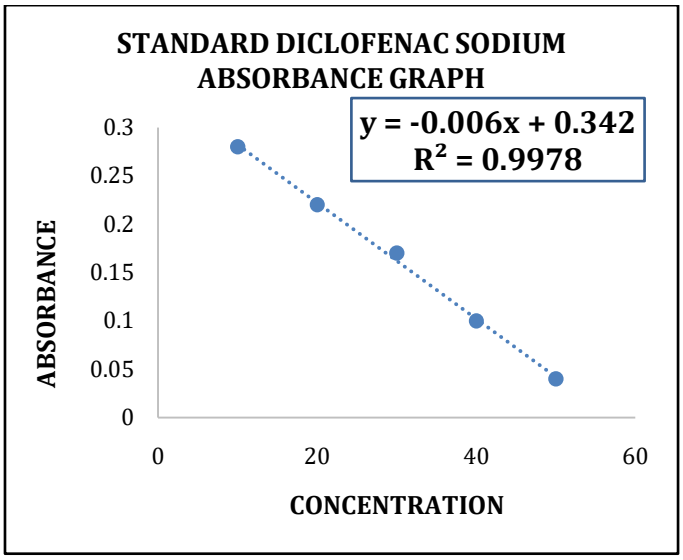


Fig-11. Regression equation graph for diclofenac sodium Absorbance at 560nm

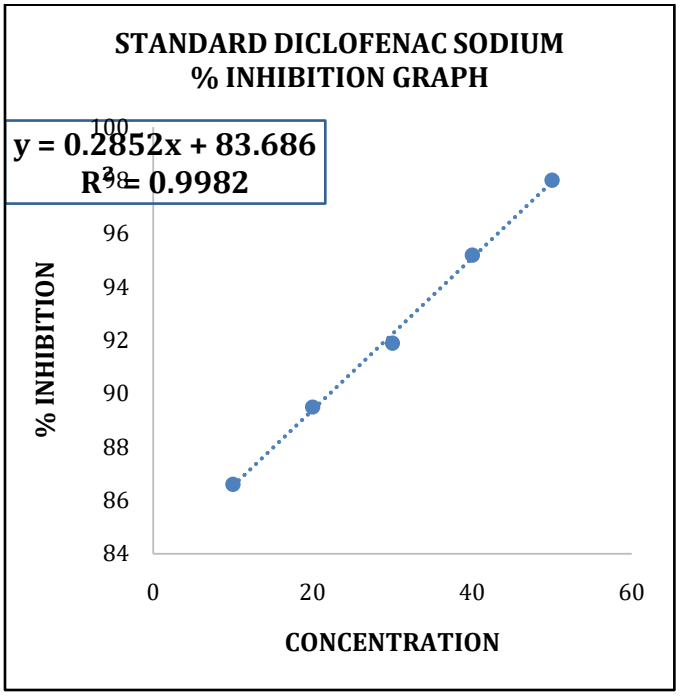


Fig-12. Regression equation graph for diclofenac sodium

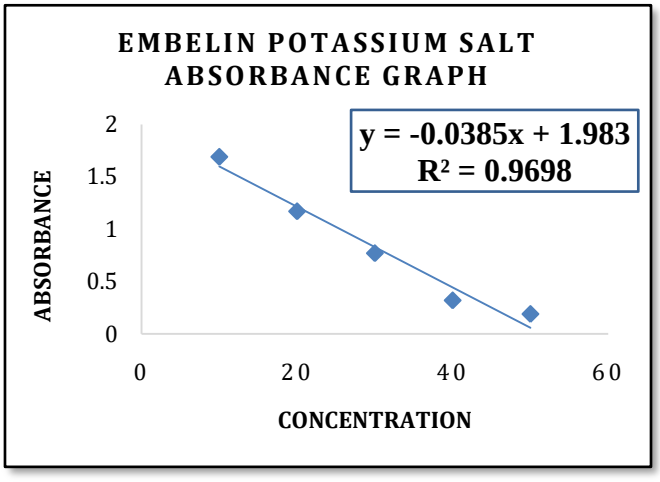


Fig.-13.Regression equation graph for embelin Potassium Salt Absorbance at 560nm

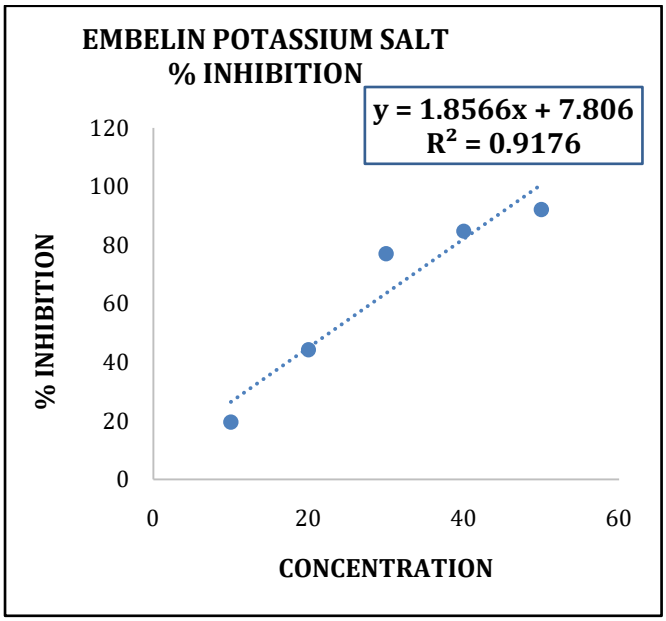


Fig-14.Regression equation graph for Embelin Potassium Salt

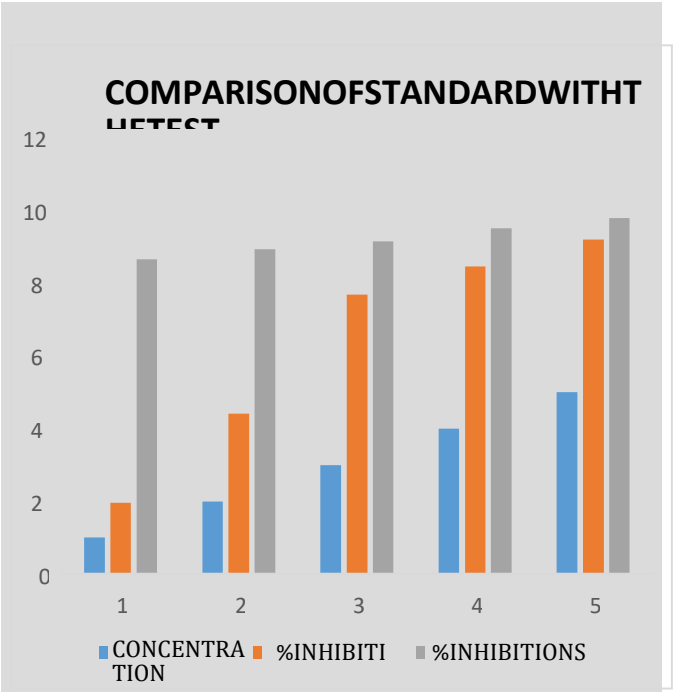


Fig -15:Comparison of anti-inflammatory activity of standard and test using heat induced hemolysis

Albumin Denaturation

Table No–9: % inhibition of Embelin Potassium Salt: *N=3 (Each conc.determined 3 times)

EMBELIN POTASSIUM SALT			
Sl.No.	**Concentration in µg/mL**	**Absorbance*: Mean±SD**	**% Inhibition**
1	10	0.014±0.000	96.3
2	20	0.01±0.005	97.3
3	30	0.008 ±0.001	97.8
4	40	0.005 ±0.001	98.6
5	50	0.003 ±0.001	99.2

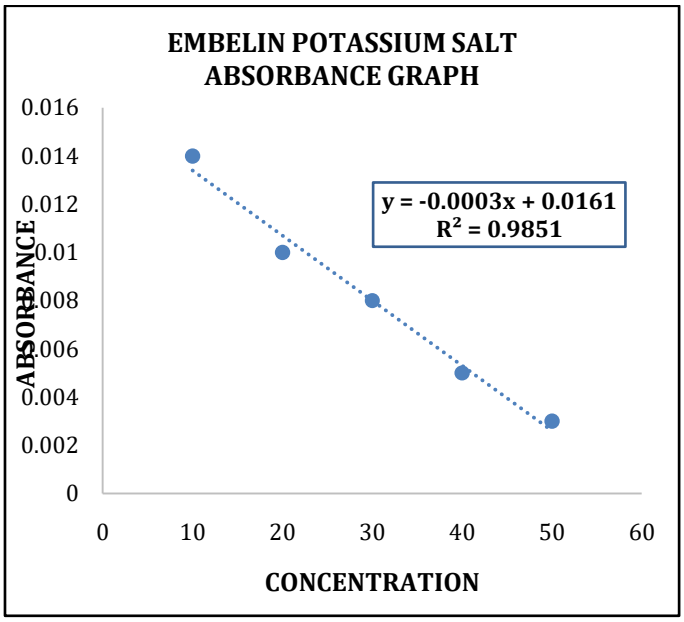


Fig-16. Regression equation graph for Embelin potassium salt absorbance at 660 nm

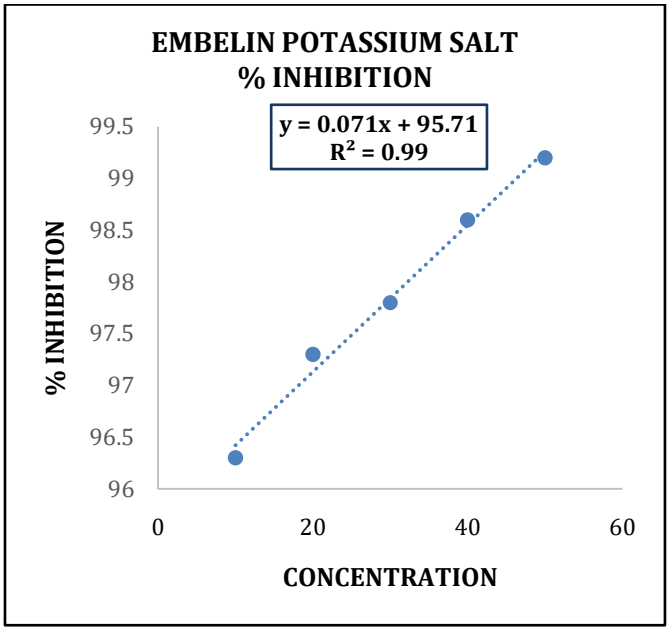


Fig-17. Regression equation graph for Embelin Potassium Salt

Docking Studies

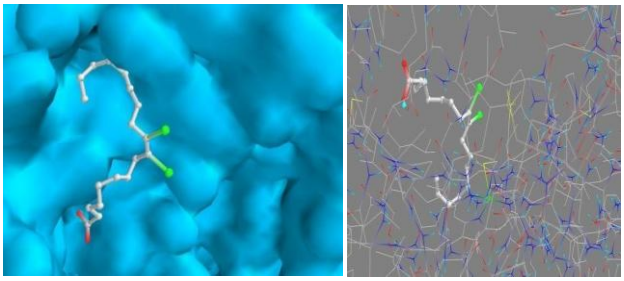
Molecular docking studies were carried out for predicting molecular interactions between ligand receptors with the help of a computer program. PASS (prediction of activity spectra for substance) has been employed as a strong potential to predict the biological potential of an organic drug like molecule. Molecular docking was carried out by PyRx, which include docking wizard with easy–to use user interface which makes it a valuable tool for computer. Smile is a freely accessible online software used for conversion into different molecularformats. In order to view ligands and protein ,BIOVIAdiscovery studio visualizer was used. Docking scores were analysed, binding affinity of Embelin with Oxidoreductase enzyme (PDB–1NR6) and that of standard Diclofenac with the same protein were compared. Binding affinity of Diclofenac was found to be –4.9

[Table 10] and that of Embelin as –5.7 [Table 11]. These results suggest that Embelin have more binding affinity with Oxidoreductase than Diclofenac. Fig 18&19 depict the molecular docking of diclofenac and embelin with oxidoreductase enzyme[PDB-1NR6]. In heat induced hemolysis, Diclofenac shows 98.01% inhibition and that of Embelin potassium salt shows 92% inhibition at 50µg/ml. Embelin potassium salt shows a comparable result with standard Diclofenac. These results show the probability of Embelin being a substitute to Diclofenac due its similar effects.

Molecular Docking of Diclofenac Vs Oxidoreductase Enzyme [PDB–1NR6]

Table No–10: Docking scores and binding affinity of Diclofenac

Ligand	Binding Affinity	rmsd/ub	rmsd/b
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	**–4.9**	0	0
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.8	33.444	31.548
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.6	33.946	32.18
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.6	34.147	32.838
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.6	31.412	28.992
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.6	3.102	2.107
1nr6prep_tclcactvs 000q1RA3o_uff_E=133.13	–4.5	8.121	5.206



(a) **(b)**

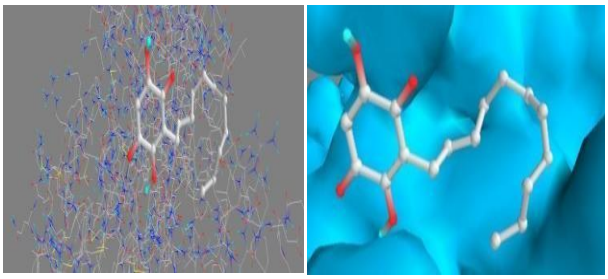
Figure No–18: Diclofenac docked against INR6 Protein

Molecular Docking of Embelin Vs Oxidoreductase Enzyme (PDB–1NR6)

Table No–11: Docking Scores and binding affinity of Embelin

Ligand	Binding Affinity	rmsd/ub	rmsd/b
1nr6prep_tclcactvs 000I6NpQS_uff_E=108. [41]	**–5.7**	0	0
1nr6prep_tclcactvs 000I6NpQS_uff_E=108. [41]	–5.6	2.758	1.144
1nr6prep_tclcactvs 000I6NpQS_uff_E=108. [41]	–5.5	21.974	20.442
1nr6prep_tclcactvs 000I6NpQS_uff_E=108. [41]	–5.4	2.94	1.226
1nr6prep_tclcactvs 000I6NpQS_uff_E=108. [41]	–5.3	22.436	20.787
1nr6prep_tclcactvs000I6 NpQS_uff_E=108. [41]	–5.2	8.362	4.821
1nr6prep_tclcactvs000I6 NpQS_uff_E=108. [41]	–5.2	21.578	19.957
1nr6prep_tclcactvs000I6 NpQS_uff_E=108. [41]	–5.2	34.932	33.215
1nr6prep_tclcactvs000I6 NpQS_uff_E=108. [41]	–5.2	8.077	5.433

Table No–11: Docking Scores and binding affinity of Embelin



(a) **(b)**

Figure No–19: Embelin docked against INR6 Protein

Preparation of Cream and Drug Determination

As per the objective, the Potassium salt of Embelin was formulated into a cream. The formulation process involved incorporating the Potassium salt into a suitable cream base to create a topical product**.** The physical evaluation of cream such as spreadability, pH and viscosity were carried out. The spreadability coefficient was estimated to be 2031.5mm^2. The pH was estimated by pH meter and was found to be 6.5. pH values are similar to skin pH which is usually 4–6. The viscocity was determined using Brookfield viscometer and was found to be at the range 30050–30075 centipoise. Table 12 illustrates physical evaluation of cream. The assay was carried out to know the percentage content of Embelin present in developed formulation which is of 1%w/w.It was found that 1000mg of prepared cream contains 95%w/w of Embelin potassium salt.Table 13provides a record of the concentration of embelin potassium salt in µg/mL and the corresponding absorbance readings obtained during the drug determination process.

Physical Evaluation of Cream

Table No–12: Physical parameters of formulated cream

Characteristics	Observation
Colour	Grape wine Colour
Spreadability–Coefficient	2031.5mm^2
pH	6.5
Viscosity	3850 centipoise

Drug Content Determination

Table No–13: Assay of Embelin Potassium Salt

Concentration in µg/mL	Absorbance
800	0.16
1000	0.25
1200	0.29
1400	0.35
1600	0.4

Conclusion

The studies revealed that the isolated potassium salt of embelin in the form of prepared cream possess anti–inflammatory activity. On analysing the docking scores binding affinity of Embelin with Oxidoreductase enzyme (PDB–1NR6) and that of standard Diclofenac were found to be comparable. These results suggest that Embelin have more binding affinity with Oxidoreductase than Diclofenac. In future *in–vivo* studies are required for the prepared topical cream to confirm its effectiveness and in comparison with commercially available anti–inflammatory cream.

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Conflict of Interest

The authors declare that there is no conflict of interest for this study

Author Contribution

All authors have contributed equally.

Informed Consent

Not required

Ethical Statement

Not required

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