"Polyherbal Suspension for Gangrene Treatment: Formulation, Evaluation, *In-Vitro* and *In-silico* Studies"

Shruthi Goyal
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Hyderabad, Telangana, India

B Narasimha
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Hyderabad, Telangana, India

K Charan
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Hyderabad, Telangana, India

Vivek Kumar Tiwari
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Hyderabad, Telangana, India

ABSTRACT:

Polyherbal suspension formulation is a type of herbal medicine that combines multiple plant extracts to achieve a specific therapeutic effect.Any medicinal preparation that just contains one or more active plant ingredients is considered an herbal medicinal product. In the current research work polyherbal suspension was prepared where three plant extracts are used *Terminalia arjuna*, *Tinospora cordifolia, Rubia cordifolia*these contain different chemical phytoconstituents which have a potent activity to treat conditions like diabetes, inflammation, wound healing properties which are the main causes of gangrene so the prepared suspension might be effective in treating the condition. They consist ofanthraquinones, alkaloids, flavonoids and many more. The oldest known type of healthcare used by humans is herbal medicine. The ideal substitutes are polyherbal formulations since they are less expensive, better for the environment, and easier to get than contemporary medications.Physical test and accelerated stability analysis was performed including nature, colour, Odor, texture,redispersibility, sedimentation volume, flow rate, viscosity, PH.The suspension was used to evaluate its antioxidant and antidiabetic activitywhich is a well-known smodel for treating gangrene by in-vitro studies like DPPH and alpha-amylase assay. *In-silico* studies like molecular docking and Swiss ADME. Molecular docking studies have provided valuable insights into the binding mechanisms of CBD and its analogues to the CB2 receptor, whereas Swiss ADME was performed for the molecular likeliness and pharmacokinetic parameters.

Keywords:*Terminalia arjuna*, *Tinospora cordifolia, Rubia cordifolia,* Polyherbal suspension, Swiss ADME, Molecular docking.

1. INTRODUCTION:

Gangrene is a clinical condition characterized by ischemic and necrotic tissue, often identified by discolored or black tissue and associated with a shed of natural tissue planes. It can be life-threatening and may require amputation in severe cases. Causes include insufficient blood supply, traumatic injury, diabetes, foot gangrene, and symptoms like numbness, pain, change in skin color to red or black, and coolness. Treatment includes antibiotics, surgery to remove dead tissue, advancements in regenerative medicine, advanced wound care, microvascular surgery, infection control, personalized medicine, telemedicine, and remote monitoring. From previous studies, we concluded that the three selected herbs *Terminalia arjuna*, *Tinospora cordifolia, Rubia cordifolia L* showed their potency and synergistic effect for the treatment of gangrene condition by*in-vitro* studies.In this research, we aimed to formulate the polyherbal suspension from the selected herbs and use it for evaluating potency to treat gangrene by *in-vitro* and *in-silico* studies.

A pharmaceutical suspension is a coarse dispersion of the medicinally active material in which the exterior phase is evenly distributed throughout the internal phase. The internal phase consists of uniformly sized insoluble solid particles (0.5–5 microns) that are maintained throughout the suspending vehicle with the aid of one or more suspending agents. When used for non-oral purposes, the external phase (suspending media) is typically aqueous but can also be an organic or greasy liquid. Examples include antacid oral suspensions, antimicrobial oral suspensions, analgesic oral suspensions, and dry powders for oral suspension.

1.1 Advantages:

- Certain drugs that are suspended may be able to preserve their chemical stability. Compared to other dose forms, the drug in suspension form for procaine penicillin G shows a better rate of bioavailability. Solutions> suspension> capsule > compressed tablet > coated tablet
- A drug harsh or bitter taste may be covered up by a suspension. Example chloramphenicol.
- The onset and duration of the activity are controllable. For instance, protamine zinc-insulin solution.

1.2 Disadvantages:

- Compaction, sedimentation, and physical stability can all be problematic.
- Due to its weight, handling and transportation require extra caution.
- It is challenging to formulate; unless the suspension is packed in unit dosage form, precise and uniform doses cannot be obtained.

1.3 Applications:

- Drugs that are poorly soluble or insoluble are typically suitable for suspension. Such as suspension of prednisolone.
- To stop the medication from degrading or to increase its stability. For instance, oxy tetracycline suspension.
- A medication suspension can be created for topical use. Consider calamine lotion.
- A suspension that regulates the rate of medication absorption can be prepared for parenteral administration. For example, penicillin procaine.
- Vaccines are frequently designed as suspensions as a means of immunization. For example, barium sulphate is used to examine the gastrointestinal tract.

1.4 Features:

- Must be pourable without being watery or gritty.
- The sediment generated must be easily re-suspended with a modest degree of shaking, and the suspended particles should not settle quickly.
- It should be easy to inject like good syringe ability.
- Must have a pleasant Odor, colour, and palatability.
- It must be stable in terms of chemical state, physical state, and microbial state.
- Sterilization of the parenteral/ophthalmic suspension is recommended.

Polyherbal suspension formulation is a type of herbal medicine that combines multiple plant extracts to achieve a specific therapeutic effect. These formulations are often used to treat various health conditions, such as inflammation, hypertension, and depression.

1.5 EXCIPIENTS USED:

The most common excipients used in polyherbal suspensions are:

1. **Suspending agents:**these helps keep the herbal extracts suspended in the formulation like sodium carboxymethylcellulose (Na CMC) and xanthum gum
2. **Sweetening agents:** these improve the taste of the suspension. Examples include sucrose and sorbitol
3. **Preservatives:** these prevent microbial growth in the suspension. Examples include methyl paraben and propyl paraben
4. **Flavouring agents:** mask unpleasant taste and improve palatability. Examples include lemon oil and peppermint oil
5. **Wetting agents:**these helps disperse the herbal extract in the vehicle. Examples include polysorbate 80 (tween 80)
6. **Viscosity modifiers:** these control the viscosity of the suspension. Examples include glycerine and propylene glycol.
7. **Tonicity modifiers:** these adjust the tonicity of the suspension. Examples include sodium chloride

2. AIM AND OBJECTIVES OF THE WORK:

2.1 Aim: To perform *In-vitro*&*In-silico* studies and prepare a suspension of polyherbal extract in treating gangrene.

2.2 Objectives:

- Prepare a suspension of the polyherbal extract by the trituration method.
- Evaluate the stability parameters of polyherbal suspension.
- Perform *In-vitro*Antioxidant activity of suspension by DPPH assay.
- Perform *in-vitro*Antidiabetic activity of suspension by Alpha-Amylase assay.

- Perform *in-silico* studies like Molecular Docking and *Swiss*-ADME.

3. MATERIALS AND METHODS:

The procedure of this method consists of a series of steps performed sequentially and systematically to achieve the objectives set under established guidelines and recommendations. Everyone became part of these steps from field trips to observation including chemical selection and collection of plant crude drugs, selection of dose values, standardization of protocols, equipment used, fabrication reagents, selection of specific solvents for extraction, preparation of protocols, and result implementation of standardized protocols. All of this requires good construction, and the concept is simple a mechanical hand that manages materials and processes in a certain way.

3.1 Plant materials:

The crude powders of *Terminalia arjuna, Tinospora cordifolia, and Rubia cordifolia* were purchased from JAIN LIFESCIENCES Pvt Ltd. In the Amazon E-Commerce platform.

3.2Methods:

3.2.1. Preparation of polyherbal suspension dosage form:

Table 1 displays the ingredients of the formulation used to make 100ml of plant extract suspension. Through trituration, the medications small particles with a mesh size of 100 is correctly combined. The medication was then combined with water and several additives, including sodium carboxymethyl cellulose (CMC), Tween 80, flavouring, and sweetening agents, to improve stability during the formulation's shelf life.

Table 1.1: Composition of polyherbal suspension

S.No.	Name of ingredients	Quantity taken
1	Polyherbal extract	3g
2	Tween-80	0.3% w/v
3	Sodium CMC	1.5%
4	Sodium benzoate	3g
5	Sucrose	0.3g
6	Lemon oil	3ml
7	Purified water q. s	100ml

Figure 1.1: Showing the requirements for carrying the suspension studies

3.2.2. Evaluation of polyherbal suspension:

1. **Physical test for polyherbal suspension:**
   The polyherbal formulation was physically tested at 45° C and room temperature (+25°C). the outcomes were noted.
2. **Accelerated stability studies:**
   For polyherbal suspension, accelerated stability investigations were conducted. For the formulation, a variety of factors were examined, including sedimentation volume, redispersibility, flow rate, viscosity, pH, and crystal development. The values were observed.

3. **Sedimentation volume:**
   The ratio of the ultimate height (Hu) of the sediment to the beginning height (H0) of the entire suspension as it settles in a cylinder under typical circumstances is known as the sedimentation volume. It was ascertained by observing that the volume of the sediment is expressed as ultimate height and maintaining a measured volume of suspension in a graduated cylinder in an undisturbed state for a certain amount of time.
4. **Redispersibility:**
   A measuring cylinder was used to allow the suspension to settle. The cylinder mouth was closed and inverted, and the quantity of inversions required to bring the suspension back to homogeneity was counted.
5. **Rheology:**
   The apparent viscosity was calculated using the equation by measuring the amount of time the suspension sample took to pass through a pipette.
   Flow rate= volume of pipette (ml) / flow time
6. **Viscosity:**
   Using spindle no.3, a Brookfield Viscometer operating at 50rpm was used to measure the samples viscosity at room temperature.
7. **pH:**
   The pH meter was used to determine the suspension pH

3.2.3. *In-vitro* studies:

3.2.3.1 Antioxidant activity by DPPH assay:

Prepare 100 µM DPPH solution and take 3.943 mg of DPPH in methanol made up to 100ml.To prepare a 3 mg/ml standard stock solution of ascorbic acid, take 0.3g of Ascorbic acid in distilled water made up to 100 ml. Take a minute quantity of polyherbal suspension in distilled water. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay is a well-known technique for determining the antioxidant capacity of plant extracts. During the process, an antioxidant sample is combined with a DPPH reagent solution and is incubated for about 50 minutes causing the purple DPPH color to fade to yellow over time. The degree of color change is monitored using a spectrophotometer at 517 nm.

The following formula is used to calculate the free radical scavenging activity of ascorbic acid, which is frequently used as a benchmark.

% inhibition= [(Absorbance of control- Absorbance of test)/ Absorbance of control] x 100.

3.2.3.2 Antidiabetic activity by Alpha-Amylase assay: (well-known model for gangrene)

Sodium phosphate buffer solution of P^H=6.9 was prepared. Alpha-Amylase (1mg/ml), taking 0.01g of Alpha-Amylase from ex-porcine pancreas was prepared in warm water to make volume up to 10ml. 1% soluble starch, dissolve the required amount of soluble starch into prepared buffer boiled and filtrated it is used as substrate. take 5g of DNSA powder in 500ml volumetric flask consisting of 100ml of 2N Sodium Hydroxide, now add 250 ml of distilled water to it, add 150 g of Sodium Potassium tartrate dissolve it completely make up the volume to up to 500ml by distilled water. A reagent was prepared.1 mg/ml standard stock solution of Acarbose was prepared, taking 0.1g of Acarbose in distilled water made up to 100 ml. Take a minute quantity of plant extract dissolved in distilled water. The DNS method is a redox reaction that converts 3, 5-dinitrosalicylic acid (DNS) into 3-amino-5-nitrosalicylic acid in an alkaline medium. It measures alpha-amylase activity using a UV spectrometric method. α-amylase, an enzyme found in saliva and pancreatic juice, attacks starch randomly and converted it into maltose which reduces 3,5-dinitrosalicylic acid (pale yellow) to 3-amino-5-nitrosalicylic acid (orange-red colored). The intensity of this reaction is measured using a colorimeter at 540nm wavelength.

Procedure: Take the polyherbal suspension sample and add it into a test tube consisting of buffer solution, then add α-amylase enzyme solution and keep it at room temperature for 20 min. After that pour the substrate solution into the test tubes and wait for 10 min. later add DNS reagent boil it for 10 minutes at a low temperature, cool it, and check absorbance at 540 nm in the colorimeter. Acarbose was used as standard. The free radical scavenging activity was determined by evaluating % inhibition by following the formula.

% inhibition = [(Absorbance of Control- Absorbance of Test)/ Absorbance of Control] × 100

3.2.4. *In-silico* studies:

3.2.4.1 Molecular docking studies:

Currently, the binding characteristics of ligand-receptor complexes are predicted using docking approaches. A type of bioinformatics modelling called "molecular docking" entails interacting two or more molecules to create a stable adduct. It forecasts the three-dimensional structure of any complex based on the binding characteristics of the ligand and target. Using the score feature of the Cb Dock 2 program, many potentials adduct structures are generated by molecular docking and subsequently ranked and classified. A ligand-receptor complex with an optimal shape and reduced binding free energy is the primary goal of molecular docking. A data bank is necessary for the actual use of molecular docking in order to search for targets with the correct PDB format.The ligand-receptor complex intermolecular interactions are significant and necessitate challenging modelling tasks. Typically, the number of ligand molecules is permitted to vary while the receptor is maintained partially or completely rigid.

1. **Protein-ligand interactions**
   Drug candidates binding orientation to their target proteins is predicted via docking simulations. To generate docking simulation experiments, Cb Dock 2 was employed.
2. **Docking stimulation on HSP-90**

Heat shock protein 90 is an essential chaperone protein that helps fold, stabilize, and modify a variety of protein substrates, which is essential for cellular protectionand upkeep.Docking stimulation on the HSP-90 protein involves the use of computational methods to predict how small molecules bind to the protein. This process is crucial for identifying potential inhibitors of HSP-90, which is a key therapeutic target for various diseases. In a study, a pharmacophore model was constructed using an X-ray crystallographic structure of HSP-90 complexed with an original ligand. The model consisted of one hydrogen bond donor, one aromatic centre, and two hydrophobic features. This model was then used for virtual screening to identify new inhibitors. The inhibitory activities of HPs 1-4 on HSP-90 were studied using fluorescent-labelled GM-FP experiments. With IC50 values ranging from 17.64 to 61.69Nm, the results demonstrated that all four drugs could competitively block GM's ATP binding site activity to Hsp90. The most powerful inhibitory effects were shown in HP-4. HSP-90 has been studied for its role in wound healing and gangrene. Specifically, HSP-90 has been identified as a key player in the wound healing process, particularly in the context of keratinocyte-secreted HSP-90 alpha (HSP-90α) which has shown promise in promoting wound closure in various animal models, including pigs and mice. The protein is upregulated during the healing process, and its administration has been shown to accelerate wound healing. While there is limited direct research on HSP-90 in gangrene, its role in wound healing and its potential therapeutic applications make it a relevant consideration for gangrene treatment. Gangrene is a severe form of wound infection that can lead to tissue death, and understanding the mechanism of wound healing and tissue repair can inform strategies for managing and treating gangrene.

3. **Ligand preparation**
   The compounds three-dimensional structures were obtained from the PubChem database in the. Sdf format. The 2D ligands that were drawn on the ligand importing side of Cb Dock 2.
4. **Protein preparation**
   Heat shock protein-90 X-ray crystalline structure (PDB ID: 6N8X) was obtained from the RCSB protein bank. The discovery studio visualizer provides attributes of the structure- based drug design (SBDD) site sphere.
5. **Ligand docking and scoring**
   Cb Dock 2 was used to facilitate flexible glide-ligand docking, which increased protein-ligand interactions. A docking score is displayed for the docked compounds.
6. **Visualization and analysis**
   The discovery studio visualizer was used to display the final docking process. To understand the binding interaction between ligand and protein, the ligand interaction was depicted. The vina scoring function was used to choose the best-docked buildings. The more negative the score the

more favourable the binding. Furthermore, the various ligand-receptor interactions were examined, and the docked ligand postures were shown.

***3.2.4.2 Swiss*-ADME studies:**

The Swiss ADME assay includes a step-by-step procedure for input molecules. The process begins with importing a drawing or 2D chemical structure using Molecular Sketcher. The settings are then converted to SMILES characters and added to the SMILES list. Clicking the "Run Button" button will start the calculation. Output panels show bioavailability radar, physicochemical properties, lipophilicity, water solubility, and pharmacokinetics. The output is then exported using graphical plots, CSV export, clipboard copy, and one-click interactivity. This allows you to create new CADD projects on other SwissDrugDesign tools.

4. RESULTS AND DISCUSSION:

4.1 Evaluation of polyherbal suspension

Table 1.2: Physical test for polyherbal suspension

S.No.	Parameter	Result
1	Nature	Liquid
2	Colour	Reddish brown
3	Odor	Pleasant
4	Texture	Suspension

S.No.	Parameter	Result
1	Redispersibility	1 inversion
2	pH	7.04
3	Flow rate	5ml / 30 seconds
4	Viscosity	107.4 cP
5	Sedimentation	0.9

Table 1.3: Accelerated stability analysis

Figure 1.2: Prepared polyherbal suspension

Figure 1.3: pH meter showing the result

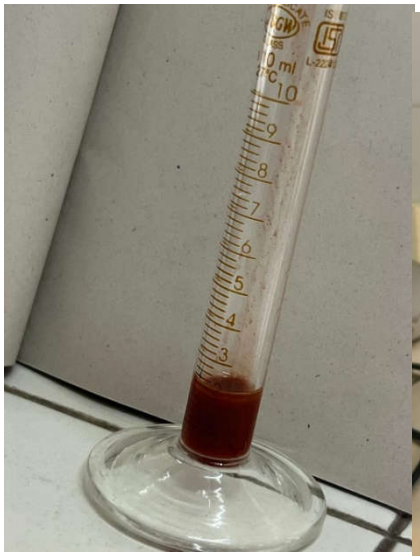


Figure 1.4: Sedimentation rate

Figure 1.5: Viscosity by Brookfield viscometer

Figure 1.6: Flow rate by using pipette

4.2 *In-vitro* antioxidant activity of polyherbal suspension:

The polyherbal suspension was subjected to in vitro antioxidant activity. Antioxidant activity was performed by using a DPPH assay.

DPPH assay: the antioxidant activity of polyherbal suspension was performed at different concentrations against standard Ascorbic acid using DPPH assay. The % inhibition and IC_{50} values are given in the table.

DPPH assay of polyherbal suspension and standard.

Table 1.4:DPPH assay of polyherbal and standard

COMPOUND	CONCENTRATION(µL)	%INHIBITION	IC_{50} VALUE
POLYHERBAL SUSPENSION	0	0	53.1
	20	20.5	
	40	40.5	
	60	61.1	
	80	75.1	
	100	86.3	
ASCORBIC ACID	0	0	42.1
	20	19.3	
	40	38.1	
	60	63.5	
	80	77.4	
	100	83.1	

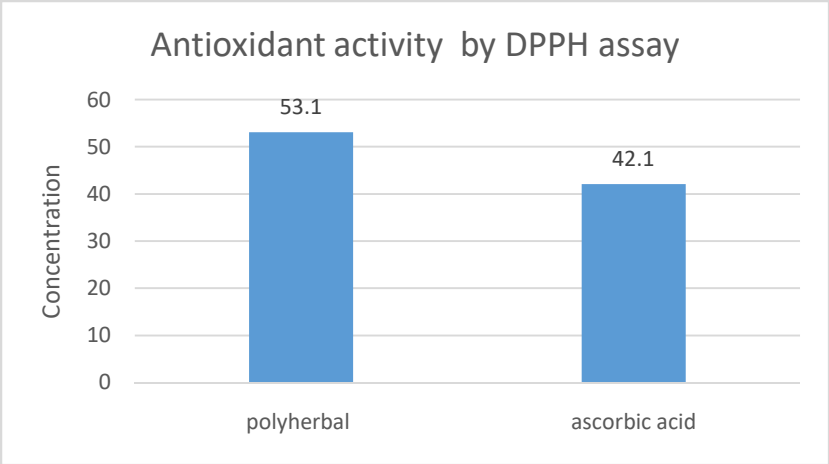


Figure 1.7: IC50 values of polyherbal and standard ascorbic acid in DPPH assay

4.3 *In-vitro* antidiabetic activity of polyherbal suspension:

The polyherbal suspension was subjected to in vitro antidiabetic activity. The antidiabetic activity was performed by using an Alpha-amylase assay.

Alpha-amylase assay: the antidiabetic activity of polyherbal suspension was performed at different concentrations against standard Acarbose using Alpha-amylase assay. The % inhibition and IC_{50} values are given in the table.

Alpha-amylase assay of polyherbal suspension and standard.

Table 1.5: Alpha-amylase of polyherbal and standard

COMPOUND	CONCENTRATION(µL)	%INHIBITION	IC_{50} VALUE
POLYHERBAL SUSPENSION	0	0	97.1
	50	28.4	
	100	53.4	
	150	91.6	
ACARBOSE	0	0	78.5
	50	35.1	
	100	61.6	
	150	78.7	

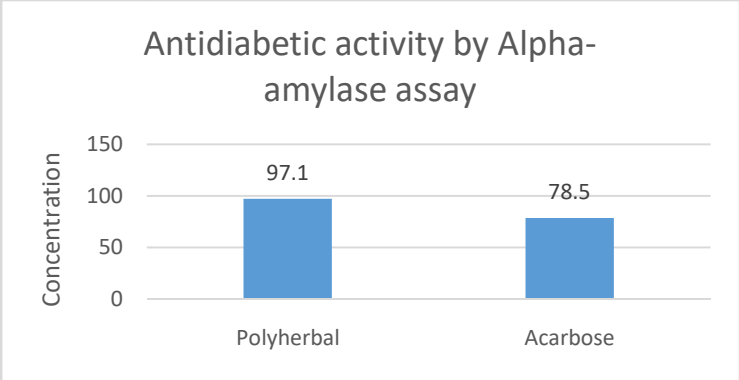
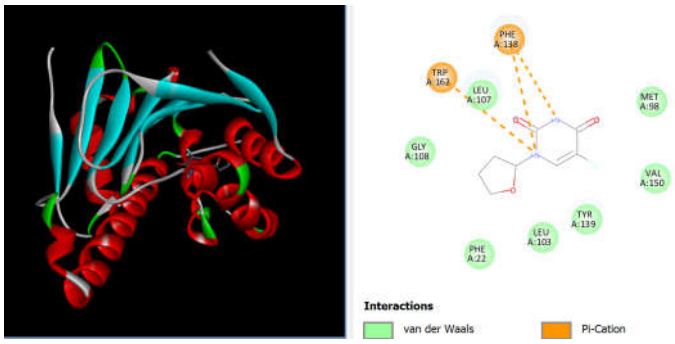


Figure 1.8: IC50 values of polyherbal and standard acarbose in Alpha-amylase assay

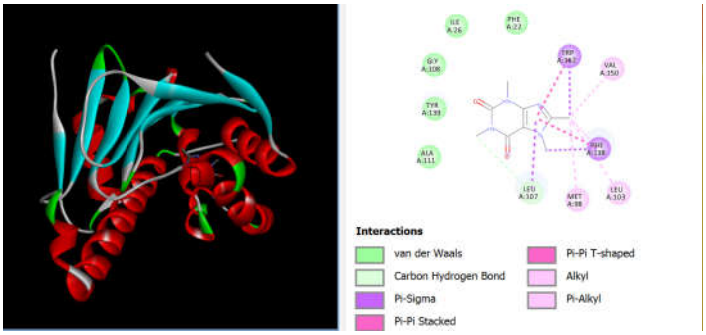
4.4 Docking studies:

First, the extra chains were removed from the protein that was retrieved from PDB. Attributes of spheres are prepared and noted. Two molecules identified from GC-MS studies were selected, and rest from the plant extract of*Terminalia arjuna, Rubia cordifolia, Tinospora cordifolia.* Later molecules drawn in Cb Dock 2 and ligprep was created. Protein is uploaded with sphere attributes and the structures were docked against 6N8X protein.

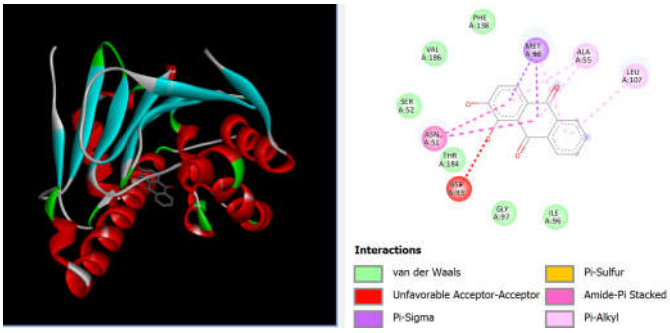
Docking indicated that some of our compounds have good binding ability with heat shock protein-90 (PDB ID: 6N8X). Following are the ligand interactions of compounds present in *Terminalia arjuna, Rubia cordifolia, Tinospora cordifolia* with 6N8X protein.



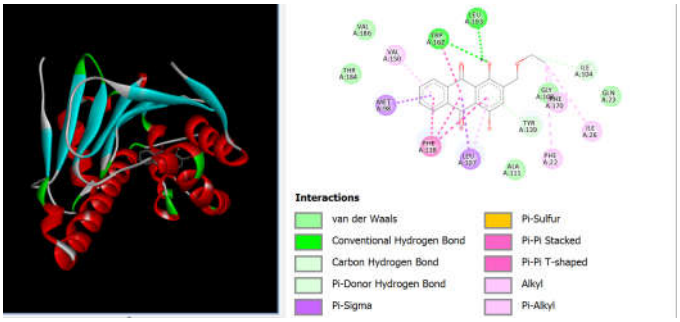
Tegafur pocket: C1 & Score: -6.6, Chain A: PHE22 ILE26 ASN51 ASP93 MET98 ASP102 LEU103 ILE104 LEU107 GLY108 ALA111 GLY135 VAL136 PHE138 TYR139 VAL150 TRP162 PHE170 THR184 VAL186



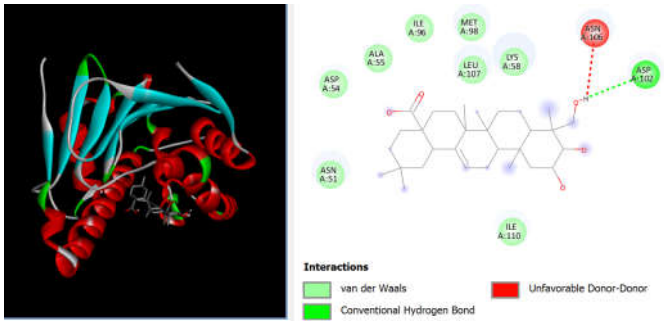
Xanthine 1,3,7,8 tetracycline pocket: C1 & Score: -7.3, Chain A: PHE22 ILE26 LEU48 ASN51 SER52 ALA55 ASP93 ILE96 GLY97 MET98 ASP102 LEU103 LEU107 GLY108 ALA111 GLY135 VAL136 PHE138 TYR139 VAL150 TRP162 THR184 VAL186



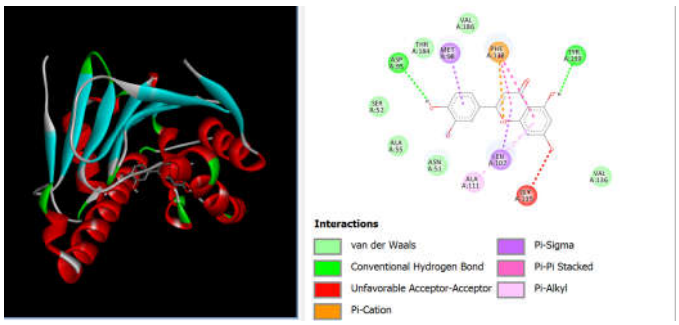
Alizarin pocket: C1 & Score: -6.9, Chain A: LEU48 ASN51 SER52 ALA55 ILE91 ASP93 ILE96 GLY97 MET98 ASP102LEU107 PHE138 THR184 VAL186



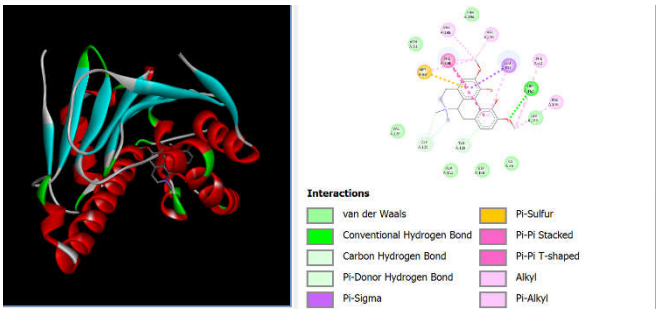
Christofin pocket: C1 & Score: -9.4, Chain A: PHE22 GLN23 ILE26 GLU47 LEU48 ASN51 SER52 ASP54 ALA55 LYS58 ILE91 ASP93 ILE96 GLY97 MET98 ASP102 LEU103 ILE104 ASN105 LEU107 GLY108 ILE110 ALA111 GLY132 GLY135 VAL136 GLY137 PHE138 TYR139 VAL150 TRP162 PHE170 THR184 VAL186



Arjunolic acid pocket: C1 & Score: -6.8, Chain A: SER50 ASN51 SER52 ASP54 ALA55 LYS58 ILE96 GLY97 MET98 THR99 ALA101 ASP102 ASN106 LEU107 GLY108 THR109 ILE110 ALA111 LYS112 SER113 GLY114 THR115 GLY132 GLN133 PHE134 GLY135 VAL136 GLY137 PHE138 TYR139 THR184



Luteolin pocket: C1 & Score: -9.1, Chain A: PHE22 GLN23 ILE26 GLU47 LEU48 SER50 ASN51 SER52 ASP54 ALA55 ASP93 GLY95 ILE96 GLY97 MET98 ASP102 LEU103 ILE104 LEU107 GLY108 ILE110 ALA111 GLY132 GLY135 VAL136 GLY137 PHE138 TYR139 VAL150 TRP162 PHE170 THR184 VAL186



Magnoflorine pocket: C1 & Score: -8.8,Chain A: PHE22 ILE26 GLU47 LEU48 ASN51 SER52 ASP54 ALA55 LYS58 ASP93 ILE96 GLY97 MET98 ASP102 LEU103 ILE104 ASN106 LEU107 GLY108 ILE110 ALA111 GLY132 GLY135 VAL136 GLY137 PHE138 TYR139 VAL150 TRP162 PHE170 THR184 VAL186

Table 1.6: Docking scores

Compounds	Score
Tegafur	-6.6
Xanthine 1,3,7,8 tetracycline	-7.3
Christofin	-9.4
Alizarin	-6.9
Arjunolic acid	-6.8
Luteolin	-9.1
Magnoflorine	-8.8

The more negative score the more favourable the binding.

***4.5 Swiss*-ADME studies:**

Predicted pharmacokinetic parameters of Swiss ADME studies.

Table 1.7:ADME properties

COMPOUND	GI ABSORPTION	SKIN PERMEATION	BIOAVAILABILITY SCORE	Log $P_{o/w}$	SOLUBILITY
ALIZARIN	High	-5.52 cm/s	0.55	1.85	3.69-02 mg/ml
CHRISTOFIN	High	-5.79 cm/s	0.55	2.66	3.26e-02 mg/ml
ARJUNOLIC ACID	High	-5.13 cm/s	0.56	3.11	1.87e-04 mg/ml
LUTEOLIN	High	-6.25 cm/s	0.55	1.86	5.63e-02 mg/ml
MAGNOFLORINE	High	-6.44 cm/s	0.55	-0.66	4.19e-02 mg/ml

5. CONCLUSION:

In the present study thePolyherbal Suspension for Gangrene Treatment: Formulation, Evaluation, and *In-Vitro* and *In-silico* Studies was evaluated, and the following conclusions were drawn:

- Stability and Formulation: The polyherbal suspension was formulated and evaluated for stability parameters, adhering to World Health Organization guidelines. The formulation included various additives like preservatives, coloring agents, and flavoring agents, which are essential for pharmaceutical suspensions.
- Antioxidant and Antidiabetic Activities: The study demonstrated significant antioxidant and antidiabetic activities in the polyherbal suspension, indicating its potential in treating gangrene.

- Molecular Docking: Molecular docking studies showed the heat shock effect of the compound, which occupied the binding site and showed interactions. This suggests that the compound can interact with heat shock proteins, which is relevant to its therapeutic activity.
- ADME Parameters: The Swiss ADME net tool became used to compute key physicochemical, pharmacokinetic, and drug-like parameters for the molecules, providing treasured insights into their potential therapeutic programs.

These findings collectively help the capability of polyherbal suspension in treating gangrene,highlighting its antioxidant, antidiabetic, as well as its stability and pharmacokinetic properties.

6. ACKNOWLEDGMENT:

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