

Evaluation of Anti-Vitiligo Potential of A Polyherbal Formulation Using In Silico, In Vitro, And In Vivo Approaches

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ABSTRACT

Vitiligo is a chronic depigmentation disorder associated with immune dysregulation and oxidative stress, with the Keap1/NRF2/ARE pathway playing a key role in melanocyte defense. This study investigated the therapeutic potential of phytoconstituents isolated from *Psidium guajava*, *Hibiscus rosa-sinensis*, and *Aloe barbadensis* Miller using in silico, in vitro, and in vivo approaches. Molecular docking studies were performed using PyRx and Biovia Discovery Studio targeting tyrosinase and the chemokine receptor CXCR3 (protein: 8HNN). Five key compounds—campesterol, taraxerol acetate, cadalene, β -sitosterol, and anthraquinone—were identified. Among them, β -sitosterol showed the highest binding affinity (-9.8 kcal/mol), followed by campesterol (-9.6 kcal/mol), outperforming the standard quercetin (-7.1 kcal/mol), indicating strong therapeutic potential. In vitro evaluation of the polyherbal formulation demonstrated significant antioxidant activity (DPPH IC₅₀: 23 ± 0.8 μ g/mL vs. standard 19 ± 1.3 μ g/mL) and moderate anti-inflammatory activity (IC₅₀: 536.9 ± 18.0 μ g/mL vs. standard 363 ± 12.0 μ g/mL). Total phenolic and flavonoid contents supported its biological activity. In vivo studies were conducted in C57BL/6 mice with vitiligo induced using 0.5% monobenzene. Animals were divided into normal control, disease control, standard (Tacrolimus 0.03% gel), and test (polyherbal gel) groups. The polyherbal formulation significantly reduced depigmentation and improved skin condition compared to the disease control. Overall, the strong correlation between in silico, in vitro, and in vivo findings highlights the polyherbal formulation as a promising candidate for topical vitiligo therapy, warranting further clinical investigation.

Keywords: Molecular docking, Vitiligo genes, *Psidium guajava*, *hibiscus rosa sinensis*, *aloebarbadensis*, anti-oxidant, anti-inflammatory.

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1. INTRODUCTION

Vitiligo is a chronic autoimmune depigmentary disorder characterized by progressive loss of melanocytes, leading to well-defined hypopigmented or depigmented skin patches. It affects approximately 0.1–2% of the global population and commonly presents between 10 and 30 years of age. Genetic factors contribute nearly 80% of cases, while environmental triggers account for the remaining 20%. The disease imposes significant psychological, social, and financial burdens due to its chronic nature, treatment resistance, and frequent relapses^{1, 2}. Oxidative stress plays a central role in melanocyte dysfunction and destruction. Excess reactive oxygen species (ROS) not only damage melanocytes but also initiate autoimmune responses mediated by cytokines such as IL-6, CXCL-9, CXCL-10, and IFN- γ , leading to further melanocyte loss. Clinically, vitiligo is classified into segmental vitiligo (SV), characterized by localized

lesions, and non-segmental vitiligo (NSV), which presents as bilateral and symmetrical depigmentation. Diagnosis is primarily clinical, supported by Wood's lamp examination and, in some cases, histopathological findings showing melanocyte depletion and T-cell infiltration.^{3,4}

1.1 History

Vitiligo has been documented for over 3000 years in ancient Vedic and Egyptian texts, where early phototherapy-like treatments were described. The global prevalence ranges from 0.5% to 2%, with higher rates (up to 4%) reported in certain populations, including South Asians. Vitiligo is also associated with autoimmune disorders, with up to 32% of patients reporting a family history. Diagnostic evaluations often include tests such as CBC, ANA, and TSH. Wood's lamp examination remains a valuable tool for early diagnosis and monitoring treatment response. Current treatment options include

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topical corticosteroids, immunomodulators, phototherapy, excimer laser therapy, and surgical interventions, though limitations such as side effects and poor patient adherence persist.⁵⁻¹²

1.2 Epidemiology

Vitiligo diagnosis is primarily based on clinical presentation, with Wood's lamp aiding detection. A recent global estimate suggests a prevalence of 0.36% for physician-diagnosed cases, increasing to 0.91% when including self-reported cases, affecting approximately 73 million individuals worldwide.¹³ Environmental factors such as chemical exposure, reduced UV radiation, and microbiome alterations contribute to disease incidence. Chemicals in cosmetics and household products can induce melanocyte damage through oxidative stress.¹⁴

Advances in melanoma immunology have enhanced understanding of vitiligo, as similar immune mechanisms target melanocytes in both conditions.¹⁵⁻¹⁸ Phenolic compounds resembling tyrosine disrupt melanogenesis, triggering oxidative stress and release of damage-associated molecular patterns (DAMPs), which amplify immune responses and disease progression.^{19,20}

1.3 Vitiligo Pathogenesis

Genome-wide studies have identified over 40 susceptibility genes, including MC1R, TYR, CD44, CD80, and HLA-A, highlighting the genetic basis of vitiligo.²¹⁻²³ The disease is primarily driven by autoimmune mechanisms, where environmental triggers and genetic predisposition lead to melanocyte destruction by CD8+ T cells. Additional contributing factors include oxidative stress, intrinsic melanocyte defects, and neurogenic influences. Current evidence strongly supports an autoimmune etiology involving complex interactions between genetic and environmental factors.²⁴⁻²⁷

2. MATERIALS AND METHODS:

2.1 Plant Selection and Description²⁸⁻³¹

Psidium guajava (guava), *Hibiscus rosa-sinensis* (hibiscus), and *Aloe barbadensis* Miller (aloe vera) are medicinal plants valued for antioxidant, anti-inflammatory, and wound-healing properties. Widely used in traditional medicine, they show potential in managing skin disorders, including vitiligo, due to their therapeutic and immunomodulatory activities.

2.2 Collection and Authentication Of Plant Materials:

Fresh leaves of *Psidium guajava*, flowers of *Hibiscus rosa-sinensis*, and leaves of *Aloe vera* were procured from local regions in Visakhapatnam authenticated by, Dr. S.B. Padal, a Botanist at Andhra University in Visakhapatnam. The plant materials were thoroughly washed with distilled water to remove dust and surface contaminants and then shade-dried at room temperature ($25 \pm 2^\circ\text{C}$) for 7–10 days. The dried plant parts were finely powdered using a mechanical grinder and stored in airtight containers until further use.

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2.3 Plant Extract Preparation:

Methanol was used as the extraction solvent in a maceration procedure. Fifty grams of powdered plant material were soaked in 200 mL of methanol and allowed to stand for 72 hours with periodic shaking. The mixture was then filtered through Whatman No. 1 filter paper, and the filtrate was concentrated on a water bath, dried, and stored in a desiccator at 4°C for further analysis, yielding a concentrated liquid extract.

2.4 Preliminary Phytochemical Screening:

Alkaloids, flavonoids, phenols, terpenoids, and saponins identified in *Psidium guajava*, *Hibiscus rosa-sinensis*, and *Aloe vera* exhibit antioxidant, anti-inflammatory, and melanocyte-protective effects, supporting vitiligo therapy³². In silico, in vitro, and in vivo studies confirmed reduced oxidative stress, decreased depigmentation, and improved skin health, validating the polyherbal formulation³³.

Table 2. Preliminary phytochemical screening of selected plant extracts

(+) = present, (-) = absent

Important bioactive substances were identified in extracts from *Psidium guajava*, *Hibiscus rosa-sinensis*, and *Aloe vera* by preliminary phytochemical screening. Alkaloids, tannins, saponins, phenolics, terpenoids, and glycosides were found in all three plants. While steroids were found in *Psidium guajava* and *Aloe vera* but not in *Hibiscus rosa-sinensis*, flavonoids were found in both plants but not in *Aloe vera*. These results suggest that the extracts contain useful medicinal phytochemicals.

2.5 Polyherbal Gel Formulation:

A polyherbal gel formulation was prepared by incorporating the previously obtained plant extracts into a suitable gel base using established pharmaceutical techniques. The extracts were uniformly integrated into the base to yield a consistent, homogeneous, and physically stable formulation intended for further experimental and pharmacological evaluation³⁴.

2.6 Computational Methods

Selected phytochemicals were subjected to computational analysis to identify potential therapeutic targets in vitiligo.

2.6.1 Disease Target Identification:

Vitiligo-associated genes were retrieved from the MalaCards database, while phytochemicals from *Psidium guajava*, *Hibiscus rosa-sinensis*, and *Aloe barbadensis*

Miller were obtained from IMPPAT. Compounds were screened using ADME and drug-likeness criteria, and their targets were predicted via Swiss Target Prediction.³²

SMILES codes were used in Swiss Target Prediction to identify probable molecular targets based on chemical similarity, generating ranked targets for further analysis.³²

2.6.2 Phytochemical Target Prediction:

Phytochemical Constituents	<i>Psidium guajava</i>	<i>Hibiscus rosa-sinensis</i>	<i>Aloe vera</i>
Alkaloids	+	+	+
Flavonoids	+	+	-
Tannins	+	+	+
Saponins	+	+	+
Phenolic compounds	-	+	+
Terpenoids	+	+	+
Glycosides	+	+	+
Steroids	+	-	+

Common targets between disease genes and phytochemical targets were identified using VENNY 2.0, highlighting overlapping genes such as CXCR3 for docking studies.³²

2.6.4 Protein and ligand preparation:

Protein structures were obtained from the Protein Data Bank, prioritizing Homo sapiens proteins with high resolution and no mutations. Ligands were retrieved from PubChem and prepared for docking.³³

2.6.3 Venn diagram analysis:

2.6.5 Molecular docking:

Docking was performed using PyRx (AutoDock Vina), with visualization in Biovia Discovery Studio. CXCR3 proteins (8HNN, 8HNN) were selected, and ligands including β -sitosterol and campesterol were analyzed. The 8HNN protein showed strong interactions with all ligands.³³

Table 3. Binding affinity of the compounds

S.no	Phytochemical (Ligand)	Pub chem Id	Binding compounds	Binding Affinity (kcal/mol)
1	Beta sitosterol	173183	8HNN 222284PDB UFF E=590.47	-9.8
2	campesterol	94225	8HNN 173183 UFF E=572.96	-9.6
3	Taraxerol acetate	10225	8HNN 94225SDF uff E=959.24	-8.0
4	cadalene	222284	8HNN 10225PDB UFF E=164.27	-6.9
5	anthraquinone	6780	8HNN 6780 PDB uff E=133.80	-6.8

Molecular docking indicated strong binding affinity of all selected compounds for the target protein from (table 2), with compound beta sitosterol and campesterol displaying (-9.8 kcal/mol) and (-9.6 Kcal/mol) from *hibiscus rosa sinensis*, *psidium guajava* and *aloe barbadensis mill* has the highest interaction and potential inhibitory activity had the

highest binding affinity among the assessed compounds, indicating a robust and sustained engagement with the receptor. Moderate binding energies were also shown by other phytoconstituents, suggesting a potential synergistic contribution to the plant extracts.

Table 4. Phytochemical constituents of *Psidium guajava*, *Hibiscus rosa-sinensis*, and *Aloe barbadensis mill*.

<i>Psidium guajava</i>								
S.No.	Name of Phytochemical	IMPAT identifier (IMPHY no)	Drug Likeness	ADME properties				
			Lipins rule	Ghose rule	Drug like score (%)	GI Absorption	BBB crossing	Bio-availability
1	Beta sitosterol	IMPHY014836	Pass (0)	Pass (0)	0.44	yes	no	0.55
2	Cadalene	IMPHY000070	Pass (0)	pass (0)	0.63	no	high	0.55
<i>Aloe barbadensis miller</i>								
1	Campesterol	IMPHY012402	Pass (1)	Pass (1)	0.47	no	low	0.55
2	Anthraquinone	IMPHY007192	Pass (1)	Pass (1)	0.57	yes	high	0.55
<i>Hibiscus rosa-sinensis</i>								
1	Taraxerol acetate	IMPHY007372	Pass (1)	Fail (3)	0.29	no	low	0.55
2	Beta sitosterol	IMPHY014836	Pass (0)	Pass (0)	0.44	yes	no	0.55

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ADME prediction methods were used to evaluate the pharmacokinetic properties of the selected phytoconstituents. The results (Table 3) indicated that most of the compounds exhibited favorable absorption, distribution, metabolism, and excretion profiles and

complied with Lipinski's rule of five. These findings suggest that the compounds possess good oral bioavailability potential and acceptable drug-like characteristics.

Table 5. Identified common genes of phytochemicals identified in plants

S.no	Medicinal Plant	Phytochemical	Number of Identified genes	Name of the genes	Gene symbol
1	<i>Psidium guajava</i>	cadalene	1	Chemokine (C-X-C motif) receptor3, tyrosinase	CXCR3, TYR
		beta sitosterol	1	Chemokine(C-X-C motif) receptor 3	CXCR3
2	<i>Hibiscus rosa-sinensis</i>	Taraxerol acetate	1	Chemokine(C-X-C motif) receptor 3	CXCR3
		Beta sitosterol	1	Chemokine(C-X-C motif) receptor 3	CXCR3
3	<i>Aloe barbadensis miller</i>	anthraquinone	1	Chemokine(C-X-C motif) receptor 3	CXCR3
		campesterol	1	Chemokine(C-X-C motif) receptor 3	CXCR3

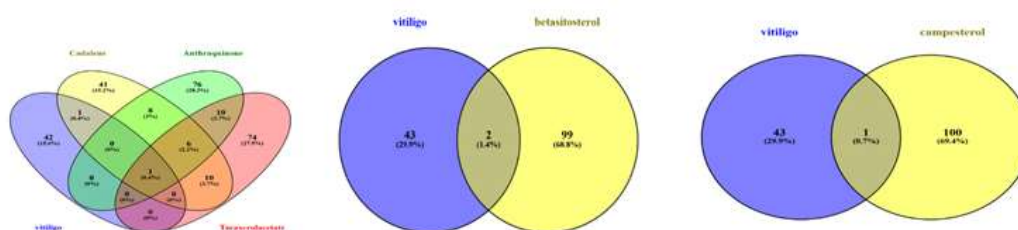


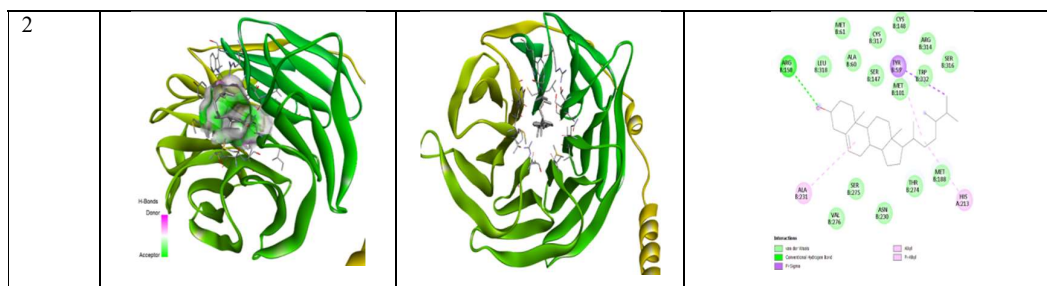
Figure 2: A) Venn diagram for identifying genes shared by the vitiligo disease genes and the plant phytochemicals anthraquinone, cadalene, and taraxerol acetate; (B) Venn diagram of genes shared by β -sitosterol and disease-associated targets; (C) Venn diagram of genes shared by campesterol and disease-associated targets.

S.No	(A) Anthraquinone h bond donor and acceptor	(B) Docking ligand	(C) 2D image of anthraquinone
1			

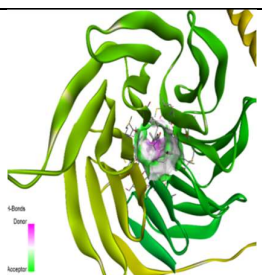

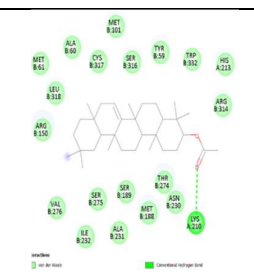
PROTEIN: 8HNN

S.No	(A)Campesterol H bond donor and acceptor	(B)docking ligand	(c) Campesterol 2D image

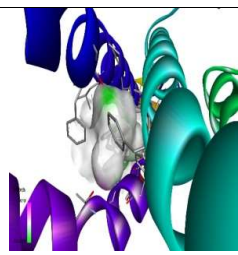
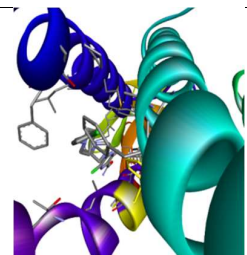
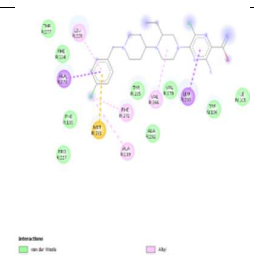
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PROTEIN: 8HNN

S.No	(A) Taraxerol acetate H bond donor and acceptor	(B) docking ligand	(C) taraxerol acetate 2D image
3			

PROTEIN: 8HNN

S.No	Cadalene H bond donor and acceptor	Docking ligand	2D diagram of cadalene
4			

PROTEIN: 8HNN

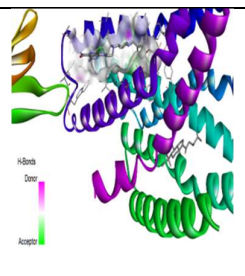
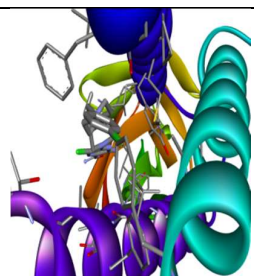
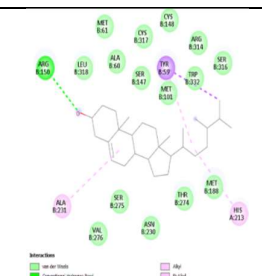
S.No	Beta sitosterol h bond donor and acceptor	Docking ligand	2D diagram of betasitosterol
5			

Figure 3: visualization analysis of ligand protein interactions with 8HNN Hydrogen bond donor and acceptor interactions, Docking ligand conformation, and 2D Interaction diagram

3.1 Determination of total flavonoid content:

Total flavonoid content of the polyherbal extract (Psidium guajava, Hibiscus rosa-sinensis, Aloe vera) was determined by the aluminium chloride colorimetric

method using quercetin as standard. Absorbance was measured at 415 nm, and results were expressed as μg quercetin equivalents per mg of extract based on the calibration curve.

% Inhibition values of polyherbal mixture and quercetin

The total flavonoid concentration (TFC) of the polyherbal mixture was estimated using quercetin as the benchmark, with results expressed in quercetin equivalents (QE). Although the overall flavonoid content of the polyherbal mixture was lower than that of the pure quercetin standard, the sample's flavonoid levels were only slightly reduced

and remained relatively close, indicating a substantial flavonoid presence in the formulation. This suggests that the polyherbal mixture contains appreciable flavonoid components, which likely contribute to its notable antioxidant capacity.

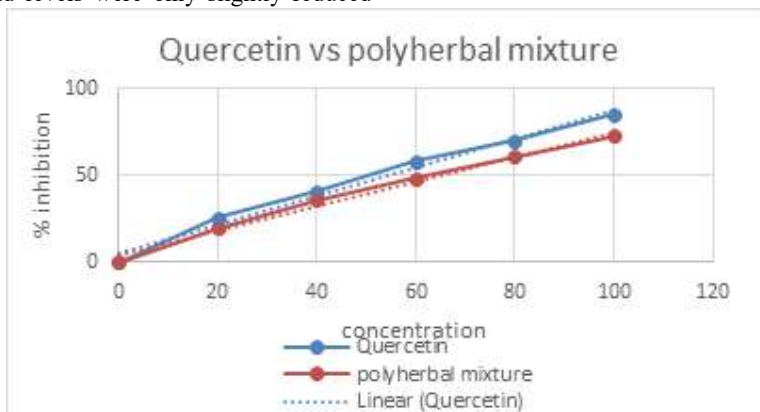


Figure 4: Inhibition of quercetin and polyherbal extract at different concentrations. The polyherbal extract and quercetin showed dose-dependent increases in percentage inhibition. At all doses quercetin continuously showed greater inhibition than the polyherbal extract. Although it was less than the norm, the polyherbal extract nevertheless showed significant inhibitory action.

3.2 Determination of total phenolic content:

Total phenolic content of the polyherbal extract (Psidium guajava, Hibiscus rosa-sinensis, Aloe vera) was determined using the Folin–Ciocalteu method. Phenolic compounds react with Folin–Ciocalteu reagent in alkaline medium to form a blue complex measured at 765 nm. Gallic acid (20–100 µg/mL) was used for calibration. Extract solutions (1 mg/mL) were treated with diluted reagent, followed by sodium carbonate, and incubated for 30 minutes. Absorbance was recorded against a blank, and phenolic content was calculated from the calibration curve and expressed as mg gallic acid equivalents per gram of extract (91.36 mg GAE/g).

% Inhibition values of polyherbal mixture and gallic acid

Gallic acid was used as the standard for the estimation of total phenolic content (TPC) in the polyherbal mixture. The results were expressed as gallic acid equivalents (GAE). The standard showed higher total phenolic content compared to the polyherbal mixture; however, the sample exhibited slightly lower but nearly comparable phenolic levels, indicating the presence of significant phenolic constituents in the formulation. This suggests that the polyherbal mixture possesses considerable phenolic content, which may contribute to its antioxidant potential

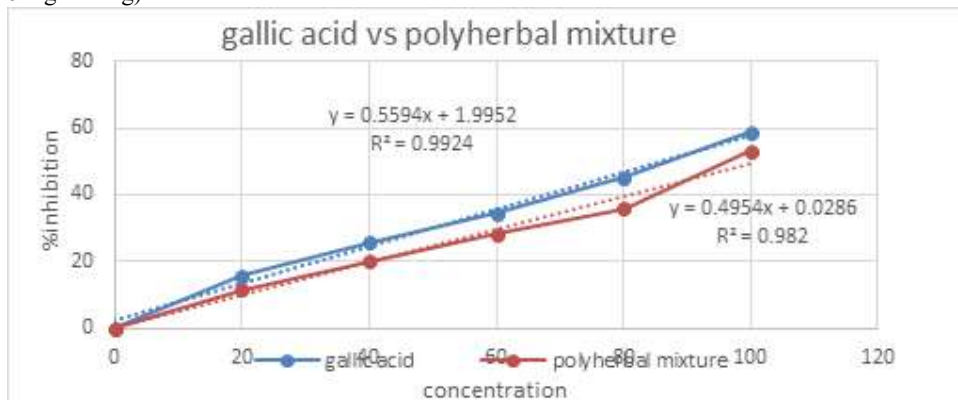


Figure 5: % inhibition of gallic acid and polyherbal mixture at different concentration. The polyherbal extract and gallic acid showed dose-dependent increases in percentage inhibition. Compared to the polyherbal extract, gallic acid showed more inhibitory action. Polyherbal exhibits substantial action and is closer to the standard.

3.3 Dpph antioxidant activity of polyherbal mixture:

Antioxidant activity of the polyherbal extract (Psidium guajava, Hibiscus rosa-sinensis, Aloe vera) was evaluated using the DPPH radical scavenging assay. The violet DPPH radical (517 nm) is reduced by antioxidants to a yellow form, with decreased absorbance indicating scavenging activity. A stock solution (1000 µg/mL) was

prepared in ethanol and diluted to obtain working concentrations. Various concentrations were tested, and absorbance reduction at 517 nm was measured using a UV-visible spectrophotometer. The assay demonstrated dose-dependent antioxidant activity of the extract, confirming its free radical scavenging potential.

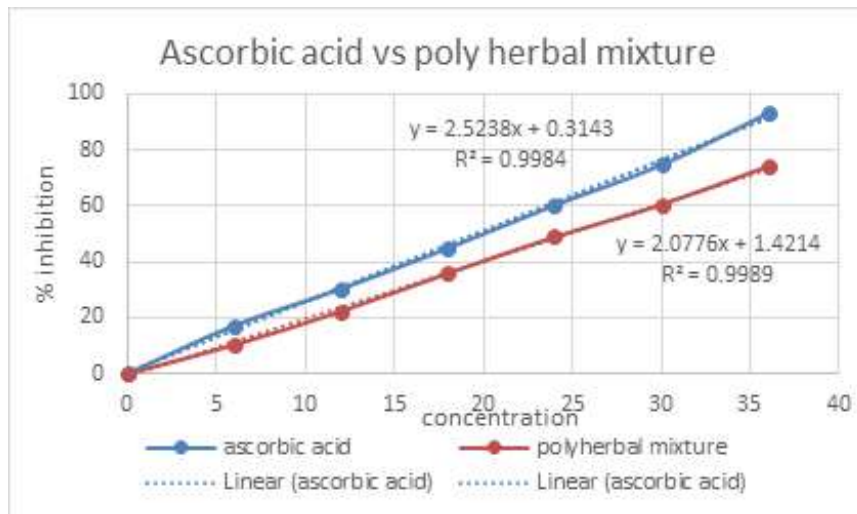


Figure 6: Both standard and poly herbal extract showed dose-dependent efficacy, ascorbic acid showed a greater percentage inhibition than the polyherbal mixture. The polyherbal mixture IC₅₀ value is moderate but significant activity was nearer to the standard.

3.4 In vitro anti-inflammatory activity (egg albumin denaturation method):

Anti-inflammatory activity of the polyherbal extract was evaluated using the heat-induced protein denaturation method, with diclofenac sodium as standard. The assay measures the ability to inhibit egg albumin denaturation, monitored by absorbance at 660 nm. Extract (50–800 µg/mL) and standard (50–400 µg/mL) solutions were incubated with phosphate buffer and egg albumin, heated, cooled, and analyzed. Percentage inhibition of protein

denaturation was calculated, indicating the extract's anti-inflammatory potential.

% Inhibition values of polyherbal extract and diclofenac sodium

The data was represented as n=3. The percentage inhibition increased with increased concentration for both standard and poly herbal mixture and the IC₅₀ value of polyherbal mixture (423.9 ± 18.0) was nearer to IC₅₀ standard Diclofenac sodium is (363.2 ± 12.0)

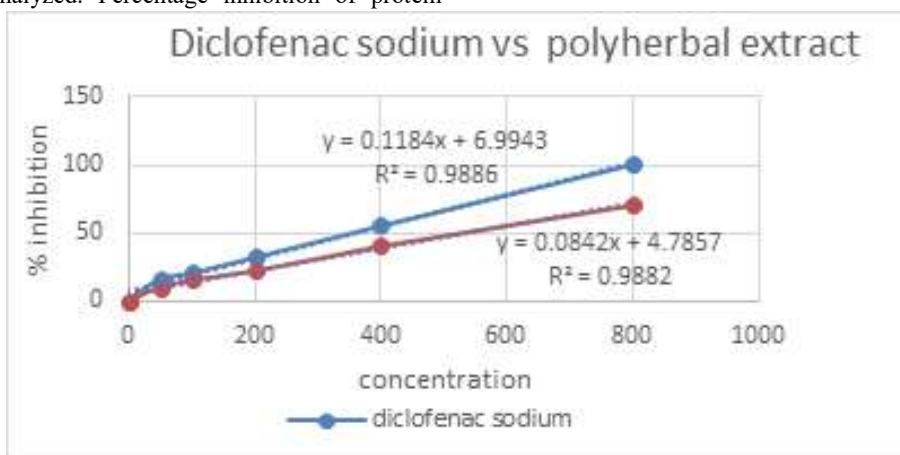


Figure 7: It showed dose-dependent anti-inflammatory efficacy, diclofenac sodium showed more inhibition than the polyherbal extract. The activity of the polyherbal extract was moderate but significant activity.

4. INVIVO STUDIES

4.1 Experimental protocol

Albino Wistar rats (150–200 g) and female C57BL/6 mice were housed under standard conditions with IAEC approval. Vitiligo was induced by topical monobenzone (0.5%) for 21 days. Animals were divided into control, disease, standard (tacrolimus gel), and test (polyherbal gel) groups. Depigmentation and tyrosinase activity were evaluated to assess repigmentation. The gel base was prepared using Carbopol 934 and propylene glycol. The study compared the polyherbal formulation with standard treatment, targeting melanocyte protection and melanin synthesis pathways.

Tacrolimus gel was prepared by dissolving the drug in propylene glycol and incorporating it into Carbopol gel, with pH adjusted to 6.0–6.5 using triethanolamine. The polyherbal gel was similarly formulated. About 100 mg was applied daily, and depigmentation was monitored periodically. The polyherbal group showed reduced depigmentation and improved pigmentation compared to disease control. Although tacrolimus exhibited superior efficacy, the polyherbal formulation demonstrated significant repigmentation, indicating its potential as an alternative or adjunct therapy for vitiligo management.

Table 10. Grouping of animals

1	Group 1	Normal control	No treatment
2	Group 2	Disease control	Monobenzone (0.5%w/v) applied topically once a day
3	Group 3	standard group	Tacrolimus gel (0.03%) applied topically once a day
4	Group 4	Test group	Polyherbal gel (100 mg) applied topically once a day

Table 10: Scoring system for assessment of depigmentation in vitiligo model

Score	Depigmentation (%)	Observation
0	0%	Normal skin
1	Less than 10%	Minimal loss of depigmentation
2	10–25%	Mild depigmentation
3	26–50%	Moderate depigmentation
4	51–75%	Marked depigmentation
5	More than 75%	Severe depigmentation

A standardized grading system based on the percentage of pigmentation loss was used for the in vivo evaluation of skin depigmentation. Higher scores indicated increasing severity of pigment loss, ranging from mild (<10%) to severe (>75%), whereas a score of 0 represented normal

skin without depigmentation. The treatment group showed reduced severity scores compared with the disease control group, indicating improved skin pigmentation and demonstrating the therapeutic potential of the polyherbal formulation.

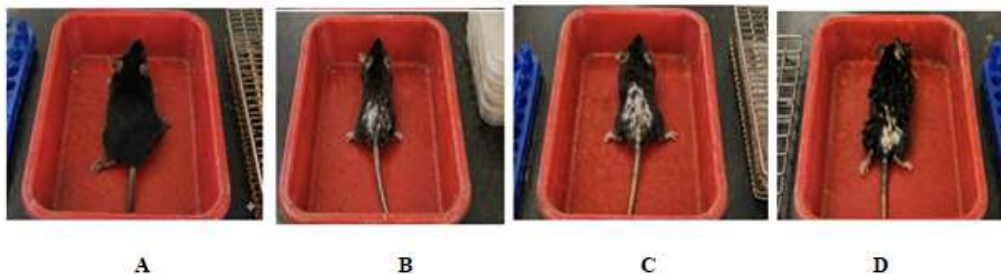


Figure 8: Images of depigmentation and repigmentation in a vitiligo model generated by monobenzone(A) Normal control with normal pigmentation(B) Disease control with severe depigmentation (C) Standard with considerable repigmentation (tacrolimus) (D) Test with moderate repigmentation (polyherbal gel).

DISCUSSION

Vitiligo involves oxidative stress, autoimmunity, and melanocyte loss. This study evaluated a polyherbal formulation using in silico, in vitro, and in vivo approaches. Docking showed strong interactions with melanogenesis targets, while in vitro studies confirmed antioxidant and anti-inflammatory activity. In vivo, the formulation reduced depigmentation and improved repigmentation in a monobenzone-induced model. Its effects, comparable to tacrolimus, suggest melanocyte-protective potential. Consistent findings across models support its therapeutic promise; however, further studies,

including clinical trials, are required to establish long-term safety and efficacy.

CONCLUSION

The current work effectively assessed a polyherbal formulation's anti-vitiligo potential using an integrated strategy that combined in vitro, in silico, and in vivo techniques. A theoretical foundation for the observed biological benefits was provided by in silico analysis, which revealed advantageous molecular interactions between the formulation's phytoconstituents and important target proteins implicated in oxidative stress and melanogenesis pathways. Significant antioxidant and anti-

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inflammatory properties were shown in vitro tests, indicating that the formulation may shield melanocytes from oxidative damage and regulate immune-mediated harm, two processes essential to the pathophysiology of vitiligo. Reduced depigmentation as contrast to the group under disease control ratings and a lower percentage of depigmentation in comparison to the disease control group demonstrated polyherbal gel therapeutic efficacy in an in vivo study utilizing a monobenzene-induced vitiligo model. The formulation's promising anti-vitiligo action was demonstrated by the degree of improvement that was comparable to that attained with the usual treatment. The reliability and interpretability of the data were improved by using a common Carbopol-based gel foundation for both the standard and test formulations, which guaranteed consistent topical drug delivery and reduced formulation-related variability. Overall, the results show that the polyherbal formulation has strong anti-vitiligo activity and may be a viable alternative or supplemental treatment option. Nevertheless, more research with larger animal cohorts and human clinical trials is necessary to completely determine its safety, effectiveness, and ideal dosage schedule. *Psidium guajava*, *Aloe vera* and *Hibiscus rosa-sinensis* contain bioactive phytoconstituents that act synergistically, highlighting their suitability for the development of polyherbal formulations. Their enhanced efficacy and multi-target pharmacological activities make such formulations widely used in herbal therapies and commercial products.

FUTURE PERSPECTIVE

Some of the clinical and toxicological research evaluations has to be performed on *aloe vera* shows that studies on vitiligo activity studies.

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INTEREST CONFLICT

Regarding the publication of this paper, the authors declare that they genuinely have no conflicts of interest.

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