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Antimicrobial and Phytochemical Studies of Hydroethanolic Extracts of *Plumbago zeylanica* (L.), a Medicinal Plant Used against Microbial Infections and Intestinal Disorders

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Antibiotic resistance remains a real public health problem. The search for new molecules to effectively combat this problem is becoming a necessity. The objective of this study was to evaluate the *in vitro* antimicrobial effect of hydroethanolic extracts of leaves and roots of *Plumbago zeylanica* and to highlight the phytochemical compounds present in these extracts. Reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853) and clinical isolates (*Escherichia coli* ESBL, *Shigella spp*, *Klebsiella spp*, *Acinetobacter spp*, *Pseudomonas aeruginosa*, *Enterococcus spp*, *Staphylococcus aureus* MRSA and *Candida albicans*) were used. The agar well diffusion and broth microdilution methods were used to evaluate the antimicrobial effect. The susceptibility of the strains varied, depending on the extract, with a best inhibitory effect of the hydroethanolic extract of *P. zeylanica* roots on *S. aureus* ATCC 29213, *S. aureus* MRSA, *Shigella spp* and *C. albicans*. The MICs obtained was between 0.63 and 5 mg/ml. The most effective antimicrobial potential was obtained with the roots extract and staphylococcal strains were the most sensitive to the tested extracts. The tested extracts contain compounds such as flavonoids, tannins, phenolic compounds, triterpenes and sterols, saponosides and cardenolipid glycosides. These results support the use of *P. zeylanica* for the treatment of microbial diseases and contribute to the search for new bioactive molecules.

Keywords: Antimicrobial resistance; Plumbago zeylanica; leaves and roots extracts; antimicrobial potential; phytochemicals.

1. INTRODUCTION

For several decades, antibiotic therapy has been the incontestable means of combating disastrous situations linked to infectious diseases and restoring health (Okou et al., 2018). The use of antibiotic molecules in human and animal health has thus been marked by relief for populations threatened with disability and sometimes death from epidemics and microbial diseases considered incurable (Vasseur, 2014). At the same time, the prescription of medicinal plants by local herbalists to combat diseases was diverted in favor of synthetic molecules promoted by conventional medicine. However, the use of these new synthetic molecules very quickly resulted in clinical failures (Kagnou et al., 2020; Ouro-Djeri et al., 2022). Their effectiveness is continually eroded following the spread of antibiotic resistance genes with the immediate effect of increasing the number of deaths and the socio-economic consequences associated with infectious diseases (Ouedraogo et al., 2017). This phenomenon, essentially accentuated by the misuse of antimicrobial molecules in human health and in the environment, favors the selection of resistant mutants and concerns all microbial strains involved in the occurrence of infectious pathologies (Gan et al., 2024). Multidrug-resistant strains have been identified in communities and in healthcare structures (Dos Santos et al., 2024). A large number of *S. aureus* responsible for post-surgical suppurations and abscesses have been shown to be resistant to cephamycins and vancomycin. Carbapenem-resistant Enterobacteriaceae have been recorded in many hospitals around the world (Epelboin et al., 2015; Sohrabi et al., 2024).

To overcome this situation, which comes on top of the relatively high cost of conventional molecules, many hopes remain placed in the secret of medicinal plants (Kagnou et al., 2020). They present, through their diversity, the greatest resource of bioactive natural substances that can act alone or in synergy on molecular targets, inhibit, interfere in cellular metabolism or sequester quorum sensing auto-inducers to thus counteract resistance mechanisms in microorganisms (Bouyahya et al., 2017; Mignanwandé et al., 2020).

Plumbago zeylanica L. (Plumbaginaceae) is a leafy subshrub native to South Asia. The leaves and roots of this plant are used by traditional medicine in decoction, dyeing or powder in Africa and Asia to combat skin diseases, microbial conditions such as leprosy, scabies, ringworm, dermatitis, abscesses, boils, itchy skin, diarrhoea, sores, leg ulcers and post-surgical suppurations (Singh et al., 2017; Shukla et al., 2021). Despite these uses in traditional medicine, little scientific work has confirmed the pharmacological effects of this plant. It is in this mindset of valorization of plant species of the African flora that the present study was undertaken with a view to evaluating the *In vitro* antimicrobial potential of hydroethanolic extracts

of the roots and leaves of *P. zeylanica* on reference strains and clinical isolates involved in the occurrence of infectious pathologies in humans.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material used consisted of leaves and roots of *P. zeylanica*. These organs, collected in Avetonou at Agou area in May 2023, were botanically identified at the Laboratory of Botanic and Plant Ecology, Faculty of Sciences, University of Lomé. These plant organs were carefully washed, dried at air condition room for two weeks, then crushed into powder (Hoekou et al., 2015).

2.2 Microbial Strains

The reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853) were provided by the National Institute of Hygiene of Lomé. The clinical germs were isolated from pus in the Bacteriology Laboratory of Saint John of God Hospital in Afagnan. The isolated microorganisms are: *E. coli* extended-spectrum beta-lactamase (ESBL), *Shigella spp*, *Klebsiella spp*, *Acinetobacter spp*, *P. aeruginosa, Enterococcus spp*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans*.

2.3 Extractions

The extraction was performed by maceration of 100 g powder from each organ in 1000 ml of hydroethanolic solution (30/70). This mixture obtained was stirred manually for 5 minutes and then left under continuous stirring for 72 hours at room temperature. The macerate obtained was successively filtered twice through hydrophilic cotton then once through Whatman N°1 filter paper and evaporated under vacuum at 40°C and at reduced pressure between 999-1000 in a rotavapor. The extracts thus collected were stored at +4°C in sterile and airtight boxes until their use (Tidiane et al., 2021).

2.4 Phytochemical Screening

The major phytochemical groups were revealed in this study by chemical tests carried out on the two hydroethanolic extracts of *P. zeylanica* according to Harbone, (1991) and EL-Haoud et al. (2018). These methods were essentially based on the principles of solubility, coloring and precipitation making it possible to highlight the presence of the main secondary metabolites.

2.4.1 Test for phenolic compounds

The reaction with ferric chloride (FeCl3) made it possible to characterize the polyphenols. To 2 ml of each hydroethanolic extract, add a drop of 2% alcoholic solution of ferric chloride. The appearance of a more or less dark blue-blackish or green color confirmed a sign of the presence of polyphenols.

2.4.2 Test for alkaloids

The alkaloids were characterized using Burchard reagents (iodo-iodide reagent) and Dragendorff reagents (potassium iodo-bismuthate reagent). 6 ml of each solution were evaporated to dryness. The residue was dissolved in 6 ml of 60° alcohol. The addition of 2 drops of Dragendorff's reagent to the alcohol solution caused a precipitate or an orange color. Adding 2 drops of Burchard's reagent to the alcohol solution caused a reddish-brown precipitate and indicated a positive reaction.

2.4.3 Test for flavonoids

Flavonoids were investigated by the cyanidin reaction. 2 ml of each extract were evaporated and the residue was taken up in 5 ml of hydrochloric alcohol diluted twice. By adding 2 to 3 magnesium shavings, heat was released followed by a pink-orange or purplish color. The addition of 3 drops of isoamyl alcohol intensified this coloring which confirmed the presence of flavonoids.

2.4.4 Test for reducing compounds

Their detection consists of introducing 2 ml of the extract into a test tube and 2 ml of Fehling liqueur is added. Then, the whole is brought to a boiling water bath for 8 min. Obtaining a brick red precipitate indicates the presence of reducing compounds.

2.4.5 Test for cardenolipid glycosides

2 ml of chloroform is added to 1 ml of the extract, the appearance of a reddish-brown color after the addition of H_2SO_4 indicates the presence of cardenolipid glycosides.

2.4.6 Test for tannins

The presence of tannins is demonstrated by adding to 1 ml of each extract, 1 ml of water and 1 to 2 drops of $Fecl_3$ solution diluted to 1%. The appearance of a dark green or blue-green color indicates the presence of tannins. The appearance of a dark green color indicates the presence of catechic tannins. The appearance of a blue-green color indicates the presence of gallic tannins.

2.4.7 Test for saponosides

To test for saponosides, 10 ml of the hydroethanolic extract were poured into a test tube. The tube was shaken for 15 secondes then left to rest for 15 min. A persistent foam height greater than 1 cm indicated the presence of saponosides.

2.4.8 Test for sterols and triterpenes

Sterols and polyterpenes were sought by the Liebermann reaction. 5 ml of each extract were evaporated on a sand bath. The residue is dissolved hot in 1 ml of acetic anhydride; 0.5 ml of concentrated sulfuric acid was added to the triturate. The appearance, at interphase, of a purple or violet ring, turning blue then green, indicated a positive reaction.

2.4.9 Test for quinones

A few drops of 1/10 NaOH were added to a tube containing 2 ml of extract to be analyzed. The presence of quinones is confirmed when the aqueous phase turns yellow, red or purple.

2.5 Inoculum Preparation

The inoculum of the strains identified for the study was prepared from 24 hours young colonies taken from Mueller Hinton or Sabouraud chloramphenicol agar and suspended into 10 ml of physiological water then adjusted to 0.5 Mac Farland. This inoculum was used to inoculate the agar plates by swabbing (Lagnika et al., 2016).

2.6 Sensitivity Tests of Strains to Plant Extracts

The agar well-diffusion method was used to investigate the sensitivity of the microbial strains to the extracts. Mueller Hinton and Sabouraud Chloramphenicol agars were used for bacteria and *C. albicans*, respectively (Hoekou et al., 2015). A solution of 20 mg/ml of each extract was prepared by dissolving the dry extract in dimethylsulfoxide 1% (DMSO). Wells of 6 mm in diameter were made in each petri dishes previously inoculated. 50 µl of each extract solution at 20 mg/ml were dispensed into these wells. Each plate was made in duplicate. The plates were then left for 30 minutes to 1 hour at room temperature then incubated at 37°C for 18 - 24 hours. Gentamicin (10 µg) and nystatin (100 µg) were used as positive controls for bacteria and yeast, respectively (Chaouche et al., 2016). DMSO 1% in sterilized distilled water served as a negative control. The sensitivity of the germs to the extracts was evaluated by measuring inhibition zone diameters around the wells.

2.7 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) was determined by microdilution method in 96 well-plates with Mueller Hinton broth for bacteria and Sabouraud broth for *C. albicans*. The microbial suspensions were diluted 10^{-2} with broth and dispensed into 96 well microplates containing a range of concentrations of each extract from 20 to 0.156 mg/ml. The plates were incubated at 37°C for 24 hours for bacteria and 48 hours for yeast. The MIC was determined as the lowest concentration of extract demonstrating no visible growth on the broth (NCCLS, 2003; Chibuzor et al., 2024).

The minimum bactericidal concentration of the extracts was determined after reading the MIC. The starting inoculum was diluted from 10^{-1} to 10^{-4}. These different solutions were inoculated onto Muller Hinton agar in 5 cm strips using a 2 µl loop, then incubated at 37°C for 24 hours. At the same time, the contents of all wells in which there was no visible growth were inoculated on Mueller Hinton agar starting from the MIC towards the highest concentrations, then incubated at 37°C for 24 hours. The MBC was determined after counting the colonies as being the lowest concentration of the extract that allowed less than 0.01% of the bacteria in the starting inoculum to survive (Sina et al., 2021).

2.8 Determination of the Antimicrobial Power of Plant Extracts (MBC/MIC)

The MBC/MIC report made it possible to highlight the modalities of action of the plant extracts

studied. A substance is said to be bactericidal when the MBC/MIC ratio ≤ 4; bacteriostatic when this ratio is greater than 4; and ineffective or tolerant on the strain studied when this ratio is greater than 32 (Sina et al., 2021).

2.9 Data Analysis

The data recorded during the antimicrobial tests were processed using Excel 2016. The results are expressed as mean ± SEM (Standard Error of the Mean).

3. RESULTS

3.1 Extraction and Phytochemical Screening

The extraction yields were 7.25% for the hydroethanolic extract of the leaves and 6.0% for that of the roots of *P. zeylanica*.

The preliminary phytochemical study revealed the presence of flavonoids, gallic and catechic tannins, phenolic compounds, sterols and triterpenes, cardenolipid glycosides and saponosides in the two hydroethanolic extracts of *P. zeylanica* studied. Reducing compounds and alkaloids were found only in the hydroethanolic extract of *P. zeylanica* leaves, whereas quinones were absent in the two extracts (Table 1).

3.2 Sensitivity of Strains to Tested Extracts

The different inhibition zone diameters obtained during the tests appear in Table 2 and Fig. 1A. The hydroethanolic extracts of the leaves and roots of *P. zeylanica* had variable effects both on the reference strains and on the clinical isolates tested. However, the root extract was more active in inhibiting the growth of all reference strains and some clinical isolates studied with inhibition zone diameters that varied from 15 to 26 mm. The highest inhibition zone was obtained on *S. aureus* ATCC 29213 (26 mm) at the concentration of 20 mg/ml. The root extract inhibited the growth of the Gram-negative bacilli isolates (*Shigella spp*, *E coli* ESBL, *P. aeruginosa*) with the exception of *Klesbsiella spp* and *Acinetobacter spp*. This extract has also been shown to be effective against *S. aureus* MRSA. Besides, the hydroethanolic extract of the leaves was only found to be active at the same concentration on *S. aureus* ATCC 29213 (19 mm) among the reference strains and on *S. aureus* MRSA (16 mm). On the other hand, it was inactive on *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. coli* ESBL, *Shigella spp*, *Klebsiella spp*, *Acinetobacter spp* and *P. aeruginosa*. Both extracts had no effect on *Enterococcus spp*. *C. albicans* used for this work was inhibited by the hydroethanolic extract of *P. zeylanica* roots while the extract of the leaves was inactive on the same isolate. The reference molecules, gentamicin (10 µg/ml) for the bacterial strains and nystatin (100 µg/ml) for *C. albicans*, also inhibited the growth of the strains used in this study with inhibition zone diameters varied from 9 to 20 mm (Table 2).

3.3 Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) of the Active Extracts

Antimicrobial parameters were presented on Table 3. The MIC of the hydroethanolic extract of *P. zeylanica* roots varied from 0.62 to 1.25 mg/ml on the reference strains. They were 0.62 to 5 mg/ml against the Gram-negative bacilli (*E. coli*, *Shigella spp*, *Acinetobacter spp*) and the Gram-positive cocci (*S. aureus*). It was 1.25 mg/ml still with the hydroethanolic extract of *P. zeylanica* roots on *C. albicans*. On the other hand, the MIC of the hydroethanolic extract of *P. zeylanica* leaves were 5 mg/ml on *S. aureus* ATCC 29213 and *S. aureus* MRSA. The roots extract of *P. zeylanica* was more active on the growth of the strains used compared to the antimicrobial effect revealed by the hydroethanolic extract of the leaves. The lowest MIC is obtained on *S. aureus* ATCC 29213 and *E. coli* ESBL with the hydroethanolic extract of *P. zeylanica* roots. The MBC of the hydroethanolic extract of *P. zeylanica* roots on bacteria ranged from 1.25 to 20 mg/ml. On *C. albicans*, the MBC was 5 mg/ml. The MBC of the hydroethanolic extract of *P. zeylanica* leaves was 20 mg/ml on the two strains of *S. aureus* studied. The roots extract showed bactericidal effect on the tested bacteria except *P. aeruginosa*. The same extract was fungicidal on *C. albicans.* The leaves extract was bactericidal on the two strains of *S. aureus.*

Table 1. Results of phytochemical screening

Secondary metabolites	Hy-Ext Ra	Hy-Ext Fe
Flavonoids	+	+
Phenols compounds	+	+
Cardenolipid glycosides	+	+
Catechical tannins	+	+
Gallic tannins	+	+
Alkaloids	-	+
Saponosides	+	+
Sterols and Triterpenoids	+	+
Quinones	-	-
Reducing compounds	-	+

+ : Presence; - : Absence, Hy-Ext Ra = Hydroethanolic Roots extract, Hy-Ext Fe = Hydroethanolic Leaves extract.

Table 2. Sensitivity of the strains studied to plant extracts

Microbial strains	Inhibition Zone Diameters (in mm)			
	Hy-Ext Ra	Hy-Ext Fe	GEN 10 µg	NS 100 µg
S. aureus ATCC 29213	26 ± 0.20	19 ± 0.00	18 ± 0.30	NT
P. aeruginosa ATCC 27853	16 ± 0.00	0 ± 0.00	16 ± 0.10	NT
E. coli ATCC 25922	15 ± 0.10	0 ± 0.00	18 ± 0.00	NT
Shigella spp	22 ± 0.00	0 ± 0.00	20 ± 0.30	NT
Klebsiella spp	0 ± 0.00	0 ± 0.00	14 ± 1.00	NT
Acinetobacter spp	0 ± 0.00	0 ± 0.00	13 ± 0.00	NT
E. coli BLSE	15 ± 0.03	0 ± 0.00	17 ± 0.10	NT
S. aureus SARM	22 ± 0.00	16 ± 0.10	19 ± 0.40	NT
Enterococcus spp	0 ± 0.00	0 ± 0.00	9 ± 0.20	NT
P. aeruginosa	15 ± 0.10	0 ± 0.00	16 ± 0.00	NT
Candida albicans	19 ± 0.10	0 ± 0.00	NT	19 ± 0.50

Hy-Ext Ra = Hydroethanolic Roots extract, Hy-Ext Fe = Hydroethanolic Leaves extract, GEN = Gentamicin, NS = Nystatin, NT = Not tested.

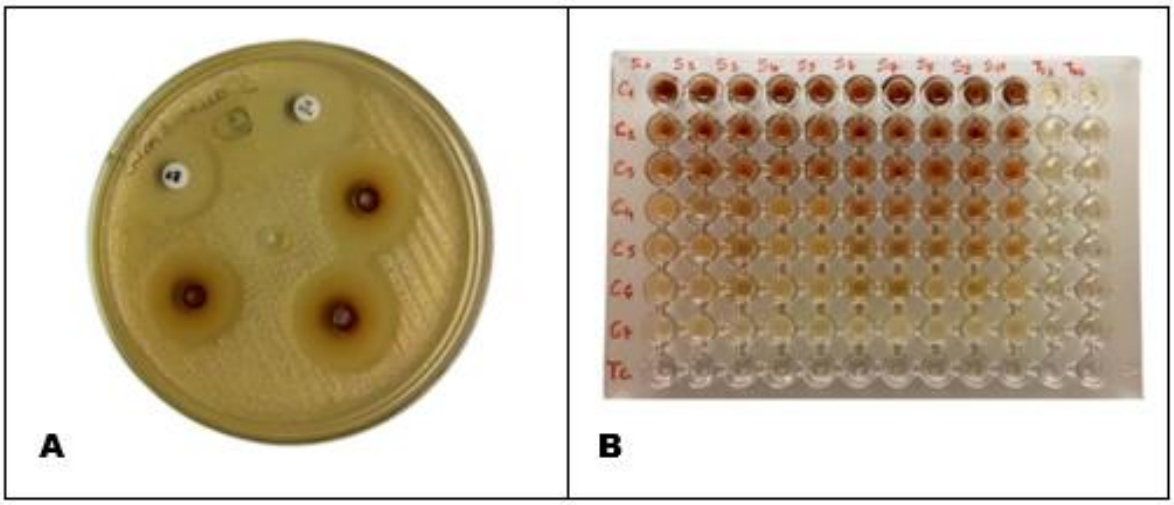

Fig. 1. Results of the sensitivity test of hydroethanolic extract of *P. zeylanica* Root on *S. aureus* ATCC 29213 at the left (A), and the determination of the MICs at the right side (B)

Table 3. Antimicrobial parameters: MIC, MBC and antimicrobial potency

Microbial strains	Types of extracts	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Activity
S. aureus ATCC 29213	Hy-Ext Ra	0.63	2.50	3.96	Bactericidal
P. aeruginosa ATCC27853	Hy-Ext Ra	1.25	5.00	4.00	Bactericidal
E. coli ATCC25922	Hy-Ext Ra	0.63	1.25	1.90	Bactericidal
Shigella spp	Hy-Ext Ra	5.00	10.00	2.00	Bactericidal
S. aureus SARM	Hy-Ext Ra	5.00	10.00	2.00	Bactericidal
E. coli BLSE	Hy-Ext Ra	0.63	2.50	3.96	Bactericidal
P. aeruginosa	Hy-Ext Ra	1.25	10.00	8.00	Bacteriostatic
S. aureus SARM	Hy-Ext Fe	5.00	20.00	4.00	Bactericidal
*S. aureus ATCC*29213	Hy-eth Fe	5.00	20.00	4.00	Bactericidal
C. albicans	Hy-Ext Ra	1.25	5.00	4.00	Fungicidal

Hy-Ext Ra = Hydroethanolic roots extract; Hy-Ext Fe = Hydroethanolic leaves extract; MIC = Minimal inhibitory concentration; MBC = Minimal bactericidal concentration.

4. DISCUSSION

Plants constitute a potential source of natural molecules essential for humans to prevent diseases, restore their health, feed and protect or even beneficial for other forms of life (Subramaniyan et al., 2018). The understanding of the pharmacological properties of phytoconstituents and the identification of new molecules of medical interest remain obvious for communities threatened with disability and sometimes death by these pests which are microorganisms. The leaves and roots of *P. zeylanica* are used in dyeing, poultices and decoctions by traditional medicine in the plateau and maritime regions of Togo to combat microbial diseases in humans and damage to the skin and mucous membranes in domestic animals and livestock. This traditional use guided the choice of hydroethanolic extraction adopted in this study using ethanol and water as solvents (Agban et al., 2020). Furthermore, this mixture of polar solvents allows the extraction of a large number of molecules depending on its affinity towards the secondary metabolites present within the plant (Tidiane et al., 2021; Bajaj et al., 2021). A fairly large yield without variability was recorded in this study during the hydroethanolic extraction carried out on the leaves and roots of *P. zeylanica*.

During the present work, carried out on the hydroethanolic extracts of the leaves and roots of *P. zeylanica*, the search for the major phytochemical groups revealed the presence of flavonoids, tannins, phenolic compounds, terpenoids and sterols in the two plant extracts while the alkaloids were found only in the hydroethanolic extract of the leaves of *P. zeylanica*. Shukla et al. (2021) reported the presence of alkaloids in the roots of *P. zeylanica*. These authors indicated that in addition to alkaloids, quinones were strongly represented within the plant. There was a difference between our results and those of these authors. Subramaniyan et al. (2018) had recorded the similar results in the ethanolic extract of *Rumex vesicarius*. This can be explained by a difference in several parameters, whether geographical, physicochemical or biological, such as: the difference in the harvest site including the environment of the plant, light, precipitation, topography, season, type of soil, harvest period, the genetic heritage of the plant or the extraction procedure used (Hoekou et al., 2015; EL-Haoud et al., 2018).

Susceptibility tests carried out with hydroethanolic extracts of the leaves and roots of *P. zeylanica* showed that the sensitivity of reference strains and clinical isolates varied from one germ to another depending on the extract. The hydroethanolic extract of *P. zeylanicaun* roots revealed a significant inhibitory effect against the growth of the majority of reference strains and clinical isolates. On the other hand, the hydroethanolic extract of leaves of *P. zeylanica* was only found to be active against *S. aureus* ATCC 29213 and *S. aureus* MRSA. The results were similar to those reported by Singh et al. (2017). These authors had meant that the alcoholic extracts of the roots of *P. zeylanica* had a greater inhibitory action compared to that revealed by the alcoholic extracts of the leaves. The variability of the antimicrobial effect between the two extracts of *P. zeylanica* on the reference strains and the clinical isolates could be explained by the variation in the concentration of active principle passing from one organ to another within the same plant. Indeed, plants

contain, in varying proportions, secondary metabolites such as phenolic compounds, alkaloids or sterols (Kagnou et al., 2020; Mignanwandé et al., 2020). These molecules with multiple properties can cross cell membranes and interact with structural constituents, interfere with metabolism in microorganisms or act by inhibiting gene products to thus sabotage their growth (Bouyahya et al., 2017). Zhu et al. (2022) reported that the two alcoholic extracts of roots and leaves of *P. zeylanica* were active against bacteria. However, these authors used the plant extracts at a higher dose (100 mg/ml) than that adopted for this study (20 mg/ml). This could explain the divergence of our results regarding the sensitivity of clinical isolates of *Klebsiella spp*, *Acinetobacter spp* and *Enterococcus spp* against the hydroethanolic extract of roots of *P. zeylanica. C. albicans* was found to be sensitive to the hydroethanolic extract of *P. zeylanica* roots. Shukla et al. (2021) reported that extracts of *P. zeylanica* were active on a large number of microscopic fungi, hence their increased uses in traditional medicine to combat skin and mucous membrane conditions in humans and domestic animals. This antimicrobial potential of *P. zeylanica* extracts was attributed by Singh et al. (2017), largely to phenolic compounds and naphthoquinones represented mainly in the roots and in lesser quantities in the other plant organs of species of the *Plumbago* genus. Plumbagin isolated from this plant is known for its effects on microbial growth. It act by inhibiting gene products to thus counteract the growth of microorganism (Shukla et al., 2021).

The highest MIC is obtained in *Shigella spp* and *S. aureus* MRSA with the root extract and the lowest is obtained in *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. The hydroethanolic extract of roots of *P. zeylanica* is found to be bactericidal on *Shigella spp*, *S. aureus* MRSA, *E. coli* ESBL, *E coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and bacteriostatic on *P. aeruginosa*. The hydroethanolic leaves extract was bactericidal against *S. aureus* MRSA and *S. aureus* ATCC29213. The same extract had no effect against the other microorganisms in this study. These results obtained are comparable to those reported by Shukla et al. (2021). According to their results, *P. zeylanica* extracts was effective on Gram-negative bacteria, Gram-positive cocci including multidrug-resistant strains of *E. coli* and certain microscopic fungi. Gentamicin and nystatin used as reference drugs respectively inhibited the growth of bacteria and fungi used for this study. The antimicrobial potential of the hyroethanolic extract of roots of *P. zeylanica* was higher than that of the gentamicin against *E. coli* ATCC25922 and *Shigella Spp*. However that of nystatin was the same as that of the hydroethanolic extract of roots against *C. albicans*. The bacteriostatic action of the hydroethanolic extract of *P. zeylanica* root against *P. aeruginosa* could be linked to the genetic variability present in this bacterium. Indeed, *P. aeruginosa* is recognized for its faculties of frequent mutations and rapid acquisition of extra-chromosomal resistance genes giving it flexibility of adaptation against antimicrobials (Pang et al., 2019; Sarkar, 2020). This work reveals the effectiveness of the hydroethanolic extract of *P. zeylanica* root compared to the hydroethanolic extract of leaves.

5. CONCLUSION

The present study has demonstrated the antimicrobial potential of *P. zeylanica* roots and leaves extracts on reference strains and clinical isolates responsible for microbial infections. Phytochemical screening revealed the presence of flavonoids, tannins, phenolic compounds, sterols and triterpenes, cardenolipid glycosides and saponosides in the two extracts studied. The extract of the roots, the most active, was found to be active against Gram-negative bacilli, Gram-positive Cocci and *C. albicans* whereas the hydroethanolic extract of the leaves inhibited only the growth of *S. aureus* strains. This antimicrobial potential could be attributed to the secondary metabolites present in the extracts analyzed. This work helped elucidate the use of *P. zeylanica* in traditional medicine to combat infectious diseases. However, further studies are required to highlight the innocuity of the roots extract of the plant, isolate and identify the bioactive molecules for the formulation of new antimicrobial drugs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Analytical Study of *Guduchyadi Rasayana*: An Ayurvedic Formulation for Academic Stress

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Guduchyadi Medhya Rasayana herb is an Ayurvedic formulation herb that is useful to improve memory. It is utilized for the stress management and improvement of cognitive health in children, advocating for the standardization of the intervention for clinical application in stress reduction among pediatric patients as well as general mental. The formulation is composed of eight herbal ingredients: *Guduchi* (Tinospora cordifolia), *Apamarga* (Achyranthes aspera), *Shankhpushpi* (Convolvulus pluricaulis), *Vacha* (Acorus calamus), *Haritaki* (Terminalia chebula), *Shatavari* (Asparagus racemosus), *Vayvihdanga* (Embelia ribes), and *Kushtha* (Saussurea lappa). This study investigates and validates the pharmacognostic and analytical properties of *Guduchyadi Rasayana*,

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with a specific focus on its efficacy in stress reduction for children. Prepared in compliance with Good Manufacturing Practices (GMP), the formulation underwent various analyses, including organoleptic, physicochemical, and thin-layer chromatography (TLC). Organoleptic testing revealed a greenish-yellow color and a pleasant fragrance. Physicochemical results indicated an alcohol-soluble extractive value of 29.94%, total ash content of 9.54%, and water-soluble extractive of 22.07%. TLC fingerprinting provided distinct Rf values (0.48, 0.51, 0.73, 0.83, 0.90), identifying several active phytochemicals that may contribute to its stress-relieving effects. The study's findings confirm that a broad spectrum of active ingredients in *Guduchyadi Rasayana* supports its standardization for clinical use. However, any clinical study can assess its efficacy in pediatric stress reduction and overall mental well-being.

Keywords: Guduchyadi rasayana; reduce stress; organoleptic Characters; physicochemical parameters; TLC.

1. INTRODUCTION

Herbal drugs possess various properties, including *Rasa, Guna, Virya, Vipaka,* and *Prabhava*, and the importance of a drug is based on these properties and its actions (Sharma, 2000). The *Sushruta Samhita* elaborates on these aspects in detail (Samhita, 2007). The action of a drug is closely linked to its chemical structure, especially the predominance of certain proto-elements (Sati, 2024). In *Ayurveda, Rasayana* drugs play a crucial role in managing age-related disorders due to their antioxidant properties. These properties help prevent free radical damage, delay aging, and rejuvenate the mind and body (Udupa and Sing, 1978). Furthermore, *Medhya Rasayana* consists of eight herbal drugs, which are particularly effective in enhancing mental functions and intellect. There is a growing demand for plant-based pharmaceuticals. However, some drug preparations lack formal standards. This study aims to demonstrate the pharmacognostic and analytical potential of *Guduchyadi Medhya Rasayana*, highlighting its beneficial effects, particularly on mental health and cognitive abilities. *Guduchyadi Medhya Rasayana* is described in authentic Ayurvedic texts such as *Chakradatta*, (2007) and *Yogaratnakara*, (2002) where it is listed under the *Rasayanadikara.* This formulation, which includes eight herbal drugs, has gained attention due to its therapeutic properties. Despite the increasing demand for plant-based pharmaceuticals, many preparations still lack proper standards. Therefore, this study seeks to explore and validate the pharmacognostical and analytical aspects of *Guduchyadi Medhya Rasayana*.

The analytical study of *Guduchyadi Rasayana* was conducted using the following parameters: organoleptic properties (appearance, color, odor, taste), physicochemical parameters (loss on drying, total ash, acid-insoluble ash, water-soluble extract, alcohol-soluble extract, pH, uniformity of weight, friability, hardness, disintegration time), as well as qualitative analysis and thin-layer chromatography (TLC).

1.1 Aims and Objectives

- Identification and authentication of raw drugs used for *Guduchyadi Rasayana*.
- This study focuses on *Guduchyadi Rasayana,* an ayurvedic formulation, and evaluates its potential to reduce academic stress in children.
- Preparation of *Guduchyadi Rasayana* at GMP-certified pharmacy.
- Organoleptic characters, Physicochemical, and TLC analysis of *Guduchyadi Rasayana*.

2. MATERIALS AND METHODS

Guduchi (Tinospora Cardifolia), Apamarga (Achyanthes Aspera), Shankhpushpi (Convolvulus Pluricaulwas), Vacha (Acorus Calamus), Haritaki (Terminalia chebula), Shatavari (Asparagus racemosus), Vayvihdanga (Embelia ribes), and *Kushtha (Saussurea lappa)* were purchased from authenticated resources at Jodhpur.

2.1 Identification and Authentication of Raw Drugs

The drug identification and authentication were done by the Department of Dravya Guna, PGIA, Karwar Jodhpur.

2.2 Methods of Preparation *Guduchyadi Rasayana*

The classical formulation of *Guduchyadi Rasayana* includes a variety of *Medhya* herbs. This section provides a comprehensive discussion of these herbs, covering their synonyms, vernacular names, constituents, properties and actions, traditional classifications, therapeutic effects, pharmacological activities, recommended dosages, and the parts used, were thoroughly discussed in this section below. The drug was prepared at the Postgraduate Institute of *Ayurved* (formerly known as the University Postgraduate Institute of Ayurved Studies and Research and University College of Ayurveda) in Jodhpur. According to classical texts, all the ingredients of *Guduchyadi Rasayana—Guduchi, Apamarga, Shankhpushpi, Vacha, Haritaki, Shatavari, Vayvihdanga,* and *Kushtha*—were taken in equal proportions of 3 kg each. A total of 24 kg of raw material was processed through a pulverizer to obtain a fine powder of *Guduchyadi Rasayan*. A loss of 600 grams was observed during this process. After complete drying in sunlight, the *Guduchyadi Rasayan* was packed into 100 gm containers. All containers were labeled and distributed to patients for 21 days.

2.3 Methods of Preparation of *Guduchyadi rasayan*

The "Protocol for Testing Ayurvedic, Siddha, and Unani Medicines,"published by the National Institute of Science Communication and Information Resources (NISCAIR), CSIR, and released by the Government of India's Department of Ayurveda, Yoga — Naturopathy, Unani, Siddha & Homeopathy (AYUSH), New Delhi, provided the foundation for the parameters used in various investigations.

2.4 Place of Work

Cultivator Phyto Lab Pvt. Ltd. Sonamukhi Nagar, Sangaria Fanta, Jodhpur.

Sample Registration No. – CPL/O/24/09/01473/1

Sample Code – CPL/ 2024/07728-N

Date of Sample sent to Lab & Sample Registration -12/09/2023

Date of start of analysis: 13/09/2024

Date of completion of Analysis-18/09/2024

Duration- 6 days

2.4.1 An analytical study was conducted under the following headings

1. Organoleptic Characters
2. Physiochemical Parameters
3. Chromatographic Fingerprint - TLC

2.4.2 Organoleptic characters

Organoleptic characteristics refer to the sensory properties of a substance that can be evaluated using the five senses: sight, smell, taste, touch, and, occasionally, hearing. In the context of herbal products and natural remedies, organoleptic analysis is crucial for assessing quality,

Table 1. Ingredients of Guduchyadi Rasayan

S.No.	Name of Drug	Latin Name	Family	Useful part
1.	*Guduchi*	*Tinospora Cardifolia*	*Menispermaceae*	Root, Stem, leaves
2.	*Apamarg*	*Achyanthes Aspera*	*Amaranthaceae*	Mula,Tandul,patra (Panchang)
3.	*Shankhpushpi*	*Convolvulus Pluricaulis*	*Convolvulaceae*	Whole plant
4.	*Vacha*	*Acorus Calamus*	*Araceae*	Mula & Bhoumik kand
5.	*Haritaki*	*Terminalia chebula*	*Combretaceae*	Fruits (dry)
6.	*Shatavri*	*Asparagus racemosus*	*Liliaceae*	Kandh
7.	*Vayvihdanga*	*Embelia ribes*	*Myrsinaceae*	Phal
8.	*Kushth*	*Saussurea lappa*	*Compositae*	Mula(Roots)

Table 2. Organoleptic Properties of Guduchyadi Rasayana

S. No.	Macroscopic Study	*Guduchyadi Rasayan*
1.	Appearance	Powder
2.	Color	Greenish Yellow
3.	Odor	pleasant

Table 3. Physio-chemical Parameters & TLC of *Guduchyadi Rasayan*

S. No.	Test Parameters	Unit	Result	Test Method
1.	Alcohol soluble extractive	%	29.94	API Part I Vol IX: 2016
2.	pH Value	-	4.81	API Part I Vol IX: 2016
3.	Total Ash	%	9.54	API Part I Vol IX: 2016
4.	Water Soluble extractive	%	22.07	API Part I Vol IX: 2016
5.	Moisture	%	8.51	API Part I Vol IX: 2016
7.	Thin-Layer Chromatography	-	0.48,0.51,0.73,0.83,0.90	API Part II Vol IV: 2017

authenticity, and overall acceptability. The color, aroma, and flavor of an herb can provide important information about its freshness, potency, and potential adulteration. This type of analysis is especially important in traditional systems of medicine, like Ayurveda, where the sensory traits of herbs are often linked to their therapeutic properties.

2.4.3 Physiochemical parameters

To ensure the safety, potency, and efficacy of the prepared formulation, several critical parameters were assessed. These included the Alcohol Soluble Extractive, which measures the amount of active constituents that can be extracted with alcohol, providing insights into the bioactive components in the formulation. The Loss on Drying at 105°C (moisture content) was evaluated to determine the level of water present, as excessive moisture can lead to microbial growth or degradation of the formulation. The pH Value was assessed to ensure the formulation maintains a stable acidic or alkaline environment suitable for its intended use. The Total Ash content was measured to determine the amount of inorganic matter left after combustion, which can indicate the presence of contaminants or adulterants. Finally, the Water-Soluble Extractive was analyzed to identify the compounds that can be extracted by water, providing additional information about the chemical profile and potential therapeutic properties of the formulation (Tamboli et al., 2021).

2.4 Alcohol-Soluble Extractive

To determine the alcohol-soluble extractive value from an Ayurvedic powder, a specific procedure is followed to accurately assess the extractive components. First, approximately 5 grams of the powdered sample are weighed with precision and placed in a glass-stoppered flask. Next, 100 ml of 90% ethanol is added to the flask, and the mixture is shaken for the first 6 hours, after which it is allowed to stand for 18 hours to ensure proper extraction. The mixture is then rapidly filtered to minimize solvent loss, and 25 ml of the filtrate is transferred into a pre-weighed evaporating dish. The solvent is carefully evaporated in a water bath, and the residue is dried at 105°C until it reaches a constant weight (Indian Pharmacopoeia Commission, 2010). Finally, the residue is weighed, and the percentage of alcohol-soluble extractive is calculated using the appropriate formula, which allows for an accurate evaluation of the extractable compounds from the Ayurvedic powder (Gyansanchay, 2022).

> Percentage of alcohol-soluble extractive= (Weight of residue / Weight of sample) ×100

This method aids in identifying the quantity of active ingredients that dissolve in alcohol, which can be vital for the effectiveness of Ayurvedic formulations (Pawar and Jadhav, 2015).

Moisture Content (Loss on Drying at 105°C): The process for determining the loss on drying (LOD) begins with sample preparation, where a clean, dry, and pre-weighed glass-stoppered shallow weighing bottle is used. Approximately 1-2 grams of the sample are carefully weighed into the bottle. Next, the bottle containing the sample is placed in a drying oven set at 105°C. The stopper is removed and placed in the oven alongside the bottle to ensure proper drying. The sample is dried to a constant weight, typically for

about 3 hours. Once drying is complete, the bottle is removed from the oven and immediately sealed with the stopper. The bottle is then allowed to cool to room temperature in a desiccator to prevent moisture absorption. After cooling, the bottle with the dried sample is reweighed (Quirino, 2023). The loss on drying (LOD) is then calculated using the formula

LOD (%) = (Initial Weight−Final weight / Initial weight) ×100

This method helps assess the moisture content and volatile substances in the sample, which is essential for quality control (Quirino et al., 2023; Brasileiro, 2023).

2.4.1 pH Value determination

To determine the pH value of an Ayurvedic powder, a specific procedure is followed to assess its acidity or alkalinity. First, 1 gram of the Ayurvedic powder is weighed accurately and dissolved in 100 ml of distilled water to prepare a 1% w/v solution. The solution is thoroughly stirred to ensure the powder is completely dissolved. The pH meter is then calibrated using standard buffer solutions, typically at pH 4.0, 7.0, and 9.0, to ensure accurate readings. After calibration, the electrode is rinsed with distilled water and blotted dry before being immersed into the prepared solution. The pH value is recorded once the reading stabilizes. After measurement, the electrode is cleaned and stored according to the manufacturer's instructions to maintain its longevity and accuracy. This method is essential for determining the pH value of the Ayurvedic powder, which provides insights into its potential effectiveness and stability, as the pH can influence the solubility and bioavailability of its active components (Indian Pharmacopoeia Commission, 2010).

2.4.2 Total ash value determination

To determine the total ash value of a churn, a specific procedure is followed. First, 2 grams of the churn are accurately weighed and transferred to a previously ignited and tared silica crucible. The crucible is then gradually heated to a temperature range of 500-600°C, ensuring the material turns white, which indicates complete combustion of the sample. Once the ignition is complete, the crucible is allowed to cool in a desiccator to room temperature to prevent moisture absorption. After cooling, the crucible with the ash is weighed. The total ash value is then calculated using the appropriate formula, providing important information about the inorganic content of the churn.

Total Ash (%) =(Weight of ash / Weight of sample)×100

This method helps assess the quantity of inorganic material in the churna after complete incineration (Eshmukh, 2024).

2.5 Water Soluble Extractive Determination

To determine the water-soluble extractive value of a churn, the following procedure is carried out. First, 5 grams of the churn are accurately weighed and placed into a glass-stoppered flask. Then, 100 ml of chloroform water (a mixture of chloroform and water) is added to the flask. The mixture is shaken frequently for the first 6 hours to ensure thorough mixing, then allowed to stand for 18 hours to facilitate proper extraction. After this maceration period, the mixture is rapidly filtered to prevent any loss of solvent. Next, 25 ml of the filtrate is transferred into a pre-weighed evaporating dish. The solvent is then evaporated using a water bath, and the residue is dried at 105°C until it reaches a constant weight. Finally, the residue is weighed, and the percentage of water-soluble extractive is calculated using the appropriate formula, which provides insight into the extractable components of the churn.

Percentage of water-soluble extractive = (Weight of residue / Weight of sample)×100

This method aids in measuring the quantity of active ingredients that dissolve in water, which is essential for the formulation's therapeutic effects (Kolhe, 20210).

2.6. PHYTOCHEMICAL PARAMETERS

2.6.1 Fingerprinting of *Guduchyadi Rasayan* by thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was used to develop a chromatographic fingerprint for Guduchyadi Rasayana, which serves as a unique profile of its chemical constituents. TLC allows for identifying key compounds present in the formulation, aiding in quality control and standardization. This technique is particularly useful in confirming the presence of active ingredients and ensuring consistency in the formulation's composition.

To perform Thin Layer Chromatography (TLC) on an Ayurvedic powder (Churna), a systematic procedure is followed. First, approximately 1 gram of the Churna is weighed, and the sample is extracted with a suitable solvent, such as methanol or ethanol, by shaking it for 30 minutes. The extract is then filtered and concentrated to a smaller volume. A pre-coated silica gel TLC plate is used for the analysis, and a baseline is drawn about 1 cm from the bottom of the plate using a pencil. Small spots of the concentrated extract are applied along the baseline using a capillary tube or micropipette, and the spots are allowed to dry completely. The TLC plate is then placed in a TLC chamber containing a prepared mobile phase, such as a mixture of chloroform and methanol, ensuring that the baseline remains above the solvent level. The chamber is covered, and the solvent rises up the plate through capillary action until it reaches about 1 cm from the top. After the development process, the plate is removed, dried, and the spots are visualized either under UV light or by spraying with a suitable detecting reagent, such as iodine vapor or anisaldehyde-sulfuric acid. Finally, the distance traveled by each spot and the solvent front are measured to calculate the Rf (retention factor) values, which provide information about the individual components in the Ayurvedic powder. Calculate the Rf value for each spot using the formula:

Rf= Distance travelled by the compound / Distance travelled by the solvent front

This procedure helps in identifying and analyzing the compounds present in the Ayurvedic formulation, enabling quality control and standardization (Chaudhary and Kumar, 2020).

TLC Profile (API, 2017):

Sample Name: *Guduchyadi Rasayan*

sample Id: CPL_2024_07728

Sample Preparation: 1ml in 10ml methanol.

Mobile Phase: Toluene: Chloroform: methanol

Derivatization: Anisaldehyde +Sulphuric Acid +Acetic Acid

Sample Injection volume: 10 µl

Solvent distance travelled: 8 cm.

Visualization after derivatization Rf Value: 0.48,0.51,0.73,0.83,0.90

3. RESULTS

The formulation of *Guduchyadi Rasayana* was prepared following standard operating procedures at the GMP-certified Nagarjuna Pharmacy of the Post Graduate Institute of Ayurveda and subjected to quantitative analysis. An analytical study of *Guduchyadi Rasayana* was conducted at Cultivator Phyto Lab Pvt. Ltd. in Sonamukhi Nagar, Sangaria Fanta, Jodhpur, to evaluate its organoleptic characteristics, physicochemical properties, and chromatographic analysis. The results of the pharmaceutical analysis are discussed below.

Organoleptic Characteristics: The organoleptic characteristics of *Guduchyadi Rasayan* in powder form include a greenish-yellow color and a pleasant odor. In contrast, the powder form of *Guduchyadi Rasayan* is characterized by a reddish-brown color and also has a pleasant odor. The typical color and odor of *Guduchyadi Rasayan* is greenish-yellow with a pleasant aroma. The justification for the powder's color and odor is that the contrasting colors and forms (powder) suggest different processing methods and possibly varying herbal constituents. The pleasant odor in form indicates a favorable aromatic profile, which is essential for patient acceptance and therapeutic efficacy.

Physicochemical Parameters: The physicochemical parameters of *Guduchyadi Rasayan* were evaluated based on criteria from API I, Vol. IX, 2016, which were shown as follows:–

1. **Alcohol-Soluble Extractive**:-The alcohol-soluble extractive was reported at 29.94%, indicating that *Guduchyadi Rasayan* effectively extracted active principles, enhancing its therapeutic efficacy. This value fell within the range of 20-30%, suggesting consistency in preparation methods.
2. **Loss on Drying (Moisture):-** A moisture content of 8.51% was deemed reasonable, ensuring that the product maintained stability without becoming overly dry, which could degrade active ingredients.
3. **pH:-** With a pH of 4.81, *Guduchyadi Rasayan* was mildly acidic, which could benefit the solubility of certain compounds

and enhance absorption in the gastrointestinal tract.

4. **Total Ash Content:-** The total ash content of 9.54% reflected the presence of inorganic minerals. This value was acceptable and suggested a quality product rich in beneficial minerals
5. **Water-Soluble Extractive:-** At 22.07%, this value indicated that a significant proportion of the compounds were soluble in water, which was crucial for the bioavailability of herbal medicines. This fell within the typical range of 15-25%, further affirming the product's quality.

Thin layer Chromatography (TLC) - The chromatographic fingerprint (TLC) analysis of *Guduchyadi Rasayan* was conducted using a 1 ml solution in 10 ml of methanol, with a mobile phase of chloroform and methanol. The solvent traveled a distance of approximately 8 cm, and after derivatization, the final Rf values obtained were 0.48, 0.51, 0.73, 0.83, and 0.90. The TLC analysis of Guduchyadi Rasayan yielded Rf values of 0.48, 0.51, 0.73, 0.83, and 0.90. When compared to typical Rf ranges for herbal extracts, it showed interpretation as

- **Rf 0.48**:- The normal range was 0.4 - 0.6, suggesting polar compounds, often critical for therapeutic effects.
- **Rf 0.51**:- Also within the 0.4 - 0.6 range, indicating similar polar constituents that could enhance the efficacy.
- **Rf 0.73**:- Fits within the 0.7 - 0.9 range, reflecting moderately polar compounds that contributed to the formulation's complexity.
- **Rf 0.83**:- This value was consistent with the 0.7 - 0.9 range, indicating non-polar to moderately polar substances that might enhance therapeutic action.
- **Rf 0.90**:- Within the 0.8 - 1.0 range, suggested less polar compounds, valuable for broadening the spectrum of activity. The diversity of Rf values confirms effective extraction methods, highlighting the presence of a rich array of phytochemicals. This complexity was essential for the synergistic effects often seen in herbal formulations, supporting the overall therapeutic potential and quality of *Guduchyadi Rasayan.* Thus, the results were not only aligned with expected norms but also underscored the formulation's efficacy.

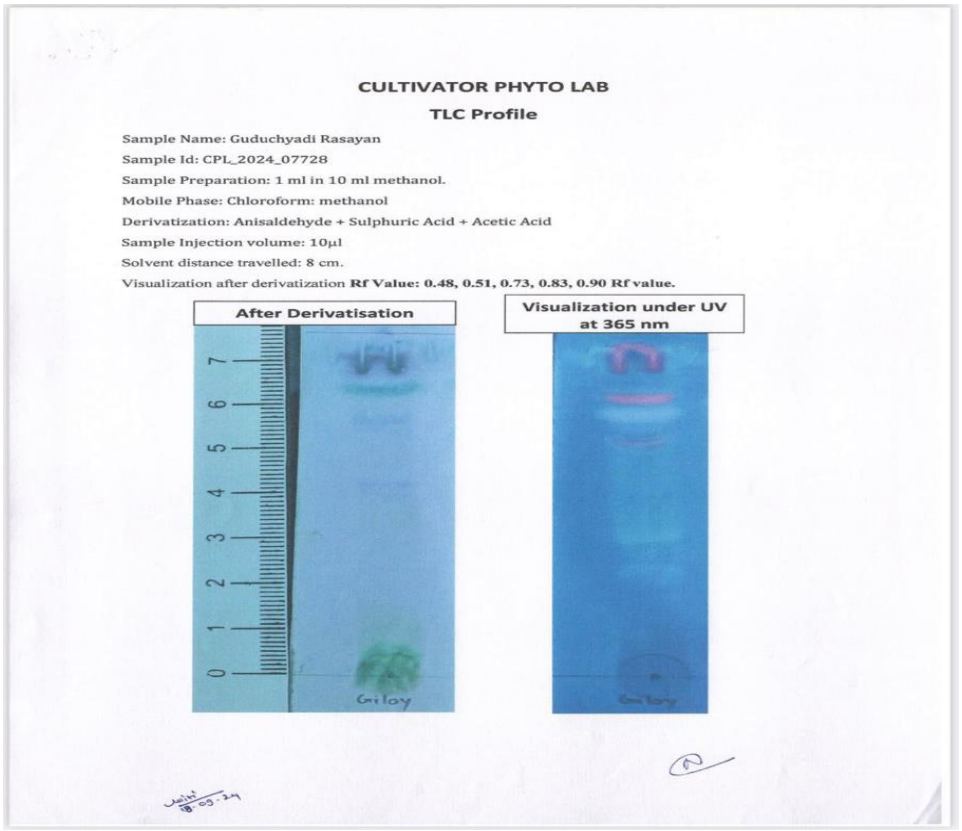

Fig. 1. (TLC Profile sample) (Cultivator Phyto Lab Pvt. Ltd, 2019)

4. DISCUSSION

The analytical study of *Guduchyadi Rasayana* was carried out to assess its organoleptic, physicochemical, and chromatographic properties, which are essential for its quality control and therapeutic efficacy. The formulation, prepared at a GMP-certified pharmacy, was subjected to rigorous testing at Cultivator Phyto Lab Pvt. Ltd. in Jodhpur. The organoleptic characteristics revealed that the powder form of *Guduchyadi Rasayana* had a greenish-yellow color and a pleasant odor. These sensory properties are important, as they reflect the quality and acceptability of the formulation, which is crucial for its therapeutic effectiveness. Physicochemical analysis provided critical insights into the formulation's composition and stability. The alcohol-soluble extractive content of 29.94% indicates a high concentration of active compounds that are likely to contribute to the therapeutic actions of *Guduchyadi Rasayana*. This result is consistent with the expected range, affirming the efficacy of the extraction process used in preparation. The moisture content of 8.51% and a pH value of 4.81 suggest that the formulation is stable and mildly acidic, which may enhance the solubility of certain compounds, thus improving their bioavailability. The total ash content of 9.54% is a reflection of the inorganic material in the formulation, while the water-soluble extractive content of 22.07% indicates a significant proportion of bioavailable compounds that dissolve in water, facilitating their absorption in the body. Thin Layer Chromatography (TLC) fingerprinting revealed multiple Rf values (0.48, 0.51, 0.73, 0.83, and 0.90), demonstrating the presence of a diverse array of chemical constituents. These values are typical for herbal formulations, indicating a mix of polar and moderately polar compounds, each contributing to the overall therapeutic efficacy. The variety of Rf values confirms the presence of a broad spectrum of active ingredients, ensuring the formulation's therapeutic potential. Overall, the findings underscore the high quality and therapeutic potential of *Guduchyadi Rasayana,* supporting its use in traditional and modern medicinal practices. The comprehensive analysis provides a strong basis for the standardization and validation of this formulation in clinical applications.

5. CONCLUSION

The Ayurvedic system of medicine is increasingly being relied upon for stress management, various health issues, and particularly lifestyle diseases. The ingredients used in these formulations have been pharmacognostically identified, authenticated, and utilized for preparation. Any plant or formulation used medicinally requires a detailed study before its use, as the therapeutic efficacy depends on the quality of the ingredients involved in the preparation of the medicinal product (Vaidya and Kulkarni, 2014). The prepared drug, *Guduchi Rasayana*, was pharmacologically subjected to physicochemical analysis, qualitative testing, and Thin Layer Chromatography (TLC). The ingredients of *Guduchi Rasayana* include *Guduchi, Apamarga, Shankhpushpi, Vacha, Haritaki, Shatavari, Vayvihdanga,* and *Kusth*, making it an herbal formulation. In this study, *Guduchi Rasayana* was prepared per classical references, following standard operating procedures at a GMP-certified pharmacy (Srinivasan and Sreevidya, 2015). The raw drugs were identified and authenticated before use. The drug underwent physicochemical analysis, qualitative testing, and TLC analysis. The present analytical study proved that This study aims to establish the groundwork for the standardization of *Guduchi Rasayana* and to prepare a monograph for the Ayurvedic Formulary of India (AFI).

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Azadirachta indica Natural Active Ingredients to Cure Mpox: *In silico* Targeting VP39

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

VP39 is an essential enzyme in the Mpox virus acting as methyltransferase for RNA capping to translate viral mRNA during human infection. This enzyme adds a 7-methylguanosine cap at the 5' end of viral mRNAs, imitating the capping mechanism of the host cells to evade immune detection. Efficient RNA capping carried out by VP39 enhances protein expression and viral replication, thereby contributing to the pathogenesis of the virus. Thus, the agent VP39 is highly attractive as a

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therapeutic target for inhibiting Mpox virus replication. In silico work were performed on the 2'-O-methyltransferase activity in VP39 and explored for inhibition by natural compounds from *Azadirachta indica* (neem). Stigmasterol, which is the most active of the phytochemicals in the neem, is a phytosterol exhibiting anti-inflammatory, antioxidant, and immune-modulatory properties. Molecular docking studies using Autodock Blow on the SeamDock web-based platform indicate a binding affinity of -7.98 kcal/mol of stigmasterol with the enzyme VP39. This negative figure indicates strong interactions and can be interpreted to put stigmasterol in contention as an antagonist for VP39-mediated RNA capping. This would disrupt viral replication. For this reason, understanding compound-protein interactions is essential in paving a better path toward neem-based therapies. Such linkages of the inhibitory action of stigmasterol and other neem compounds to the activity of VP39 would form part of their therapeutic relevance. Linking findings of molecular docking with drug discovery clearly show the need to find compounds with strong binding affinities to prime viral targets. However, many hurdles need to be crossed before all this interesting progress can be translated into a fairly clinical setting, including verifying efficacy and safety at the level of pharmacokinetics, standardizing different compounds, and obtaining regulatory approvals by means of robust clinical trial programs. All of these phases are necessary for pre-discovery in therapeutic applications against the Mpox virus.

Keywords: 2'-O-methyltransferase VP39; Mpox; SeamDock; Azadirachta indica; AutoDock.

1. INTRODUCTION

The Mpox virus is native to Central and West Africa, where it can be transmitted from animals to humans. There is growing concern around the world about the potential of the virus to trigger a pandemic. The Democratic Republic of Congo reported the first documented case in 1970 (Upadhayay et al., 2022). The exact number of cases remains unclear. The signs of Mpox are similar to those of a milder form of smallpox (Skvara et al., 2023). Cases of Mpox have been detected in more than 100 countries, raising concerns of a possible pandemic. Despite these fears, the mortality rate of the latest outbreak is significantly lower than originally thought. Although Mpox remains a public health concern, the lower mortality rate suggests that the disease may not be as dangerous as previously thought. This positive development suggests that efforts to control the spread of the disease are effective (Duarte et al., 2024). However, vigilance remains crucial to prevent further transmission and mitigate the impact on public health. To date, there are two main variants of the virus: the Central African strain (Clade-I) and the West African strain (Clade-II) are identified around the world (Inungu et al., 2019; Americo et al., 2023. Clade-I spreads more easily, leads to more severe disease, and has a mortality rate of up to 11%. Clade-II has a mortality rate of less than 1%. It does not appear to be transmissible from person to person. The current global epidemic has heightened concerns that Clade II could spread and develop into a pandemic. This shows how important it is to promote knowledge and monitor the situation. The World Health Organization (WHO) has declared a public health emergency of international concern (PHEIC) in response to the global Mpox outbreak. This declaration, made under the International Health Regulations (2005), underscores the serious nature of the outbreak and the need for coordinated international action to prevent further spread and mitigate the impact (World Health Organization, 2024). Recently, the Clade II variant of the current outbreak has also been observed in India. The Indian government has prepared for it and taken all important measures to control the spread of the virus infection (Pal et al., 2024; MoHFW, 2024). As Li et al. (2023) stated, the mortality rate for this disease was between 2% and 7% in the past. But the viral disease is spreading rapidly. Global health organizations such as the WHO and the CDC have become increasingly concerned with it in recent years (Li et al., 2023; CDC, 2022).

The 2'-O-methyltransferase VP39 (2'o-MT-VP39) of the Mpox virus is an important enzyme. It methylates the 2'-hydroxyl group of the ribose in the viral mRNA. In this way, it alters the viral copying process. This methylation process stabilizes the viral genes. It contributes to their proper folding and ensures efficient translation in the host cells. VP39 mimics the host's mRNA (Zgarbová et al., 2023). This helps the virus to evade the host's immune defenses (Kumar et al., 2024; Kmiec & Kirchhoff, 2022). It enables viral replication and survival. Inhibition of VP39 could interrupt this process. This would impair viral replication and reduce disease symptoms. Thus,

VP39 is a promising target for antiviral drugs against Mpox (Saghazadeh & Rezaei, 2023). The scientists want to understand how VP39 causes methylation. This could lead to new drugs that block its activity. These therapies could stop the viral progression of Mpox. To this end, they prevent the enzyme from stabilizing the viral genome. They offer a new way to fight this infectious disease (Silhan et al., 2022; Lu et al., 2023; Kumar et al., 2024; Silhan et al., 2023).

Azadirachta indica, or neem, is a medicinal plant. Researchers know it for its antiviral properties. Neem is rich in bioactive compounds such as azadirachtin and nimbin. Researchers have studied it for its potential to treat various viral infections. Its compounds have an antiviral effect via several mechanisms. They are therefore a versatile remedy against viral diseases (Alzohairy, 2016). Research shows that the extract of the plant is highly effective against HSV, HIV, the dengue virus, and hepatitis B. For example, quercetin, a flavonoid in neem, inhibits viral replication. It does this by impairing the synthesis of viral proteins (Wylie & Merrell, 2022). Gedunin and azadirachtin strengthen the immune system. They help the host fight viral infections. Neem extracts can prevent viruses from entering the host cells. This disrupts the early stages of the infection. Neem can inhibit viral replication and enhance the immune response. It therefore offers a promising natural approach to antiviral therapies (Foka et al., 2022). This is important for combating new and re-emerging viral threats such as Mpox. Its broad antiviral activity and low toxicity make it a good candidate for research. Here, the study proposed using molecular docking to find new drugs for the Mpox virus. This method can rapidly assess compounds for their ability to bind to viral targets. It may reduce viral virulence.

2. MATERIALS AND METHODS

2.1 PDB Retrieval, Protein Structure, and Ligand Validation

The 3D file 8B07.pdb shows the 2'-O-methyltransferase VP39 (2'o-MT-VP39) of Mpox. This viral protein supports the poly(A) polymerase in its work and has a small subunit. The RCSB is a global library of structural data on biological macromolecules. These were discovered using techniques like nuclear magnetic resonance (NMR). The RCSB has made this file available. Two chains, A and B, form the structure 8B07:pdb. The pdb file structure is a heterodimer consist of chain A and chain B. Here, chain A preferred over the chain B for our study (Silhan et al., 2022; Szabadka & Grolmusz, 2006). Analysis of the tertiary structure 2'o-MT-VP39 (8B07: chain A) showed high model quality (Szabadka & Grolmusz, 2006). An excellent ERRAT score supports its reliability. We used a tool, PROCHECK, to check the model's quality and stereochemical properties. ERRAT and PROCHECK are critical in protein model accuracy assessment. ERRAT assesses the non-bonded interactions between atoms from a view of actual versus expected atomic distances, giving a quality factor, which assigns high model reliability when above 90%. This tool can help identify erroneous modelling and improve the very protein model. PROCHECK assesses stereochemical quality based on the two dihedral angles φ and ψ displayed on a Ramachandran plot. A Ramachandran plot shows four regions: most favoured, additionally allowed, generously allowed, and disallowed. More residues in the most favoured regions mean better structural accuracy. This analysis shows how the protein in three-dimensional space has been mapped along torsion angles and the positioning of side chains concerning the protein backbone. This information is key to identifying structural errors and investigating conformational stability. Therefore, ERRAT and PROCHECK would guarantee the reliability of the structural model, which becomes an irreplaceable aid in molecular docking and drug design. The tools improve the quality of computational models, which helps locate the following lead compound against a given target. In this work, the Ramachandran plot was instrumental in tracking amino acid residues' distribution concerning different structural regions (Kumar et al., 2017; Kumar et al., 2020); this information is needed in understanding protein interaction with other molecules. Instead, with molecular docking, the natural compounds from *Azadirachta indica* were studied for a potential role as chemotherapeutics against the Mpox virus. This strategy seeks new treatments by focusing on a few key viral proteins and impairing their functions. We targeted the binding pockets in 2'o-MT-VP39. To start this study, we obtained the 3D structures of all-natural inhibitors in "*.sdf" format from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2019). Our literature search found 13 bioactive compounds in *A. indica*. We then included them in our study. The compounds included: azadiradione, campesterol, epoxyazadiradione, gedunin, linoleic acid, meliantriol, nimbin, oleic acid, quercetin, palmitic

acid, scopoletin, stigmasterol, and umbelliferone. To further check these compounds, we used the SwissADME tool. It assesses their ADMET properties: absorption, distribution, metabolism, excretion, and toxicity. This analysis lets us choose the compounds with the best docking scores (Daina et al., 2017; Kumari et al., 2021). It identified promising candidates for further study. We aim to find new ways to treat Mpox infections. We will investigate how natural compounds bind to the 2'o-MT-VP39 protein. This research could help develop new cancer drugs. It may improve treatment outcomes for those affected by the disease.

2.2 Molecular Docking

Molecular docking is a computer method. It predicts how proteins and other molecules, such as ligands, interact in 3D. It helps to find binding sites and understand their interactions. This method uses search algorithms and scoring functions. They find the best 3D position of the ligand in the target protein and estimate its binding affinity. SeamDock, a web server designed for ease of use, simplifies this. It displays a 3D grid of the active site of the target protein. It also visualizes the ligand-protein interactions (Murail et al., 2021; Jyoti et al., 2023. This tool allows you to view the stereochemistry of the binding site. This includes the binding pocket, the surrounding amino acids, the hydrogen bonds, and the polar contacts. It also shows how these things affect the shape of the protein. SeamDock's user interface and NGL viewer allow for interactive visualization (Murail et al., 2021; Jyoti et al., 2023; Bugnon et al., 2024). They make the program simple even for non-experts. No complex installations or configurations are required. The docking process starts as soon as we have defined the molecules (ligand and receptor), the search area (docking box), and the simulation parameters (Mahato et al., 2017). In AutoDock, the process involves two steps: First, prepare a grid parameter file using prepare_gpf4.py to define the search space of the receptor. Then you use autogrid4 to calculate the energy landscape for potential binding sites. The prepare_dpf4.py script generates a docking parameter file. AutoDock4 uses this together with the ligand and receptor structures. It completes the simulation and finds the best ligand-receptor interaction (Bugnon et al., 2024; Hetényi & Bálint, 2020).

3. RESULTS AND DISCUSSION

We used the user-friendly online server SeamDock for molecular docking, despite our limited knowledge of biophysics and computer science. SeamDock facilitated the analysis and 3D visualization of ligand-receptor interactions. The predicted docking score was based on the binding energies and affinities of the ligand-receptor complexes. This was done during the docking process. We used AutoDock 4 to dock the ligands to the protein binding sites. We then used evolution to find the best way for the ligand and protein to bind. To this end, we found the configuration with the lowest energy. Factors such as binding energy, solvent effects, entropy, and molecular flexibility must be considered to evaluate the interaction between a ligand and a protein. This information can generate a 3D interaction model and an affinity value. The ERRAT analysis, indicating that 94.815% of residues were above the 95% error cutoff, confirms the reliability of the protein structure used in the modelling (Fig. 1).

The ProSA energy profile confirmed this result. It gave a Z-score of -7.22 kcal/mol, showing that the model was reliable (Fig. 2). The energy profile compared to the amino acid residues confirmed the structure and accuracy of the model.

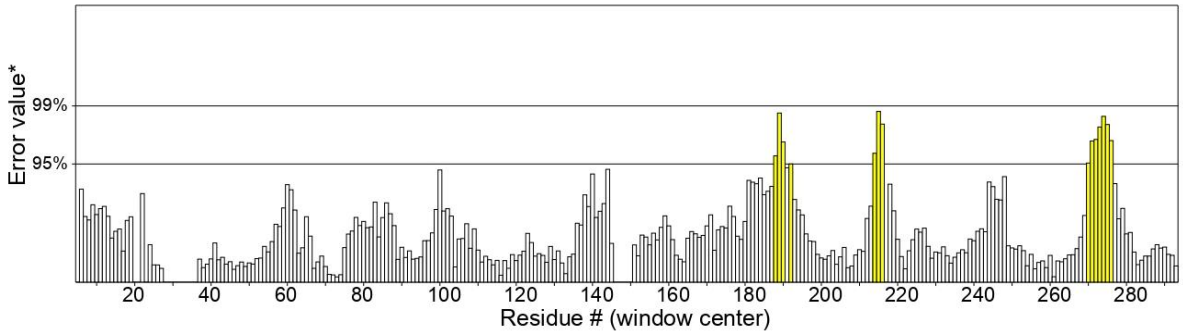


Fig. 1. ERRAT has checked the general quality

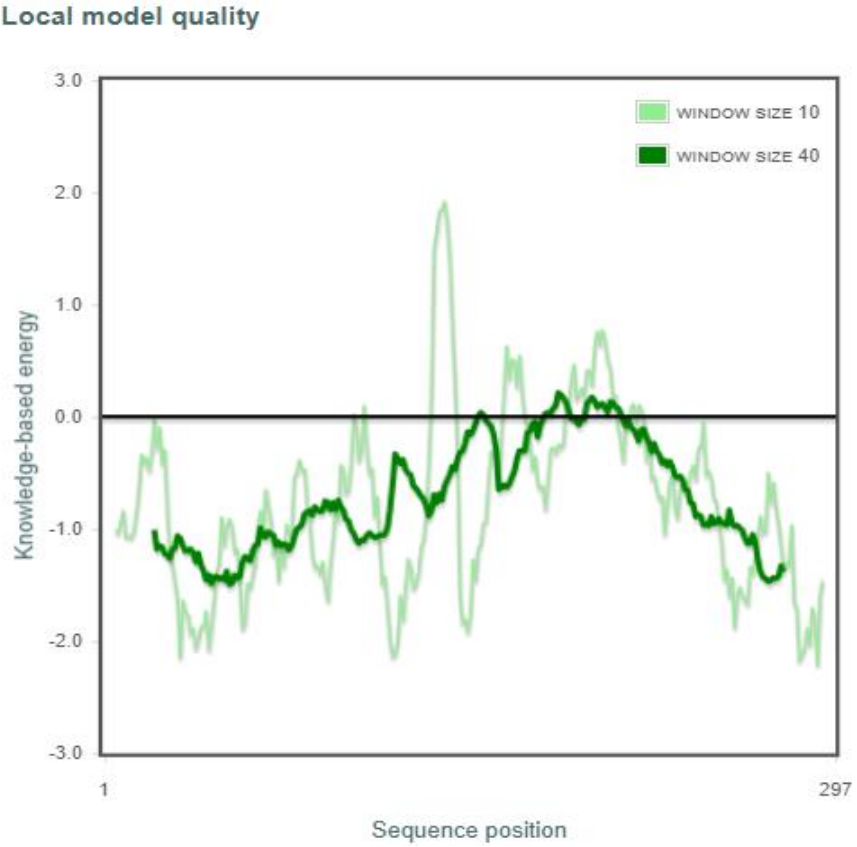


Fig. 2. ProSA energy profile of the enzyme

In addition, the PROCHECK tool generated a Ramachandran diagram. It showed that 92.5% of the residues were in favored regions and 7.5% in allowed regions. No residues were found in the allowed or disallowed regions (Fig. 3). This ensures the quality and precision of the modeled structure. These analyzes confirm the validity and reliability of the proposed 3D model. It is now suitable for further molecular docking and interaction studies. The high accuracy and reliability of the model provide a solid understanding of the 2'o-MT-VP39 binding mechanisms. This may provide information for new therapies.

We found that stigmasterol binds strongly to the enzyme 2'-O-MT-VP39. The docking value of -7.98 kcal/mol indicates the relative stability of the interaction. Structural analysis showed that stigmasterol forms hydrogen bonds with Asn273. The N-group of Asn273 interacts with the O1 of stigmasterol. It is crucial for the stabilization of the protein-ligand complex. This suggests that Asn273 is a key amino acid in the binding mechanism of the enzyme. The docking results show that the active site of the enzyme is a good ligand interaction site. This indicates its potential as a target for drug development. The strong binding affinity of stigmasterol makes it a good drug target for Mpox [35]. This research provides important insights for the development of better Mpox treatments. It aims to overcome resistance to existing drugs. The results are promising for the search for new antiviral drugs. These results underline the importance of Asn273 for the binding process. They also highlight the potential of stigmasterol as a lead molecule for new drug discovery. This work shows how the technique can be used to find new targets and drugs for mumps treatment.

The Graph 1 analyzes molecular docking results for various natural compounds from *A. indica*. It focuses on their affinity values and interactions with specific amino acids. The results show a range of binding strengths. Some compounds bind with considerable strength and permanence. Others show weaker affinities. Stigmasterol has the strongest binding. Its affinity value is -7.98 kcal/mol. It forms hydrogen bonds with Asn273. It has hydrophobic interactions with Arg76, Tyr77, and Phe293. This strong, stable

binding to the protein suggests that stigmasterol may be a good candidate for drug discovery. Azadiradione and campesterol also have strong affinities.

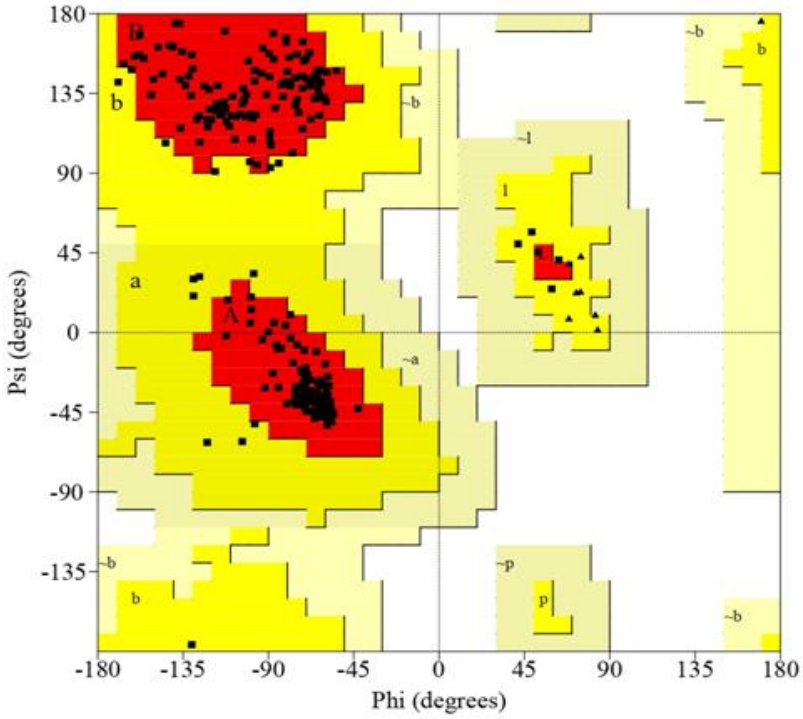


Fig. 3. Ramachandran diagram of the model protein: residues in the red area are allowed, while residues in the allowed area, residues in the yellow area are generous

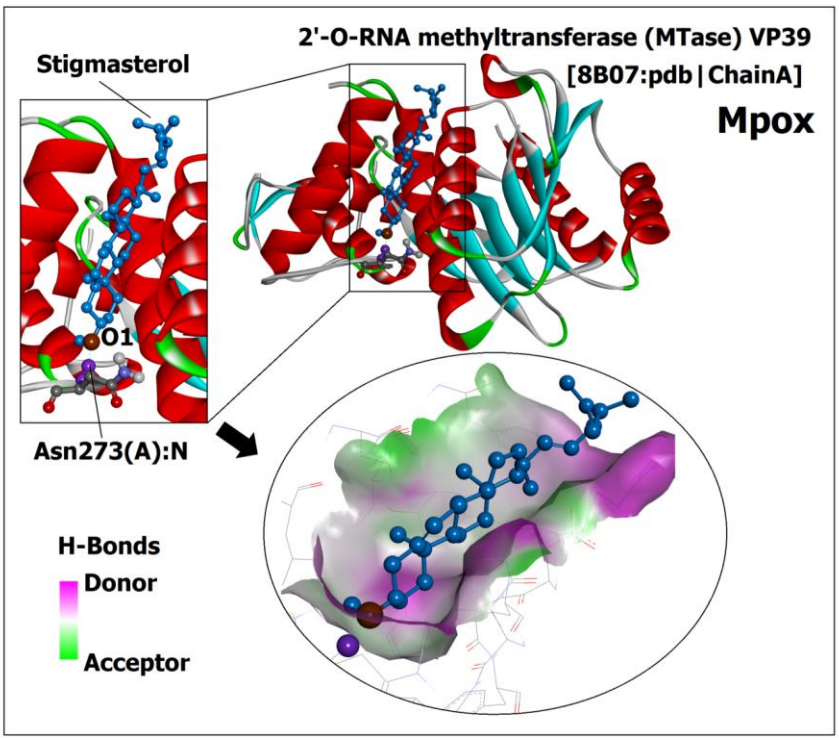
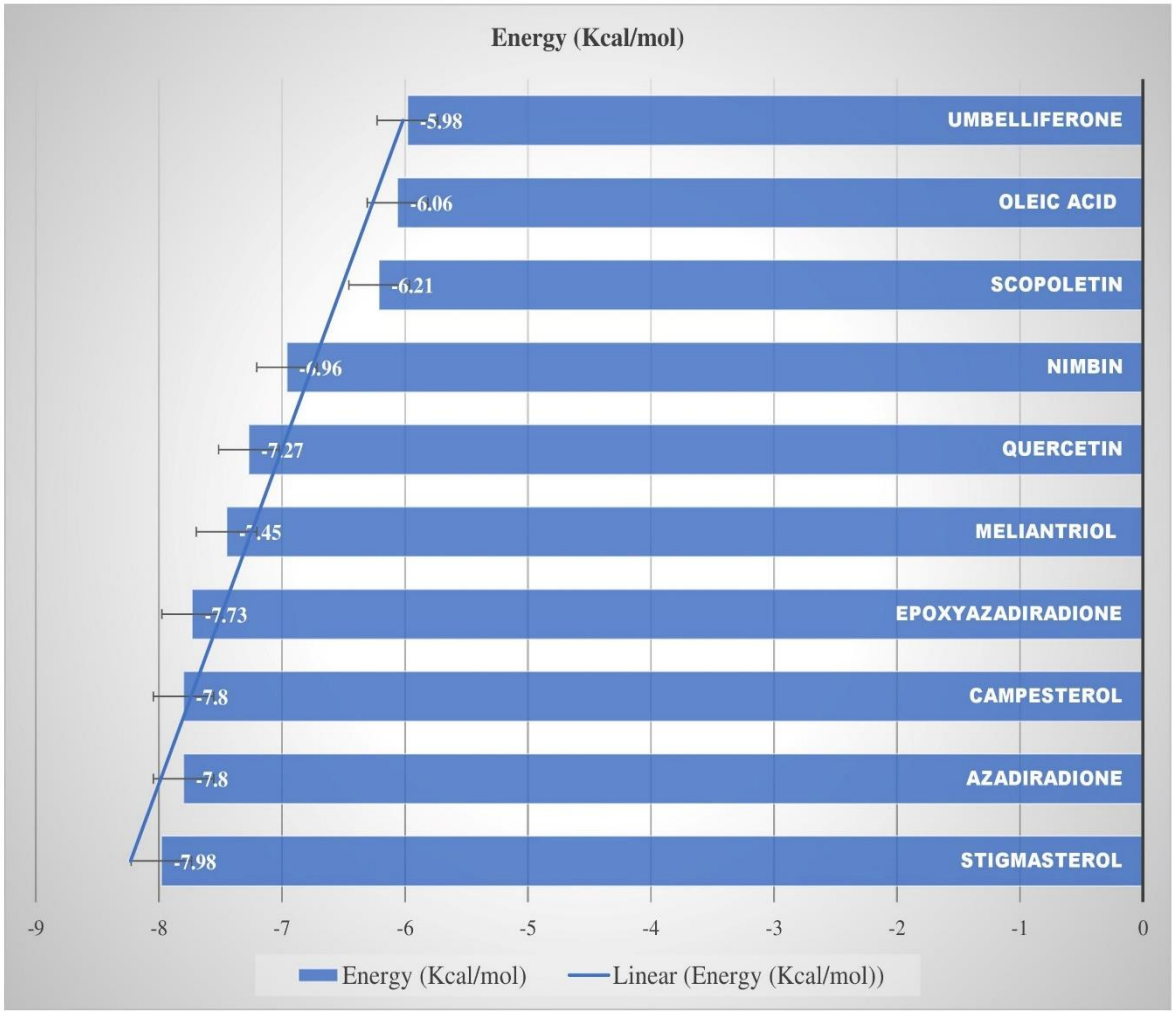
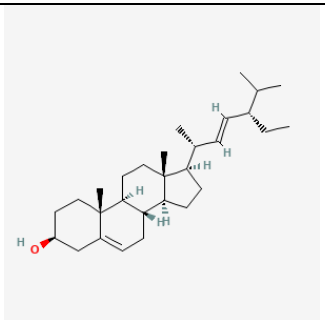
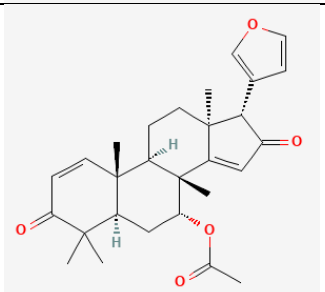
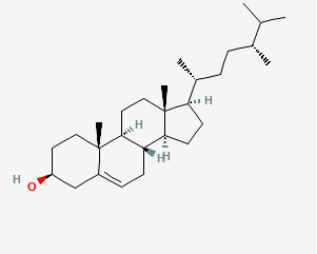


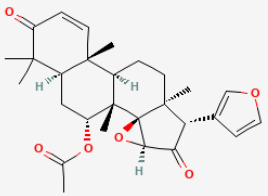
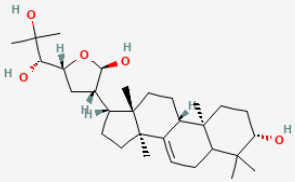
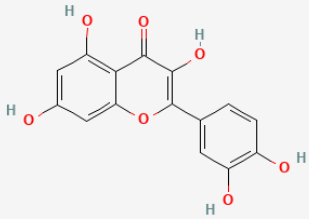
Fig. 4. Molecular interaction of 2'-O-methyltransferase with stigmasterol

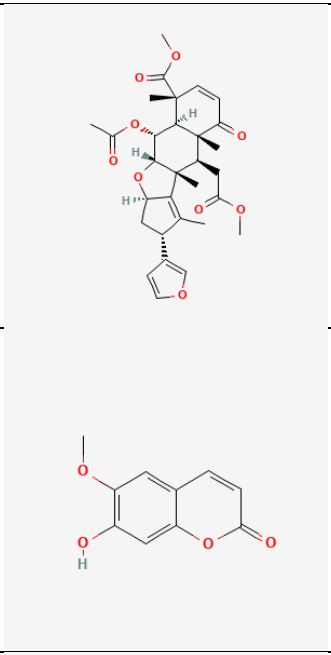


Graph 1. Provide energy released by different natural compounds from *Azadirachta indica* during molecular docking

Table 1. Amino acid residues of different ligands involved in the hydrogen bond and hydrophobic bond during molecular docking

Compound (5281426)	Structure	Affinity (kcal/mol)	Amino acid residues (H-Bond)	Amino acid residues (Hydrophobic-Bond)
Stigmasterol (5280794)		-7.98	Asn273	Arg76, Tyr77, Asp80, HIsIle102, Phe293
Azadiradione (12308714)		-7.80	Tyr77, Asp80, His81	Tyr77, Asp80, Asn84, Asn273, Lys274, Phe285
Campesterol (173183)		-7.80	Asp95	Phe115, Asp117, Val139, Ala158, Leu159

Compound (5281426)	Structure	Affinity (kcal/mol)	Amino acid residues (H-Bond)	Amino acid residues (Hydrophobic-Bond)
Epoxyazadiradione (49863985)		-7.73	His99, Asn104, Thr10, Gly105	Leu111
Meliantriol (185586)		-7.45	Asn199, Asn218, Arg220	Tyr12, His192, Ile213, Tyr214
Quercetin (5280343)		-7.27	Gly68, Ile94, Asp95, Val,116, Asp138, Arg140	Val139, Arg140

Compound (5281426)	Structure	Affinity (kcal/mol)	Amino acid residues (H-Bond)	Amino acid residues (Hydrophobic-Bond)
Nimbin (108058)		-6.96	Tyr22, Tyr204, Ala206	Tyr22, Phe180, pro202, Tyr204
Scopoletin (5280460)		-6.21	Ile94, Arg114, Val116, Ser141	Phe115
Oleic Acid (445639)		-6.06	Tyr204, Asn245	Lys33

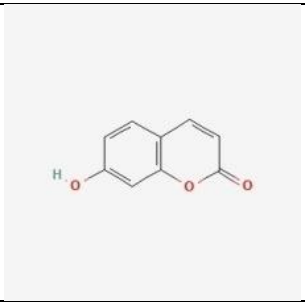
Compound (5281426)	Structure	Affinity (kcal/mol)	Amino acid residues (H-Bond)	Amino acid residues (Hydrophobic-Bond)
Umbelliferone (5281426)		-5.98	Asn218, Arg220	His192, Tyr214, Thr215

Table 2. ADMET profiling of a 2'o-MT-VP39 protein inhibitor from *Azadirachta indica* chosen as a bioactive molecule

Properties↓	Sinefungin (Control)	Stigmasterol	Azadiradione	Campesterol	Epoxyazadiradione	Meliantriol
iLOGP	0.67	5.08	3.17	4.97	3.33	4.10
XLOGP3	-4.31	8.56	4.82	8.80	4.24	5.01
WLOGP	-2.38	7.80	5.42	7.63	4.63	4.81
MLOGP	-5.13	6.62	3.28	6.54	2.54	3.84
Silicos-IT Log P	-3.03	6.86	5.00	6.63	4.84	4.09
Consensus Log P	-2.84	6.98	4.34	6.92	3.92	4.37
ESOL Log S	0.73	-7.46	-5.58	-7.54	-5.31	-5.84
ESOL Solubility (mg/ml)	2.03e+03	1.43e-05	1.17e-03	1.16e-05	2.26e-03	7.08e-04
ESOL Solubility (mol/l)	5.32e+00	3.46e-08	2.60e-06	2.90e-08	4.84e-06	1.44e-06
ESOL Class	Highly soluble	Poorly soluble	Moderately soluble	Poorly soluble	Moderately soluble	Moderately soluble
Ali Log S	-0.54	-8.86	-6.10	-9.11	-5.76	-6.64
Ali Solubility (mg/ml)	1.32e+03	5.71e-07	3.59e-04	3.13e-07	8.12e-04	1.12e-04
Ali Solubility (mol/l)	3.46e+00	1.38e-09	7.98e-07	7.80e-10	1.74e-06	2.27e-07
Ali Class	Highly soluble	Poorly soluble	Poorly soluble	Poorly soluble	Moderately soluble	Poorly soluble
Silicos-IT LogSw	0.34	-5.47	-6.31	- 5.79	-6.03	-3.88
Silicos-IT Solubility (mg/ml)	8.25e+02	1.40e-03	2.23e-04	6.42e-04	4.35e-04	6.53e-02
Silicos-IT Solubility (mol/l)	2.16e+00	3.39e-06	4.94e-07	1.60e-06	9.33e-07	1.33e-04

Properties↓	Sinefungin (Control)	Stigmasterol	Azadiradione	Campesterol	Epoxyazadiradione	Meliantriol
Silicos-IT class	Soluble	Moderately soluble	Poorly soluble	Moderately soluble	Poorly soluble	Soluble
GI absorption	Low	Low	High	Low	High	High
BBB permeant	No	No	No	No	No	No
Pgp substrate	No	No	Yes	No	Yes	Yes
CYP1A2 inhibitor	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No	No
CYP2C9 inhibitor	No	Yes	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No	No
log Kp (cm/s)	-11.69	-2.74	-5.63	-2.5	-6.14	-5.74
Lipinski #violations	2	1	0	1	0	0
Ghose #violations	1	3	Yes	2	Yes	3
Veber #violations	1	Yes	Yes	Yes	Yes	Yes
Egan #violations	1	1	Yes	1	Yes	Yes
Muegge #violations	3	2	Yes	2	Yes	1
Bioavailability Score	0.17	0.55	0.55	0.55	0.55	0.55
PAINS #alerts	0	0	0	0	0	0
Brenk #alerts	0	1	0	1	1	1
Lead likeness #violations	1	3	2	2	2	2
Synthetic Accessibility	4.74	6.21	5.89	6.17	6.34	6.96

Continue…

Properties↓	Quercetin	Nimbin	Scopoletin	Oleic Acid	Umbelliferone
iLOGP	1.63	3.69	1.86	4.01	1.44
XLOGP3	1.54	2.28	1.53	7.64	1.58
WLOGP	1.99	3.92	1.51	6.11	1.50
MLOGP	-0.56	2.04	0.76	4.57	1.04
Silicos-IT Log P	1.54	3.96	1.94	5.95	1.97
Consensus Log P	1.23	3.17	1.52	5.65	1.51
ESOL Log S	-3.16	-4.20	-2.46	-5.41	-2.54
ESOL Solubility (mg/ml)	2.11e-01	3.45e-02	6.70e-01	1.09e-03	5.66e-01

Properties↓	Quercetin	Nimbin	Scopoletin	Oleic Acid	Umbelliferone
ESOL Solubility (mol/l)	6.98e-04	6.38e-05	3.48e-03	3.85e-06	3.49e-03
ESOL Class	Soluble	Moderately soluble	Soluble	Moderately soluble	Soluble
Ali Log S	-3.91	-4.40	-2.39	-8.26	-2.25
Ali Solubility (mg/ml)	3.74e-02	2.14e-02	7.79e-01	1.54e-06	9.12e-01
Ali Solubility (mol/l)	1.24e-04	3.96e-05	4.06e-03	5.46e-09	5.62e-03
Ali Class	Soluble	Moderately soluble	Soluble	Poorly soluble	Soluble
Silicos-IT LogSw	-3.24	-5.44	-3.17	-5.39	-3.03
Silicos-IT Solubility (mg/ml)	1.73e-01	1.97e-03	1.31e-01	1.14e-03	1.53e-01
Silicos-IT Solubility (mol/l)	5.73e-04	3.64e-06	6.81e-04	4.04e-06	9.42e-04
Silicos-IT class	Soluble	Moderately soluble	Soluble	Moderately soluble	Soluble
GI absorption	High	High	High	High	High
BBB permeant	No	No	Yes	No	Yes
Pgp substrate	No	No	No	No	No
CYP1A2 inhibitor	Yes	No	Yes	Yes	Yes
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	Yes	No
CYP2D6 inhibitor	Yes	No	No	No	No
CYP3A4 inhibitor	Yes	No	No	No	No
log Kp (cm/s)	-7.05	-7.98	-6.39	-2.60	-6.17
Lipinski #violations	0	1	0	1	0
Ghose #violations	Yes	3	Yes	1	1
Veber #violations	Yes	Yes	Yes	1	Yes
Egan #violations	Yes	Yes	Yes	1	Yes
Muegge #violations	Yes	Yes	1	3	1
Bioavailability Score	0.55	0.55	0.55	0.85	0.55
PAINS #alerts	1	0	0	0	0
Brenk #alerts	1	2	1	1	1
Lead likeness #violations	Yes	2	1	2	1
Synthetic Accessibility	3.23	6.54	2.62	0.85	2.56

Azadiradione interacts with Tyr77, Asp80, and His81. Campesterol forms key bonds with Asp95 and hydrophobic residues like Phe115 and Leu159. This indicates it is very stable. It forms hydrogen bonds with His99 and Asn104. This lowers its binding potential; with an affinity of -7.73 kcal/mol. Meliantriol and quercetin also bind strongly to important residues, like Asn199 and Gly68. They form hydrogen bonds with them. Nimbin and scopoletin form fewer interactions, resulting in moderate binding strength. Oleic acid and umbelliferone show weaker affinity, with limited hydrogen and hydrophobic bonds. The results show the different levels of molecular interactions. They suggest that stigmasterol, azadiradione, and campesterol are good drug candidates. They bind strongly to target proteins and stay bound. We can further investigate these compounds for their potential uses. We may need to modify the weaker binding partners to improve their affinity (Topno et al., 2023; Kumar et al., 2024). The docking results shed light on how these natural compounds bind. This may help us develop new drugs and therapies. Identifying amino acid residues that bind helps develop targeted therapies (Kumar et al., 2024). This study shows that compounds from *A. indica* could help make new drugs. It shows the value of molecular docking studies in finding new drugs.

The Table 2 shows various compounds and their properties. It lists their solubility, absorption, and bioavailability. The compounds are: Sinefungin, stigmasterol, azadiradione, campesterol, epoxyazadiradione, meliantriol, quercetin, nimbin, scopoletin, oleic acid and umbelliferone. These compounds have different lipophilicity (LogP), solubility, permeability, and drug likeness. These are crucial for the evaluation of their pharmacokinetic and pharmacodynamic behaviour. Sinefungin, a control substance, is unique. It is hydrophilic and has low LogP values in all models (iLOGP of 0.67, XLOGP3 of -4.31, and WLOGP of -2.38). According to the ESOL, Ali, and Silicos-IT models, it is also very soluble. Its high solubility, especially in water, makes it highly bioavailable despite its low gastrointestinal absorption and lack of blood-brain barrier (BBB) permeability. On the other hand, stigmasterol has a very high lipophilicity (iLOGP 5.08, XLOGP 3 8.56), but does not dissolve well and is not well absorbed by the gastrointestinal tract, making it less bioavailable. It also violates several drug-likeness rules, including those of Lipinski, Ghose, and Veber. It has moderate permeability and poor aqueous solubility.

Azadiradione, campesterol, epoxyazadiradione, and meliantriol have moderate lipophilicity and solubility profiles. Azadiradione and epoxyazadiradione have moderate solubility and high gastrointestinal absorption. They are therefore good candidates for oral drug formulations. However, they still violate some important rules of drug-likeness (Ghose, Veber, Egan) and have moderate bioavailability. Campesterol is lipophilic (iLOGP 4.97) but suffers from poor solubility and low GI absorption, which limits its therapeutic potential. Not all compounds in this group can cross the blood-brain barrier (BBB), and some, such as azadiradione and meliantriol, act as P-glycoprotein (Pgp) substrates, which could alter drug transport.

4. CONCLUSION

The enzyme 2'-O-methyltransferase VP39 (2'-O-MT-VP39) was selected as a therapeutic target due to its crucial role in the replication process of the Mpox virus. This enzyme catalyzes the addition of a 7-methylguanosine cap to the 5'-end of viral mRNAs, mimicking the capping mechanism of the host cell. This modification protects the viral RNA from recognition by the immune system, stabilizes the viral mRNA and increases the efficiency of translation, which enables robust viral replication. The lack of specific antiviral treatments for Mpox underscores the urgent need for targeted therapeutic strategies. Inhibition of VP39 activity could block the viral replication cycle and is therefore an attractive candidate for the development of antiviral drugs. Using advanced bioinformatics methods, the researchers identified the crystal structure of VP39 and discovered it as a viable target for drug intervention. The enzyme model was validated by computational tools such as Ramachandran plot analysis, ERRAT and ProSA, confirming its structural accuracy and reliability. Among the natural compounds tested, stigmasterol showed the strongest inhibitory potential and exhibited the highest binding affinity at the active site of the enzyme. Compared to sinefungin, the reference compound, stigmasterol showed a superior ability to interfere with the activity of VP39, suggesting that it could effectively disrupt viral replication. These results make stigmasterol a promising candidate for further research. Future studies should include comprehensive in vitro and in vivo studies to evaluate its antiviral efficacy, mechanism of action, pharmacokinetics (absorption, distribution, metabolism and

excretion) and potential cytotoxicity. In addition, the evaluation of its specificity towards VP39 and potential off-target effects is crucial to ensure therapeutic safety. This research lays the foundation for the development of targeted antiviral therapies against Mpox and opens up new possibilities for more effective and precise treatment strategies

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Systematic Implementation of Quality by Design (QbD): A Perspective from Generic Pharmaceutical Industries

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This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Quality by Design (QbD) is a structured approach to pharmaceutical development that ensures predefined product quality by understanding and controlling manufacturing processes from the outset. Unlike traditional methods focusing on end-product testing, QbD emphasizes building quality into the product design itself, enhancing manufacturing efficiency and regulatory compliance. This review highlights the application of QbD in developing generic solid oral drug products, emphasizing tools like risk assessment, process design, and control strategies to achieve consistent quality. Key components include identifying and managing Critical Quality Attributes (CQAs), Critical Process Parameters (CPPs), and Critical Material Attributes (CMAs), which

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influence formulation, process development, and overall performance. The integration of Design of Experiments (DoE) to systematically study the effects of multiple variables on product and process performance, enabling optimization and robust development and effective control strategies are also discussed in this article. Addressing regulatory expectations, particularly those from the International Council for Harmonisation (ICH), this review outlines how QbD principles help generics meet bioequivalence standards, ensuring consistent quality and performance. Applying QbD not only enhances product robustness and manufacturing efficiency but also improves patient safety through better process understanding and continuous improvement. This review article outlines the various steps involved in the development of generic drug products using the QbD approach from analysis of brand product to product lifecycle management and continual improvement.

Keywords: Quality by design; critical quality attributes; risk assessment; critical material attributes; critical process parameters; control strategy.

1. INTRODUCTION

In recent decades, the pharmaceutical industry has faced challenges in quality assurance and regulatory compliance, highlighting the need for structured approaches to development. Historically, fixed manufacturing processes and extensive testing were maintained product quality. However, the limitations of the Quality by Test (QbT) approach have become evident. Under QbT, materials and products failing to meet specifications must be discarded. This approach's lack of process understanding often results in unrecognized variability, leading to inconsistent quality, batch rejections, low patient acceptance, and increased costs (Simões, Veiga, & Vitorino, 2024). Any changes to the formulation composition or manufacturing process require a lengthy and costly post-approval change submission (L. X. Yu, 2008; Zhang & Mao, 2017).

In the context of Quality by Design (QbD), Dr. Janet Woodcock emphasizes that “Product and process performance characteristics are scientifically designed to meet specific objectives, not merely empirically derived from performance of test batches” (Woodcock, 2004) this statement aligns perfectly with the fundamental principle of QbD "Quality cannot be tested into the product; it must be designed into it." Quality by Design (QbD) is a systematic approach to pharmaceutical development, involving the design and development of formulations and manufacturing processes to ensure predefined product quality. According to ICH Q8, QbD (Quality by Design) is defined as a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009). QbD implementation into the pharmaceuticals product development reducing post-approval changes (Pramod, Tahir, Charoo, Ansari, & Ali, 2016).

The concept of Quality by design (QbD) is mentioned in ICH Q8 guidance. The fundamental principle of QbD is that "Quality cannot be tested into the product; it must be designed into it.". Quality by Design (QbD) is a systematic approach to pharmaceutical development, involving the design and development of formulations and manufacturing processes to ensure predefined product quality. Implementing QbD transforms the chemistry, manufacturing, and control (CMC) review of generic products into a science-based pharmaceutical quality assessment (L. X. Yu, 2008).

According to ICH Q8 (Quality by Design), QbD is defined as a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009). The pharmaceutical industry knows the product's quality, safety and effectiveness. Product quality has increased by implementing QbD scientific tools (Rathore, 2009).

The FDA's emphasis on Quality by Design (QbD) stems from recognizing that more extensive testing does not inherently improve product quality—a principle well-established in other industries. The following equation (Lionberger, Lee, Lee, Raw, & Yu, 2008) highlights the foundation of quality:

Pharmaceutical Quality = f (drug substance, excipients, manufacturing, packaging)

Improving quality requires embedding it directly into the product. This is achieved by gaining a comprehensive understanding of how formulation and manufacturing process variables influence product quality, as represented by the function in the equation.

Products developed using QbD ensure quality by incorporating risk assessment and addressing potential risks through formulation and process optimization. This approach plays a vital role in improving product efficacy, which, in turn, enhances patient safety.

The goal of pharmaceutical development is to create a quality product and a manufacturing process that consistently achieves the desired performance. Regardless of the circumstances, products should be designed to satisfy patients' needs and deliver the intended performance. Development strategies differ between companies and products, with varying approaches and extents of development that should be detailed in the submission. An applicant may opt for an empirical approach, a systematic approach, or a combination of both for product development.

Process Analytical Technology (PAT) serves as a fundamental element of Quality by Design (QbD), facilitating real-time monitoring and control of manufacturing processes. By identifying and regulating critical process parameters, it ensures consistent product quality, minimizes variability, and enhances overall efficiency.

The Food and Drug Administration (FDA) (Nasr, 2013; Woodcock, 2004; L. Yu, 2013) and the pharmaceutical industry (Ganzer, Materna, Mitchell, & Wall, 2005; Glodek et al., 2006) are discussing Quality by Design (QbD).

From available USFDA database, Quality by design for ANDA: An example for Immediate-release dosage form (U.S. Food and Drug Administration (FDA), April 2012) and An example of Modified Release dosage form (U.S. Food and Drug Administration (FDA), December 2011), this review article explains steps for QbD implementation in generic solid oral product.

2. STEP BY STEP QBD IN GENERIC DRUG PRODUCT

Below sub-subsections outline the methodology for applying QbD principles in the development of a generic solid oral drug product.

2.1 Analysis of The Brand Product

A detailed outline for the Reference Listed Drug (RLD) analysis is to be performed as follows.

2.1.1 Clinical

Clinical information for brand products can be accessed through the label provided in the Drugs @ FDA section database. The following key categories should be taken into account for the development of generic drug products.

- Therapeutic Indication: This refers to the specific disease or condition that the drug is intended to treat.
- Mechanism of Action: This describes how the drug works at a molecular level to produce its therapeutic effect.
- Immediate Release or Extended Release: Immediate-release (IR) formulations release the active ingredient quickly, while extended-release (ER) formulations release it slowly over time.
- Dosing Frequency: This indicates how often the drug should be taken, such as "once daily" or "twice daily."
- Number of Strengths: This refers to the different dosages available for the drug, like 5 mg, 10 mg, and 20 mg.
- Reference Standard for Bioequivalence Studies: This is the strength and formulation used as a benchmark in bioequivalence studies to ensure generics are equivalent to the brand-name drug.
- Score or Un-score Tablets: Scored tablets have a line or notch to help split them into smaller doses, while unscored tablets do not.
- Label Warning for Potential Risk of Dose Dumping When Consumed with Alcohol: This is a warning that should be included if consuming the drug with alcohol can lead to a rapid release of the drug, potentially causing adverse effects.

2.1.2 Pharmacokinetics

Pharmacokinetics are essential for therapeutic efficacy and must align with the brand product to qualify as an AB-rated generic product, meeting the bioequivalence criteria.

- Tmax (Time to Maximum Concentration): This is the time it takes for the drug to reach its highest concentration in the bloodstream after administration.

- Cmax (Maximum Concentration): This is the highest concentration of the drug in the bloodstream.
- AUC (Area Under the Curve): This represents the total drug exposure over time, essentially the integral of the concentration-time curve.
- Elimination Half-Life: This is the time it takes for the concentration of the drug in the bloodstream to be reduced by half.

These parameters help in understanding the drug's absorption, distribution, metabolism, and excretion (ADME) properties.

2.1.3 Drug Release

Drug release in FDA-recommended media (if available) to be performed along with multimedia dissolution for extended release drug product. In cases where the FDA has not specified dissolution media, the generic applicant must develop media with sufficient discriminatory power to enhance the likelihood of achieving in vitro-in vivo correlation. Details of dissolution method development mentioned in sub section 2.4.

2.1.4 Physicochemical characterization

- **Description:** Detailed appearance and physical attributes of the product.
- **Batch Number:** Unique identifier for the specific production batch.
- **Expiry Date:** The date until which the product is expected to remain effective and safe for use.
- **Strength:** The amount of active ingredient per dosage form (e.g., 50 mg, 100 mg).
- **Average Tablet Weight:** The mean weight of a single tablet.
- **Score/Unscore:** Indicates whether the tablet has a line for splitting.
- **Coated/Uncoated:** Specifies if the tablet has a coating or not.
- **Diameter:** The width of the tablet, usually measured in millimeters.
- **Thickness:** The height of the tablet, usually measured in millimeters.
- **Hardness:** The force required to break the tablet, measured in kP or Newtons.
- **Disintegration Time:** For immediate-release tablets, the time taken for the tablet to break down into smaller fragments in a specified liquid medium.
- **Assay:** The measurement of the active ingredient content within the tablet.
- **Related Compounds:** Analysis of impurities and degradation products that may be present.
- **Composition:** Identify the qualitative RLD composition based on the brand product labeling, and the quantitative RLD composition based on patent literature and reverse engineering

2.2 Quality Target Product Profile

The quality target product profile (QTPP) is a prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009).

Considering the clinical and pharmacokinetic (PK) properties, along with the in vitro dissolution and physicochemical characteristics of the brand product, a QTPP should be established for the generic dosage form. The following aspects should be targeted based on the brand product:

- Dosage form
- Dosage design
- Route of administration
- Dosage strength
- Pharmacokinetics
- Stability
- Container closure system
- Administration/Concurrence with labeling
- Drug product Critical Quality Attributes (CQA)

2.3 Critical Quality Attributes

A critical quality attribute (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009). Determining a CQA from the QTPP involves assessing the potential harm to a patient if the product exceeds the acceptable range for that attribute. The following CQAs should be targeted, considering safety and efficacy:

- **Assay**: Ensures the correct potency of the drug in the dosage form.

- **Content Uniformity**: Ensures each dosage unit contains the intended amount of drug substance.
- **Dissolution**: Ensures the drug is released at the intended rate in the body.
- **Degradation Products**: Specifies acceptable levels of impurities and degradation products to ensure safety. The ICH Q3B (R2) guideline specifies thresholds for impurities in drug products based on the maximum daily dose of the drug. These thresholds ensure that impurities are controlled to maintain product safety and efficacy.

2.4 Dissolution Method Development

The biopharmaceutics drug classification system seeks to establish a connection between in vitro drug product dissolution and in vivo bioavailability. It highlights that drug dissolution and gastrointestinal permeability are key factors determining the rate and extent of drug absorption. This classification system provides recommendations for establishing standards for in vitro drug dissolution testing methods that align with in vivo processes. These methods must be based on the physiological and physicochemical factors that influence drug absorption. The analysis identifies situations where in vitro-in vivo correlation may not be expected, such as for rapidly dissolving drugs with low permeability. It also suggests that for very rapidly dissolving drugs with high solubility (e.g., 85% dissolution within 15 minutes), a simple one-point dissolution test may be sufficient to ensure bioavailability. Conversely, for slowly dissolving drugs, a detailed dissolution profile with multiple time points is necessary, incorporating conditions such as low pH, physiological pH, and surfactants to simulate in vivo processes (Amidon, Lennernäs, Shah, & Crison, 1995).

Understanding the relationship between in vitro drug release and in vivo performance is essential for several reasons:

- Assessing the impact of formulation and process variable changes on drug product quality during development.
- Predicting the performance of commercial batches using bioequivalence data from the exhibit batch.
- Facilitating the evaluation of post-approval changes.

To achieve this, a predictive dissolution method should be developed to establish an in vitro-in vivo relationship (IVIVR) that links in vitro drug release with in vivo performance. This predictive dissolution method should accurately forecast the in vivo performance of the drug product and distinguish between formulations with different performance characteristics.

Moreover, the dissolution method must be discriminative to effectively detect differences in the performance of drug products (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Guidance for Industry, August 1997). A discriminative dissolution method helps identify any variations in the formulation or manufacturing process that could impact the drug's release performance (Ashokraj et al., 2016). For immediate release dosage form, dissolution method to be developed based on drug substance aqueous solubility and absorption window.

For modified-release dosage forms, the effectiveness of the dissolution method in predicting performance for both brand and generic drug products will be assessed by:

- Testing at various pH levels (e.g., water, 0.1 N HCl, pH 4.5 phosphate buffer, and pH 6.8 phosphate buffer) to evaluate the pH-dependent solubility of the drug substance and the behavior of the extended-release polymer at different pH levels.
- Using different volumes of dissolution medium (e.g., 250 mL, 500 mL, 900 mL) to determine the impact of medium volume on the dissolution rate.
- Testing at different stirring speeds (e.g., 25 rpm, 50 rpm, 75 rpm) while ensuring that coning does not occur at low speeds during dissolution testing.
- Employing different USP apparatus (e.g., Apparatus I [Basket]Ae, Apparatus II [Paddle], Apparatus III [Reciprocating Cylinder]).

During these evaluations, one variable will be altered at a time, keeping all other variables constant, to assess the predictive power of the dissolution method.

After establishing discriminating dissolution conditions, the subsequent step will be to assess whether any of these conditions can reasonably

predict the in vivo performance of the drug product.

2.5 Pilot Bioequivalence Study

Pilot bioequivalence (BE) studies are invaluable to demonstrate that the *in vitro* dissolution used is appropriate. For modified release dosage form, Establishing an IVIVC (*in vitro-in vivo* correlation) is one of the more robust methods to ensure the continued bioequivalence (BE) of commercial lots. It provides control over post-approval changes to critical material attributes (CMAs) and critical process parameters (CPPs), thus maintaining consistent product quality and BE. However, establishing an IVIVC can be challenging. A product designed and developed using QbD principles should result in the establishment of a predictive *in vitro* dissolution method. Although less robust than an IVIVC, establishing a predictive *in vitro* dissolution method may be sufficient to ensure product quality when combined with a thorough understanding of the product and process. Additionally, such an *in vitro* method will be valuable for assessing post-approval changes. It is well acknowledged that an extended-release product developed at the pivotal batch scale may not always scale up to commercial scale and yield a drug product that is bioequivalent (BE) to the Reference Listed Drug (RLD). Both fed and fasting bioequivalence studies may be necessary to ensure that the commercial batches are BE to the RLD. It is also expected that the risk assessment of a product developed using Quality by Design (QbD) principles will identify all Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs) and include adequate controls; one of these controls will be a discriminating in vitro method. While the bioequivalence study of commercial batches will ensure the BE of the drug product, a discriminating in vitro method will assure product quality throughout its lifecycle (including post-approval changes).

The 90% confidence Interval meets the bioequivalence threshold of 80-125% for Cmax and AUC under fasted, fed, and other conditions as specified in the product-specific guidance for the brand product (U.S. Food and Drug Administration (FDA) Draft Guidanace, August 2021).

2.6 Component of Drug Product

Drug substances and excipients are components of drug products.

2.6.1 Drug substances

The assessment of various physical properties of the drug substance, such as appearance, particle morphology, particle size distribution (d10, d50, d90), and solid-state form (including the polymorphic form and its stability in the final drug product), this should be conducted using XRPD by comparing the spectra of the drug substance, drug product, and individual excipients. Additionally, properties such as melting point, solubility in different pH environments, hygroscopicity, density, and flow characteristics should also be evaluated. Chemical properties such as pKa and the stability of the drug substance in both solid state and solution, as well as biological properties like partition coefficient, Caco-2 permeability, and biopharmaceutical classification, should also be assessed. A risk assessment of drug substance critical attributes is essential for ensuring the quality and safety of a drug product.

Solid-state form: The solid-state form of a drug substance refers to its physical state and structure as a solid. The solid-state form is important because it can significantly impact the drug's performance, including its solubility, stability, and bioavailability. During the manufacturing process, the solid-state form of a drug substance may change, potentially impacting the stability of the drug product (Zhou et al., 2024). Understanding and controlling the solid-state form is crucial in drug development to ensure consistent quality and efficacy.

Particle size distribution: Particle size distribution refers to the range and proportion of different particle sizes present in a sample. It is a critical parameter in pharmaceutical development as it can significantly influence the drug's behavior and performance, including its dissolution rate, bioavailability, and stability. Ensuring a consistent particle size distribution is essential for maintaining the quality and efficacy of the drug product. Manufacturers often optimize the milling and granulation processes to achieve the desired particle size distribution. Understanding and controlling particle size distribution is crucial for developing high-quality pharmaceutical products that perform consistently and effectively.

Hygroscopicity: Hygroscopicity refers to the ability of a drug substance to absorb moisture from its surrounding environment. This characteristic is crucial in the pharmaceutical

field as it can significantly impact the drug's stability, manufacturing process, and overall performance. During formulation development, the hygroscopic nature of the drug substance must be considered to ensure product stability. Suitable excipients may be selected to stabilize the drug substance, and moisture-protective packaging might be employed. Understanding and managing the hygroscopicity of a drug substance is essential for maintaining the quality and efficacy of pharmaceutical products. It aids in designing formulations that remain stable and effective throughout their shelf life.

Solubility: Solubility is the capacity of a drug substance to dissolve in a solvent, typically water or other biological fluids. It is a crucial parameter in drug development because it affects the drug's absorption, bioavailability, and overall effectiveness. Solubility can vary with changes in the pH of the medium and can be influenced by the presence of other compounds or excipients in the formulation. To measure solubility, an excess of the drug substance is added to a solvent and allowed to reach equilibrium. Analytical techniques such as High-Performance Liquid Chromatography (HPLC) or spectrophotometry are then used to quantify the dissolved drug. Solubility impacts the rate at which the drug dissolves in the gastrointestinal tract, influencing its absorption. Poorly soluble drugs may have limited bioavailability, necessitating formulation strategies to enhance solubility. Understanding solubility is essential for designing suitable dosage forms and selecting appropriate excipients. Techniques such as salt formation, particle size reduction (Khan et al., 2022), the use of solubilizing excipients (Karataş, Yüksel, & Baykara, 2005), and creating solid dispersions (Chiou & Riegelman, 1971; Sareen, Mathew, & Joseph, 2012) can be employed to improve the solubility of poorly soluble drugs.

Moisture content: It refers to the amount of water present in a drug substance. This property is crucial as it can impact the stability, quality, and performance of the drug product. It is typically measured using techniques such as Karl Fischer titration, thermogravimetric analysis (TGA), or loss on drying (LOD). High moisture content can lead to hydrolysis or degradation of the drug substance, reducing its potency and shelf life. It can also affect the physical properties of the drug, such as particle size, flowability, and compressibility. Drug substances with high moisture content may require special storage conditions to prevent moisture absorption. Desiccants and moisture-resistant packaging are commonly used to protect the drug substance from humidity. During formulation development, moisture content can Influence the manufacturing process, particularly operations like granulation and compression. Ensuring the appropriate moisture content in a drug substance is vital for maintaining its stability and efficacy."

Residual solvents: They are the trace amounts of solvents that remain in a drug substance after the manufacturing process is complete. These solvents are typically used during synthesis, purification, or formulation stages and must be minimized to ensure the safety and quality of the drug product.

- **Class 1 Solvents**: Solvents to be avoided due to their toxicity (e.g., benzene).
- **Class 2 Solvents**: Solvents to be limited due to potential toxicity (e.g., methanol, acetonitrile).
- **Class 3 Solvents**: Solvents with low toxic potential that are less stringently regulated (e.g., ethanol).

Regulatory agencies, such as the International Council for Harmonisation (ICH), provide guidelines on acceptable levels of residual solvents in pharmaceutical products (ICH Q3C guidelines) (International Conference on Harmonisation of Technical Requirements for Registration Of Pharmaceuticals For Human Use, April 2021). These limits are based on safety assessments and are intended to minimize health risks. High levels of residual solvents can pose toxicity risks to patients and can also affect the stability, efficacy, and shelf life of the drug product."

Chemical stability: It refers to a drug substance's ability to maintain its chemical integrity and labeled potency within specified limits throughout its shelf life (Yadav, Yadav, & Mishra, 2023). This stability involves resistance to chemical changes such as degradation, oxidation, hydrolysis, and photolysis over time. Chemical instability can lead to reduced potency, formation of toxic degradation products, and alterations in the drug's physical properties. Ensuring chemical stability is crucial for maintaining the drug's efficacy and safety throughout its shelf life. Formulation strategies, such as using stabilizing excipients, optimizing pH, and employing antioxidants, can enhance chemical stability. Proper packaging, like airtight and light-resistant containers, protects the drug

substance from environmental factors. Maintaining the chemical stability of a drug substance is vital for delivering safe and effective pharmaceutical products to patients.

Flow properties: It refers to how well a powder can move under specified conditions without clumping, sticking, or causing blockages in manufacturing equipment. Smaller particles may lead to poor flow due to increased surface area and cohesion. Irregularly shaped particles and rough surfaces can also hinder flow, while excess moisture can cause particles to stick together, reducing flowability. Powders with higher density generally exhibit better flow properties. Common techniques for assessing flow properties include the angle of repose, compressibility index (Carr's index), and Hausner ratio (Sharma, Sharma, Deep, & Sharma). Good flow properties are essential for uniform mixing, accurate dosing, and consistent compression into tablets or filling into capsules. Understanding and optimizing the flow properties of a drug substance is crucial for ensuring efficient and consistent manufacturing of high-quality pharmaceutical products.

Evaluate how each drug substance attribute might impact the drug product's critical quality attributes (CQAs), including assay, degradation products, content uniformity, and dissolution. The relative risk of each attribute will be classified as high, medium, or low. High-risk attributes will need further investigation, while low-risk attributes will not require additional examination. Medium-risk attributes will be considered acceptable based on current knowledge, although further investigation may be necessary to mitigate the risk. The rationale behind the assigned risk levels should be detailed. Systematically evaluating and categorizing the risk of each attribute helps identify and address potential issues, thereby maintaining the quality and safety of the drug product.

2.6.2 Excipient

Excipient, as the second component of the drug product, must be evaluated through an excipient compatibility study. Drug substance in a formulation comes into close contact with one or more excipients, there is a possibility of physical and/or chemical interactions. These interactions could negatively affect the physical properties, stability, or performance of the drug product (Crowley & Martini, 2001) (McDaid, Barker, Fitzpatrick, Petts, & Craig, 2003). Therefore, selecting the right excipients is crucial to avoid such negative effects and develop a robust and effective formulation (Makai, Bajdik, Erős, & Pintye-Hódi, 2008; Tiţa, Fuliaş, Bandur, Marian, & Tiţa, 2011; Tita, Jurca, Fulias, Marian, & Tita, 2013). Screening for excipient-API compatibility is a key aspect of formulation development. Furthermore, the USFDA's 21st century current Good Manufacturing Practices (cGMP) initiative and the International Council on Harmonization (ICH) Q8 guidelines encourage pharmaceutical manufacturers to apply Quality by Design (QbD) principles in their drug development processes (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009). These guidelines emphasize the importance of understanding interactions between formulation components. Recent advances in both thermal and non-thermal analytical techniques have enhanced the efficiency of detecting, monitoring, and preventing incompatibilities early in the drug development process (Marini et al., 2003).

Drug excipient compatibility study should involve:

- A binary mixture of the drug substance and individual excipients, approximately in a 1:1 ratio.
- A mixture of the drug substance with all excipients.
- These mixtures should be stored in the solid state at 25°C/60% RH and 40°C/75% RH in both open and closed containers for one month.
- The assay and degradation products should then be checked to evaluate compatibility.

The excipient grade should be chosen based on the results of the excipient compatibility study. Additionally, excipient types identical to those in the RLD formulation should be selected for generic product development, provided there is no infringement on the brand product's patent.

2.7 Drug Product Development

In a QbD approach, the risk assessment tool aims to identify high-risk factors requiring detailed investigation and control. The results enable action plans to mitigate these risks, converting them into low-risk factors and minimizing threats to critical quality attributes (European Medicines Agency (EMA) - U.S. Food and Drug Administration (FDA), 2014;

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, May 2023; Tomba, Facco, Bezzo, & Barolo, 2013). Conducting a risk assessment of critical material attributes (CMAs) and critical process parameters (CPPs) should be performed before initiating formulation and process development. This should be followed by optimizing the formulation and process using a design of experiment (DOE) in which design space to be identified. Sequence of QbD approach shown in Fig. 1.

Conducting a risk assessment of critical material attributes and critical process parameters should be performed before initiating formulation and process development. This should be followed by optimizing the formulation and process using a design of experiment (Chowdary & Shankar, 2016; N. Politis, Colombo, Colombo, & M. Rekkas, 2017), known as design space development, and establishing a control strategy.

Critical Material Attribute (CMA): A physical, chemical, biological or microbiological property or characteristic of an input material that should be within an appropriate limit, range, or distribution to ensure the desired quality of output material (Maguire & Peng, 2015).

Critical Process Parameter (CPP): A process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, August 2009).

Design space means multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval (ICH Q8).

2.7.1 Formulation development

A risk assessment of formulation variables, e.g. drug substance particle size distribution, disintegrant level, glidant level, diluent level, binder level, and lubricant level to be conducted to evaluate the potential impact of each attribute on drug product CQAs, such as assay, degradation product, content uniformity, and dissolution. In the relative risk of each attribute to be categorized as high, medium, or low. High-risk attributes will require further investigation, while low-risk attributes will not need additional examination. Medium-risk attributes will be considered acceptable based on current knowledge, although further investigation may be needed to mitigate the risk. The justification behind the assigned risk level to be explained in detail. In Table 1 below, the initial risk assessment of the formulation variables is presented for illustrative purposes only.

Formulation development to be focused on evaluation of the high-risk formulation variables identified in the initial risk assessment. Initial formulation development study to be conducted at laboratory scale (Approximately 1.0 kg to 3.0 kg based on available equipment). During initial formulation development, standard manufacturing process is to be used (Process parameters do not require to be optimized during this time). Analyzing the impact of a single factor at a time is not only labor-intensive but often unproductive. Instead, one should consider multiple factors (independent variables) simultaneously in various settings through different experiments, observing their effects on the output or response (dependent variable). This approach is a more efficient and effective method for conducting or simulating experiments (Chakraborty, 2023). Design of experiment which will include formulation variables at different levels to be used to evaluate combinations effect of formulation variables on drug product critical quality attributes as well as in-process quality tests e.g. Hardness, friability, disintegration time. In Table 2 below, the optimization design for three formulation variables (classified as high or medium risk) is presented at two different levels using a 2^3 factorial design, including one center point level for each formulation variable trial in replication. Meanwhile, Table 3 showcases the number of trials and experimental results of the design for illustrative purposes only.

All tables in this review article are presented solely for illustrative purposes. For further details, please refer to following example Quality by design for ANDA: An example for Immediate-release dosage form (U.S. Food and Drug Administration (FDA), April 2012) and an example of Modified Release dosage form (U.S. Food and Drug Administration (FDA), December 2011).

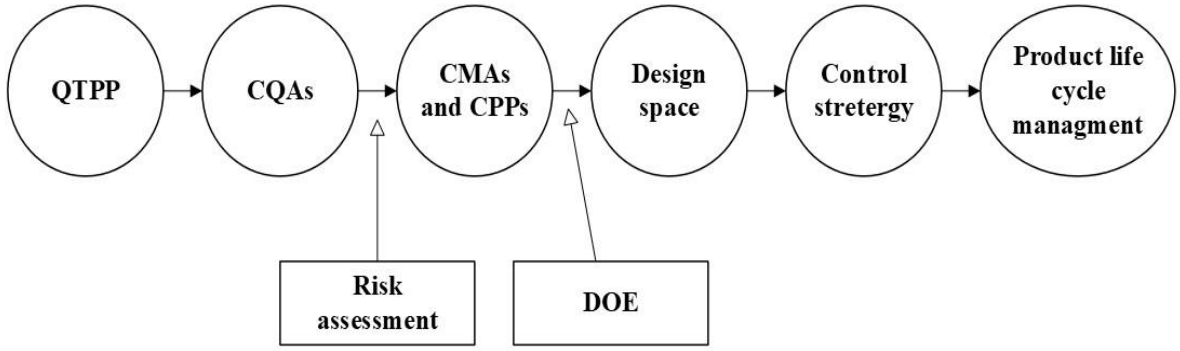

Fig. 1. Sequence of QbD approach

Table 1. Initial risk assessment of the formulation variables

Drug product CQA	Formulation Variables			
	Variable # 1	Variable # 2	Variable # 3	Variable # 4
Assay	High	Medium	Low	Low
Content uniformity	High	High	Low	Low
Dissolution	Low	Low	Low	High
Degradation products	Low	Low	Low	Low

Table 2. Design of 2^3 full factorial DOE to study formulation variable

Factors:	Level	
Formulation Variables	-1	1
Variable 1	Low	High
Variable 2	Low	High
Variable 4	Low	High
Response	**Target**	
Response 1	*	
Response 2	*	
Response 3	*	
Response 4	*	

** Record value*

Table 3. Trials and experimental results of the design

Trial	Factors: Formulation Variables			Responses			
	Variable 1 level	Variable 2 level	Variable 3 level	Response 1	Response 2	Response 3	Response 4
1.	Low	Low	Low	*	*	*	*
2.	Low	Low	High	*	*	*	*
3.	Low	High	Low	*	*	*	*
4.	Low	High	High	*	*	*	*
5.	High	Low	Low	*	*	*	*
6.	High	Low	High	*	*	*	*
7.	High	High	Low	*	*	*	*
8.	High	High	High	*	*	*	*
9.	Middle	Middle	Middle	*	*	*	*
10.	Middle	Middle	Middle	*	*	*	*
11.	Middle	Middle	Middle	*	*	*	*

** Record value*

Acceptable ranges for high and medium risk formulation variables to be established during formulation development optimization and to be included in the control strategy. Additionally, risk assessment of the formulation variables will be updated with justification as shown in Table 4.

Following the intensive application of factorial design during formulation development, a tentative composition of the generic product will be selected for optimizing the manufacturing process.

Table 4. Updated risk assessment of the formulation variables

Drug product	Formulation Variables			
CQA	**Variable # 1**	**Variable # 2**	**Variable # 3**	**Variable # 4**
CQA 1	Low	Low	Low	Low
CQA 2	Low	Low	Low	Low
CQA 3	Low	Low	Low	Low
CQA 4	Low	Low	Low	Low

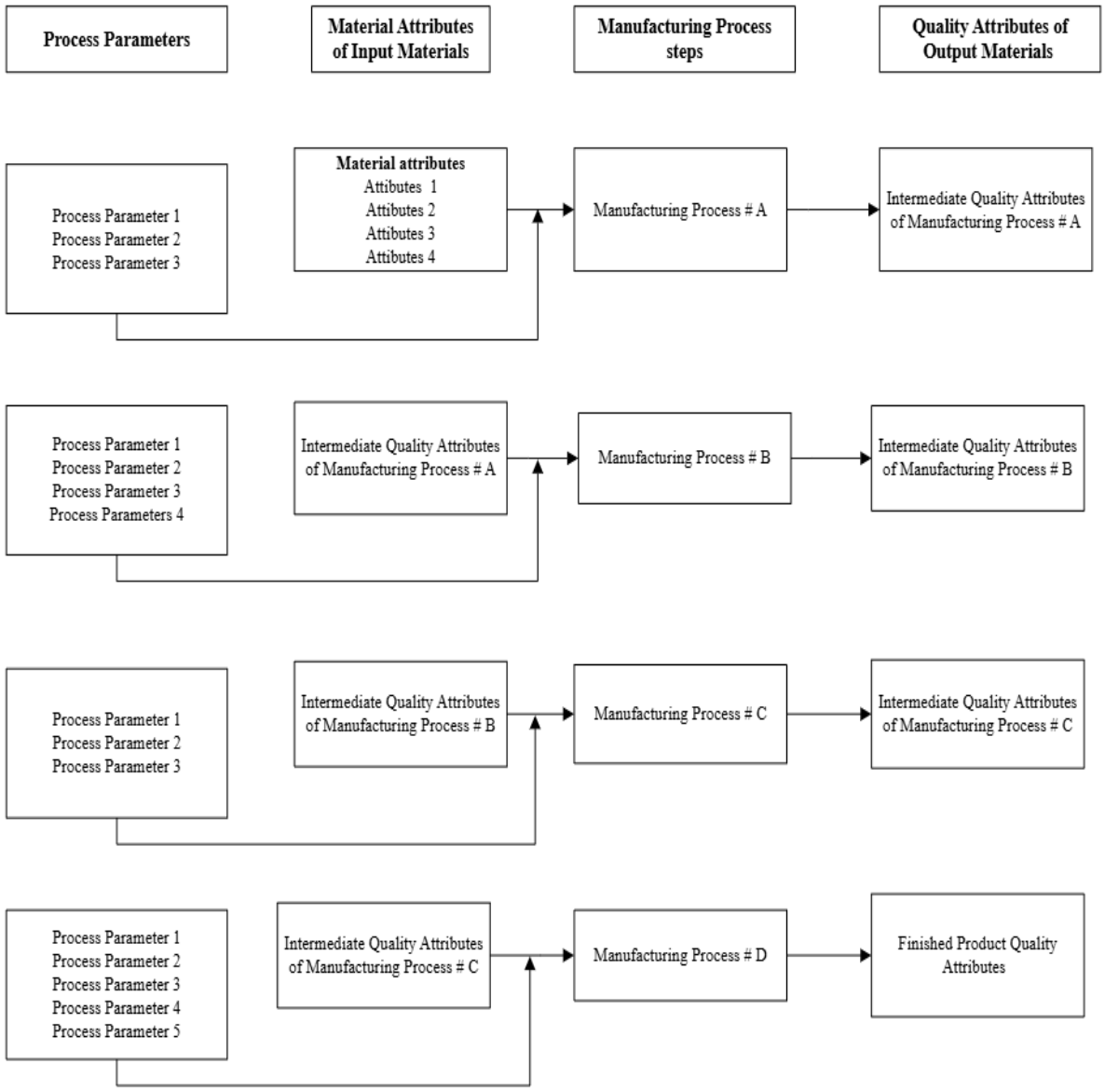

Fig. 2. Process Map Illustration

2.7.2 Manufacturing process development

Prior to starting the manufacturing process, a process map for the finalized formulation will be established. Each step in the manufacturing process will be listed in the order of occurrence. This map will illustrate the material attributes and process parameters that can potentially impact the quality attributes of intermediate and finished products. The material attributes of the input materials and the process parameters used at the initial step determine the quality attributes of the output material (intermediate) produced at this step. The material attributes of the intermediate from this step and the process parameters of the subsequent step in the manufacturing process will determine the quality attributes of the next intermediate and, eventually, the finished drug product. This cycle continues until the final step, where the finished drug product is manufactured, and its quality attributes are evaluated. The process map will guide the risk assessments performed during process development.

A risk assessment of overall manufacturing process, e.g. granulation (dry or wet), milling, blending, lubrication and compression to be conducted to evaluate the potential impact of each attribute on drug product CQAs, such as assay, degradation product, content uniformity, and dissolution. In addition to the overall manufacturing process, each variable in the manufacturing process will be evaluated for risk assessment. The relative risk of each attribute will be categorized as high, medium, or low. High-risk attributes will require further investigation, while low-risk attributes will not need additional examination. Medium-risk attributes will be considered acceptable based on current knowledge, although further investigation may be needed to mitigate the risk. The justification behind the assigned risk level to be explained in detail. In Table 5 below, the initial risk assessment of the formulation variables is presented for illustrative purposes only.

Manufacturing process development to be focused on evaluation of the high-risk process variables identified in the initial risk assessment.

Design of experiment which will include manufacturing process variables at different levels to be used to evaluate combinations effect of process variables on drug product quality attributes of output materials as mentioned in process map. This assessment will continue through to the final steps of the manufacturing process, where the quality attributes of the finished product will be evaluated. In Table 6 below, three variables for Manufacturing Process # A is presented at two different levels using a 2^{3-1} fractional factorial design with one center point where number of run can be minimized. Table 7 showcases the number of trials and experimental results of the design for manufacturing process # 1 for illustrative purposes only.

Table 5. Initial risk assessment of the overall manufacturing variables

Drug product CQA	Manufacturing Process Variables			
	Process # A	**Process # B**	**Process # C**	**Process # D**
CQA 1	High	Medium	Low	Low
CQA 2	High	High	Low	High
CQA 3	Low	Low	High	High
CQA 4	Low	Low	Low	Low

Table 6. Design of 2^{3-1} to study Manufacturing Process # A

Factors:	**Level**	
Manufacturing Process Variables	**-1**	**1**
Variable 1	Low	High
Variable 2	Low	High
Variable 3	Low	High
Response	**Target**	
Response 1	*	
Response 2	*	

** Record value*

Table 7. Tirials and experimental results of the design for Manufacturing Process # A

Trial	Factors: Manufacturing Process Variables			Responses	
	Variable 1 level	Variable 2 level	Variable 3 level	Response 1	Response 2
1.	Low	Low	High	*	*
2.	High	Low	Low	*	*
3.	Low	High	Low	*	*
4.	High	High	High	*	*
5.	Middle	Middle	Middle	*	*

** Record value*

Table 8. Updated risk assessment of the Manufacturing process variables

Drug product CQA	Manufacturing Process Variables			
	Variable # 1	Variable # 2	Variable # 3	Variable # 4
CQA 1	Low	Low	Low	Low
CQA 2	Low	Low	Low	Low
CQA 3	Low	Low	Low	Low
CQA 4	Low	Low	Low	Low

Similar to Manufacturing Process # A, all other manufacturing processes will be optimized using design of experiments.

Acceptable ranges for high and medium risk manufacturing process variables to be established during process development optimization and to be included in the control strategy. Additionally, risk assessment of each process variables will be updated with justification.

2.8 Scale-Up from Lab to Pilot Scale and Commercial Scale

After the formulation and process are optimized at the lab scale, they are scaled up to pilot and commercial production. Various scale-up principles and mathematical equations are employed to facilitate the manufacturing process scale-up. For instance, wet granulation (Alves, Simões, Simões, & Gomes, 2024), roller compaction (Nesarikar et al., 2012), final lubrication blending (Kushner IV & Moore, 2010), tablet compression press speed scale up (Dumpala, Bhavsar, & Patil, 2020) and tablet coating scale up (Pandey, Turton, Joshi, Hammerman, & Ergun, 2006) all utilize specific scale-up principles and mathematical equations.

2.9 Control Strategy

The control strategy is “a planned set of controls, derived from current product and process understanding, that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control (U.S. Food and Drug Administration (FDA), April 2009).

The control strategy will be developed from comprehensive studies on product and process understanding. These studies will investigate material attributes and process parameters that pose high risks to the CQAs of the drug product during the initial risk assessment. Variables with medium risk levels will also be evaluated in some instances. By systematically examining these factors, CMAs and CPPs will be identified, and acceptable operational ranges will be established. All variables deemed high risk in the initial risk assessment will be included in the control strategy, as the experimental conclusions will depend on the range(s) studied and the complex multivariate relationships between variables. This control strategy will thus provide a comprehensive overview of quality assurance based on current process and product knowledge. The strategy may be refined further based on experiences gained during the commercial lifecycle of the product. However, any post-approval modifications will be reported to the agency in line with CFR 314.70 and will follow guidelines for scale-up and post-approval changes.

Table 9. Control strategy example

Factor	Attributes or Parameters	Range study (Lab scale)	Set point for Pilot batch	Set point for verification batch (Commercial scale)	Proposed range for commercial scale	Purpose of control
Raw material attributes						
API	CMA 1	*	*	*	*	*
	CMA 2	*	*	*	*	*
Excipient 1	CMA	*	*	*	*	*
Excipient 2	CMA 1	*	*	*	*	*
	CMA 2	*	*	*	*	*
Excipient 3	CMA	*	*	*	*	*
Excipient 4	CMA	*	*	*	*	*
Manufacturing Process # A						
Process step	CPP 1	*	*	*	*	*
	CPP 2	*	*	*	*	*
	CPP 3	*	*	*	*	*
Manufacturing Process # A In-Process specification						
Manufacturing Process # B						
Process step	CPP 1	*	*	*	*	*
	CPP 2	*	*	*	*	*
Manufacturing Process # B In-Process specification						
Manufacturing Process # C						
	CPP 1	*	*	*	*	*
Process step	CPP 2	*	*	*	*	*
	CPP 3	*	*	*	*	*
Manufacturing Process # C In-Process specification						

** Record value*

The control strategy should encompass the critical material attributes of both drug substances and excipients, in-process controls, high-risk process parameter ranges to be investigated during development, proposed operating ranges for commercial manufacturing, and finished product release specifications.

2.10 Product Lifecycle Management and Continual Improvement

Once approved, the manufacturing process for the generic dosage form will be validated using a lifecycle approach that incorporates risk-based decision-making throughout the drug product lifecycle, as outlined in the FDA's process validation guidance (U.S. Food and Drug Administration (FDA), January 2011).

Table 10. Finished product release specification

Test	Acceptance criteria
Test A	*
Test B	*
Test C	*
Test D	*

** Record value*

The concept of validation was initially introduced by two Food and Drug Administration (FDA) officials, Ted Byers and Bud Loftus, in the mid-1970s with the aim of enhancing pharmaceutical quality. Its primary objective is to ensure that quality is integrated into the system at every stage, rather than being assessed solely at the final step (Anju & Pandey, 2017). This concept underwent further development in the United States in 1978.

The QbD approach to be adopted during the pharmaceutical development of the generic dosage form that will support product and process understanding aligned with Stage 1 (Process Design) of process validation. In Stage 1, the commercial manufacturing process will be defined based on knowledge acquired during development and scale-up activities, and control strategy will be established. The objective of Stage 2 (Process Qualification) will be to confirm the process's ability to achieve reproducible commercial manufacturing. The manufacturing facility will be designed in compliance with cGMP regulations for Buildings and Facilities (USFDA). Activities will be undertaken to verify that utilities and equipment are fit for their intended purpose and function correctly. The process performance qualification (PPQ) protocol will be drafted, reviewed, approved, and executed to demonstrate that the commercial manufacturing process operates as intended. The objective of Stage 3 (Continued Process Verification) will be to ensure the process consistently remains in a state of control (the validated state) during commercial production. Stage 3 may be best classified into Stage 3a (Data Collection and Monitoring During Routine Production) emphasis on collecting data during routine production to ensure the validated process remains in control and capable of consistently producing products of the desired quality. It mainly focuses on early routine production batches where more intensive data collection and monitoring may be performed to confirm the process is stable post-validation whereas Stage 3b (Ongoing Process Verification) emphasis on the long-term, ongoing verification of the process throughout the lifecycle of the product (Pazhayattil et al., 2018). Process validation cycle illustrated in the Fig. 3.

As part of ongoing process verification, the performance of the manufacturing process will be continuously monitored throughout the product's lifecycle to ensure it operates as expected and delivers the desired quality attributes. Statistical techniques will be employed to measure and assess process performance and capability. If any unexpected variability is detected, appropriate actions will be taken to correct, anticipate, and prevent future issues, ensuring the process remains under control. Furthermore, insights gained during routine manufacturing will be used to adjust process parameters, contributing to the ongoing improvement of the drug product.

Following recommendations by the FDA and EMA (QbD), a design space is determined early in product development through pilot-scale experiments. The commercial process is carried out within a specific area of this design space, known as the Normal Operating Range (NOR), which is close to the target operating conditions. The NOR for a commercial process is established after assessing potential scale-up effects. Continuous Process Verification takes place within the NOR. Additionally, the continuous monitoring plan for quality attributes and/or process parameters, as part of the routine control strategy, will further enhance a QbD-based product submission (Alsmeyer & Pazhayattil, 2014).

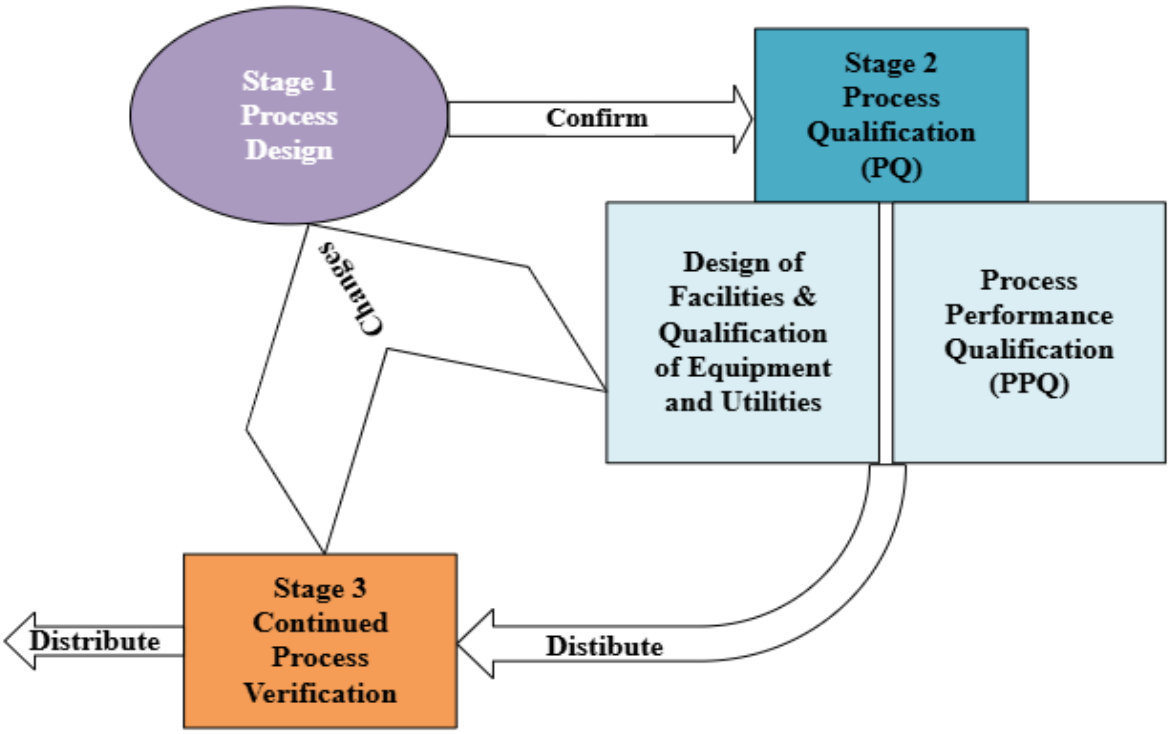

Fig. 3. Process validation cycle

3. CONCLUSION

The implementation of Quality by Design (QbD) in the development of generic solid oral drug products represents a paradigm shift in pharmaceutical manufacturing and quality assurance. By leveraging a systematic, scientific, and risk-based approach, QbD enables a deeper understanding of product and process characteristics, ensuring consistent quality while meeting regulatory expectations. The integration of key elements, such as critical quality attributes (CQAs), critical process parameters (CPPs), and design space, empowers manufacturers to achieve robust processes with reduced variability. Additionally, regulatory guidance, including ICH Q8(R2) through ICH Q14, has provided a clear framework to support practical adoption.

Although challenges persist, such as the need for specialized expertise and resource allocation, the benefits of QbD—enhanced product quality, optimized processes, and greater regulatory flexibility—are undeniable. As industry continues to evolve, the broader adoption of QbD principles will further elevate the standards of generic drug development, ultimately benefiting both manufacturers and patients through improved access to high-quality medicines.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Herbal Formulations in Management of Dermatitis

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Dermatitis, a persistently harmful skin condition, impacts millions of people globally. Redness, itching, and skin lesions are the hallmarks of dermatitis. The need for innovative approaches to therapy is highlighted by the significant side effects and poor efficacy of indigenous treatments, such as topical corticosteroids and immunomodulators. Herbal compositions offer a natural way to treat dermatitis. The compilation of the most recent information on the mechanisms, safety, and longevity of herbal formulations for the treatment of dermatitis is the aim of this thorough and extensive review. Collectively it indicates that skin lesions, irritation, and itching may be substantially minimized by using such medicines that comprise ingredients like turmeric, mango, ginkgo, and aloe vera, among others. These medications are a viable alternative to conventional medicines because of their typically good tolerance and minimal adverse reactions. The mechanisms behind the therapeutic advantages of herbal remedies in dermatitis include wound healing, antioxidant action, preventing microbial multiplication, and altering inflammatory cascades.

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These formulations contain bioactive compounds such as flavonoids, phenolic acids, and terpenoids that have been responded to skin disorders associated with dermatitis. This review highlights the potential of Phyto-pharmaceutically tailored herbal formulations as a secure, all-nature, and effective alternative to treat dermatitis. More studies are timely demand better understanding the mechanisms of action and to standardize the production and quality control of natural means in different dermatological treatments.

Keywords: Herbal formulations; dermatitis; natural remedies; wound healing; inflammation; antioxidant activity.

1. INTRODUCTION

"Dermatitis" is dubbed with inflammation of the skin accompanying erythema and discomfort. The term "dermatitis" pertains to a gamut of skin rashes and irritations triggered by a myriad of impacts, such as allergies, irritants, a hyperactive immune system, and heirlooms. Combining the words "derm" for skin and "itis" for inflammation, it results in the term "dermatitis." Contact dermatitis, atopic dermatitis, seborrheic dermatitis, and nummular dermatitis comprise the four categories of dermatitis. In contrast to atopic dermatitis, which is a pervasive, itchy skin condition that afflicts these individuals with asthma, high fevers, and a family history of them, contact dermatitis is a rash brought on by direct liaison with a tailored substance. The chronic illness known as seborrheic dermatitis is typified by red, flaky, scaly, and itchy skin. A coin-shaped rash and inflammation, nummular dermatitis is typified by tiny blisters, scabs, and scales (Kimber et al.,2002; Bonamonte et al.,2013).

Erythema, vesicles, pruritus, persistent irritation, scratching, and skin thickening are all manifestations of dermatitis, which is exacerbated by the skin's inflammatory reaction. Utilizing non-prescription skin lotion, applying cool, damp dressings, avoiding local irritation, and utilizing corticosteroids and anti-pruritic are all aspects of the treatment. Since ancient times, people have utilized herbal remedies to treat skin constraints. A return to organic produce, a spur to reengage with nature, the use of natural medicines as part of the green revolution, and the realization of the negative effects of chemical pharmaceuticals have all contributed to the current revival of herbal use. Patients are increasingly using herbal treatments, particularly those for skin conditions. Aloe Vera, Neem, Tulsi, and other plants are used to cure dermatitis. Topical dosage forms are creams, gels, ointments, pastes, suspensions, and solutions that contain one or more active chemicals that have been dissolved or equally split in a feasible framework. The dosage form design is chosen based on the intended therapeutic effect and the stipulate of the disease (Lachman et al., 1991).

The choice of excipient is mostly determined by the dosage form being designed, which is the most important step in the development of a topical dosage form since it ascertain the final product's qualities, which directly affect the effectiveness of the active ingredient and the aesthetics of the product (Banker et al., 1979). Over the past several decades, pharmaceutical research has become a lot more intrigued in skin formulation (Topical Semisolid Dosage form) due to better comprehension of skin morphology and penetration route (Chater, 2001; Aulton, 1995).

In this article an attempt has been made to review and evaluate current herbal alternatives in the management and treatment of dermatitis. Several keywords such as herbal formulations, dermatitis, natural remedies etc. had been employed to fetch relevant findings in this context. Further, few additional phrases like wound healing, inflammation, antioxidant activity etc. had also been utilized to make the study more relevant and appropriate.

2. TREATMENT APPROACHES IN DERMATITIS

1. By controlling the inflammatory response, coordinating immune system activities, and boosting antioxidant activities, herbal medicine and its active components illustrate both safeguarding and therapeutic aptitude against dermatitis (Alenazi, 2023).
2. Scientific evidence endorses the safety and efficacy of natural products, such as flavonoids, alkaloids, terpenes, glycosides, and other chemicals, in the treatment of dermatitis (Sasseville, 2008).
3. By trimming inflammation through anti-inflammatory compounds like flavonoids, tannins, and polysaccharides that can

hinder the release of inflammatory mediators like histamine and cytokinin, herbal formulations may be able to help manage contact dependent dermatitis. This will help to soothe irritated skin and lessen redness and itching (Bonamonte et al., 2013). In order to combat oxidative stress, which contributes to skin damage in contact dermatitis, several herbs also have antioxidant qualities (Slodownik et al., 2008).

4. Herbal resources for seborrheic dermatitis predominantly works by implementing their antifungal competencies to target an abundance of Malassezia yeast, a major cause of the state of affairs, while also providing anti-inflammatory effects to mitigate redness, itching, and resizing. These effects are frequently retrieved by using substances like flavonoids, terpenes, and phenolic acids (Tao et al., 2021; Adalsteinsson et al., 2020).

3. ROLE AND IMPORTANCE OF PHYTO-CONSTITUENTS

Herbal remedies are more effective in acute and chronic diseases which have fewer side effects, and are more reasonably priced; they have become the alternative implementation for the treatment and management of skin disorders (Malik et al., 2019). The chemical constituents contribute to a basic metabolic process separates them into primary and secondary metabolites. Primary metabolites usually indicate the basic biological behaviours that make them equivalent in all living cells, whereas secondary metabolites follow secondary pathways and can be the main ingredients in pharmaceutical production (Hussein et al., 2019). Because of the way plant-based compounds functioning, professionals were enthusiastic in developing natural needs to cure a range of conditions, most notably skin constraints. Patients can utilise supplements to treat and alleviate skin issues such as C and E, tea tree oil, and honey. These can restore skin health and reduce the symptoms of skin conditions. The overview aims to provide recent studies on the effects of natural compounds, such as mangiferin, lutein, curcumin, resveratrol, embelin, naringenin, quercetin, gingerol, and apigenin, on dermatological disorders (Petrova et al., 2011; Samraj et al., 2014). Numerous natural species have particular chemical components and Fig. 1 is showing the maximum available chemical component presenting plant source.

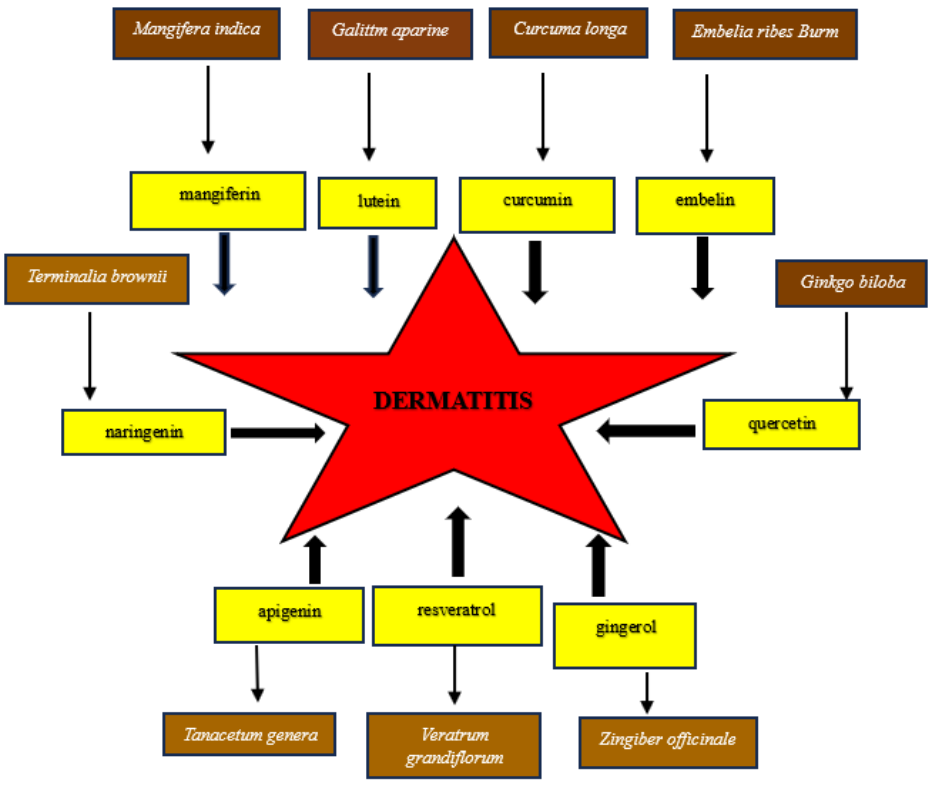

Fig. 1. Scientific names and sources of natural products reported against dermatitis

4. ELABORATION OF VARIOUS NATURAL PRODUCTS AGAINT SKIN DISORDERS

4.1 Mangiferin

A well-known substance called Mangiferin is mostly derived from the Anacardiaceae family's *Mangifera indica* (mango). It has potent chemopreventive, anti-inflammatory, and antioxidant properties. In addition, Mangiferin has hepatoprotective and gastro-protective effects, is used as a lipid-lowering drug, and has antimicrobial, antipyretic, antiviral, and antibacterial qualities (Ochocka et al., 2017).

4.1.1 Activities on skin

Mangiferin has significant impact on skin aging, enabling it to avoid wrinkles and irritation. The most prevalent inflammatory skin condition is contact dermatitis, which can be difficult to treat because it results in skin lesions and the breakdown of the skin barrier. Mangiferin is highly effective at promoting skin recuperation and wound healing (Pleguezuelos-Villa et al., 2019). For skin cancers or melanoma, mangiferin is advantageous owing to its anti-angiogenic activity, which can prevent tumours from producing their own blood cells. Mangiferin is additionally acceptable to treat human herpes viruses, such as the herpes simplex virus (HSV), and bacteria that cause skin infections (Jie et al., 2004).

4.1.2 Mechanism of actions

1. By blocking elastase, collagenase, and water loss, mangiferin helps protect the skin and prevent wrinkles, which are induced by exposure to sunlight that contains ultraviolet (UV) B or UVR radiation. Mangiferin can strengthen the collagen bonds in the skin, shielding it from harmful UVB rays (Tundis et al., 2015).
2. Oxidative stress triggers a decrease in collagen, which contributes to skin ageing. The matrix-degrading enzyme matrix metalloproteinase (MMP) is inclined to act when collagen degradation occurs. The ageing process of the skin is accelerated by the rise in MMP activity, which causes photoaging. Despite the fact that there are numerous varieties of MMP, MMP-1 is crucial and accountable for the oxidative stress-induced reduction of collagen, which is controlled by JUN-N-terminal kinases (JNK) and extracellular signal-regulated (ERK). When hydrogen peroxide is applied to human epidermal keratinocyte line (HaCat) cells, mangiferin inhibits the MEK and SEK pathways in alongside hindering the ERK and JNK pathways, which in turn prevents the expression of MMP-1. Mangiferin, however, suppresses MMP-9 activity, which is similarly produced through the ERK and MEK pathways (Kim et al., 2012).
3. Mangiferin improves wound healing and skin inflammation while lowering transcutol-P (TPA)-induced skin damage (Pleguezuelos-Villa et al., 2019).
4. When mangiferin is executed, it also suppresses the consequences of inflammatory mediators including tumour necrosis factor alfa (TNF-α) and its precursors like inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, and IL-6 that cause dermatological conditions like dermatitis and psoriasis (Zhao et al., 2017).
5. Mangiferin shows promise as an antioxidant. Through promoting fibroblast migration and cell proliferation amid the wound healing process and decreasing myeloperoxidase (MPO) activity, an enzyme entangled in inflammation, it accelerates wound healing closure.
6. Mangiferin's anti-angiogenic effect can prevent tumours from generating their own blood cells, which is why it is beneficial for skin cancer or melanoma. In addition, according to Ingenuity Pathway analysis (IPA) enrichment, mangiferin inhibits the expression of IL6, TNF, PLAU, kinase insert domain receptor (KDR), vascular endothelial growth factor receptor 2 (VEGFR2), interferon gamma (IFN-γ), fibroblast growth factor 1 (FGF1), chemokine ligand 2 (CCL2), MMP19, and placental growth factor (PGF) to hinder angiogenesis, metastasis-invasion motility, cell number growth, and viability in cancer signalling processes (Delgado-Hernández et al., 2020).

4.1.3 Various formulations with drug delivery system

1. Mangiferin, taken orally, minimises the aging-causing wrinkles mediated by UVB rays (Song et al., 2013).
2. Mangiferin nano emulsions are utilised to address skin regeneration and

inflammatory ailments (Pleguezuelos-Villa et al., 2019).
3. The hydrogel delivery mechanism of mangiferin facilitates the growth of skin flap regeneration and increases survival.
4. Mangiferin is an electrospray nanoparticle designed to combat integumentary disorders (Mao et al., 2019).

4.2 Quercetin

Fruits, vegetables, tea, spring onions, tomatoes, grapes, apples, brassica, berries, and onions are all natural sources of quercetin. The herb that possesses a majority amount of quercetin is *Ginko biloba*, which is a member of the Ginkgoaceae family. Flavonoids encompass quercetin. Furthermore, the most prevalent form of quercetin is rutin, which is predominantly glycosylated, whereas aglycone is a yellow sugar-free structure of quercetin (Ulusoy et al., 2020). In regard its anti-inflammatory and antioxidant attributes, quercetin has been revealed in countless studies to have anti-tumour, antibacterial, anti-angiogenic, anti-diabetic, anti-obesity, and anti-allergic rentals. It is also aiding for neurological and cardiovascular instances (Yang et al., 2020).

4.2.1 Activities on skin

Quercetin has anti-inflammatory and anti-aging capabilities. The cell endurance, expansion, and survival of fibroblasts are similarly impacted. One of its anti-cancer qualities is that it can help retard the proliferation of tumours and reduce cell invasion (Brown et al., 2011). Dermatological conditions that impact quality of life include cellulitis, folliculitis, impetigo, furuncles, and erysipelas. Inhibiting *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Streptococcus mutants,* and *Escherichia coli* through numerous avenues, quercetin possesses antibacterial attributes against distinct pathogens. It is also beneficial in Atopic dermatitis (prolonged erosion of the skin and inflammation), as well as contact dermatitis (an allergic reaction on the dermis that causes eruptions and distress) (Weng et al., 2012). This phytoconstituent promotes wound healing due to its anti-inflammatory and antioxidant activities, and it may mitigate keloid, an extensive dermal scar triggered by skin trauma. An *in vitro* study points to the combination of quercetin with supplements such as morin and rutin, along with the use of some antibiotics, is synergistic against MRSA (Unahabhokha et al., 2015).

4.2.2 Mechanism of actions

1. In human skin, quercetin suppresses the breakdown of collagen, COX-2, and MMP-1 induced by UV radiation. Also, owing to PKC-delta (PKCδ) and Janus kinase-2 (JAK2) are fundamental regulators of inflammation; quercetin protects the skin against UV-induced skin ageing by suppressing these two aspects (Shin et al., 2019).
2. One pertinent factor in quercetin's anti-aging properties is its inhibition of AP-1 and NF-κB activation.
3. The Nrf2 pathway allows quercetin to trigger the proteasome, which intensifies the antioxidant effect and aids prevent skin withering (Sajadimajd et al., 2020).
4. By reducing cyclin D1 and MMP-2 production, quercetin may hinder the triggering of signal transducer and activator of transcription (STAT3) via IL-6. This dampens cell proliferation thru cell aggregation, especially at the S and G2/M stages.
5. Quercetin can be beneficial in tackling melanoma by grabbing edge of tyrosinase expression, promoting p53 expression, and regulating ROS, which ultimately results in cell death and apoptosis (Vargas et al., 2011).
6. Quercetin prevents atopic dermatitis by limiting pro-inflammatory aspects and inflammatory cytokines.
7. By reducing IL-6, IL-8, and TNF-α, quercetin prevents contact dermatitis and photosensitivity, revealing its potency as a mast-cell inhibitor.
8. Quercetin seeps into the fibroblast, a deeper layer of membrane which is the prime focus for wound healing, making a good quercetin formulation that can aid boost skin penetration a must.
9. By inhibiting the transfer of Smad's complex (Smad2/3/4) and transforming growth factor-beta (TGF-β), quercetin can ameliorate keloid, a severe dermal scar caused by skin wreckage (Hatahet et al., 2016).

4.3 Curcumin

Originating from turmeric, or *Curcuma longa*, curcumin is an ingredient of the Zingiberaceae family. Curcumin, or more precisely, diferuloylmethane (75%), desmethoxycurcumin (20%), and bisdemethoxycurcumin (5%), are

curcuminoids abundant in turmeric. Meanwhile to getting used as an antimicrobial, additive, and anticancer agent, curcumin is also used to treat autoimmune diseases, respiratory conditions, depression, premenstrual syndrome, dyslipidaemia, osteoarthritis, diabetes, metabolic syndrome, endothelial dysfunction, non-alcoholic fatty liver disease, and hyperuricemia. Furthermore, curcumin possesses anti-inflammatory and antioxidant qualities. When it comes to dermatitis, curcumin does wonders.

4.3.1 Activities on skin

One useful and successful treatment for psoriasis depends on curcumin. Curcumin can be used in phototherapy for psoriasis because it is phototoxic at low concentrations against *Salmonella typhimurium* and *Escherichia coli* (Aggarwal et al., Heng et al., 2000). Additionally, curcumin works well against eczema or atopic dermatitis, reducing and improving dermatitis symptoms like thickness, erythema, scaling, and itching (Rawal et al., 2009). When used in a cream containing turmeric and sandalwood oils, this phytoconstituent can lessen radiodermatitis. After two weeks of dosing, curcumin relieves the skin damage and lessens the intensity of illuminated skin. Curcumin is beneficial in mending wounds spurred on by inflammation and oxidative damage. Its antioxidant activity triggers the cytoprotective signalling, and it suppresses lipid peroxidation, safeguarding the skin from oxidative stress. Additionally, curcumin confers human fibroblasts and keratinocytes some protection against hydrogen peroxide (Phan et al, 2001). Curcumin improves with skin ageing, particularly in older adults. It succeeds well against fungal and bacterial infections as well. Curcumin illustrates impact against methicillin-resistant *Staphylococcus aureus* (MRSA) when stipulated separately and displays some synergistic benefits when consumed in combination with other antibiotics. Curcumin is operative concerning *Acne vulgaris* (Mun et al., 2013).

4.3.2 Mechanism of actions

1. Curcumin suppresses inflammation by a direct latching mechanism that impedes with TNF-α and its receptor's signal transduction. Curcumin gel can also prevent imiquimod-induced inflammation that resembles psoriasis by blocking TNF and specific interleukin (IL) such as IL-22, IL-1β, IL-17A, and IL-17F.
2. By inhibiting NF-κB signalling, which emits inflammatory cytokines, it blocks the endosomal toll-like receptor (TLR), assisting to cause psoriatic inflammation. This diminishes the levels of IL-17 and IL-22 (Lai et al., 2017).
3. *Curcuma longa* is one of the herbs incorporating p-hydroxycinnamic acid (HCA), a phytocomponent that may tweak the protein kinase C-θ (PKC-θ) pathway by hindering PKC-θ from being phosphorylated. This can have an immunosuppressive effect on T-cells whilst avoiding the activation of T-cells triggering the development of various autoimmune disorders, including dermatitis (Vollono et al., 2019).
4. It also dampens inflammation by decreasing the transcription factor protein-1 (AP1), and NF-κB, as demonstrated in a wound model, lowers the expression of inflammatory cytokines and modifies the expression of pro-inflammatory gene products.
5. *C. longa*'s hot water extract prevents the rise in UVB-induced TNF-α and IL-1β as well as the elevated hyaluronan production that comes with ageing and contributes to dry skin.
6. In mouse keratinocyte cell lines, curcumin decreases the phosphorylation of the insulin receptor substrate-1 (IRS-1), S6K, AKT, and 4EBP1 receptors. This suggests that curcumin has an anticarcinogenic effect by inhibiting IGF-1 signalling (Asada et al., 2019).
7. By inhibiting STAT3 expression and the signalling pathway, a high curcumin dosage reduces the invasion of squamous cell A431 cells.
8. In an intradermal infection model, this component, a photosensitiser, is employed in photodynamic treatment to combat MRSA infection.
9. By generating reactive nitrogen species (RNS) and ROS linked to fungal death through apoptosis, curcumin nanoparticles prevent fungal growth (Almeida et al., 2017).

4.3.3 Available marketed formulations

1. Oral curcumin C3 Complex has the ability to lower the severity score of radiation dermatitis (radiodermatitis) and moist desquamation.
2. Herbavate® is a herbal extract cream that contains curcumin and can reduce and

improve dermatitis symptoms such as itching, scaling, thickening, and erythema.

3. Vicco® is a curcumin cream that contains turmeric oil and sandalwood oil and is used for radiodermatitis (Vaughn et al., 2016).

4.4 Lutein

Lutein, a lipidsoluble compound from the xanthop hyll family of carotenoids, is derived from the leaf y part of *Tagetes erecta* and *Galittm aparine* flow ers. It is also found in dark and leafy green veget ables and is primarily used to treat cataracts and agerelated macular degeneration (AMD). It also has some positive consequences on the connecti ve tissue (Shao et al., 2006).

4.4.1 Activities on skin

Some anti-inflammatory qualities have been demonstrated by lutein. It has been demonstrated that lutein and zeaxanthin are beneficial to the skin for improving its colour, tone, and brightness. Additionally, lutein shields the skin from UV rays from the sun, which damages skin. Lutein is an excellent treatment for a number of skin conditions, including photo dermatoses, oxidative stress, premature ageing, and skin ageing, which can lead to a skin rash. Zeaxanthin and lutein lengthen the tumour-free survival period, which is the period of time after the initial cancer therapy is finished when the patient has no more cancer indications and their tumour has shrunk in size and diversity. Psoriasis and cutaneous erythema are two conditions that lutein can reduce. With the growth of blood vessels, it has a wound-healing function (Souyoul et al., 2018; Balić et al., 2029).

4.4.2 Mechanism of actions

1. Lutein can protect against gene expression caused by UVA, UVB, and UVA1 on individual skin. While oxidative stress and photo dermatoses, which can result in skin rash, are indicators of premature ageing, lutein and tomato nutrient complex offer a good defence against UVR that damages skin.
2. Lutein and zeaxanthin can elevate the processing of hyaluronan, which is useful in wound healing. Non-sulphate glycosaminoglycans with wound healing condominiums can hydrate the skin and have a high-water binding capacity due to a hyaluronan component.
3. Carotenoids can improve skin elasticity, hydration, and boost surface lipids by reduce UV radiation which produce skin damage. Both topical and oral administration routes can endorse the skin condition, especially in skin ageing (Balić et al., 2019).
4. Sun exposure causes inflammation of the skin, where damaging UVA and UVB rays can lower serum and skin carotenoid levels and induce oxidative stress. Furthermore, whereas long wave length UV, like UVA1, produces gene expression that results in skin erythema, short wavelength UV, like UVA and UVB, induces oxidative stress. By filtering UV rays, lutein can improve skin health and reduce skin rash, also known as skin erythema (Aziz et al., 2020).
5. By preventing sunburn cells from forming and reducing epidermal hyper-proliferation, lutein and zeaxanthin help to relieve sunburn.
6. Skin cancer is a deadly skin condition. One is squamous cell carcinoma (SCC), and the other is basal cell carcinoma (BCC). Both have high incidence rates in the US, Europe, and Australia. Skin cancer is typically caused by UV radiation, which 1) damages DNA and the immune system and 2) can create free radicals with prolonged exposure to sunshine. As a result, antioxidants can help shield the skin from ultraviolet light. Similar to β-carotene, lutein is a potent antioxidant that can protect skin from UV-induced oxidative damage (Heinen et al., 2007).

4.5 Apigenin

Apigenin is a glycoside that belongs to the flavonoid class and primarily contains aglycones. It is mostly found in species of the Lamiaceae (which includes *Siderites* and *Teucrium*) and Fabaceae (which includes *Genista*) and Asteraceae (which includes *Artemisia*, *Matricaria*, *Achillea*, and *Tanacetum* genera). Furthermore, apigenin, in its glycosylated form, is present in vegetables like celery, parsley, and onion; herbs like thyme, basil, chamomile, and oregano; and plant-based drinks like wine, tea, and beer. Red and white sorghum, oranges, wheat sprouts, rutabagas, cilantro, and kumquats are other sources of apigenin (Zari et al., 2015). The biological activity of apigenin, which include antioxidant, anti-tumour, anti-allergic, anti-inflammatory, cardioprotective, neuroprotective, antibacterial, and anti-genotoxic properties, are

good and its toxicity is minimal. Additionally, it can provide protection against hypertension, autoimmune myocarditis, cardiac hypertrophy, and antidermatitic. It additionally exhibits antihyperglycemic, antiapoptotic, anti-atherogenic, and antiparasitic properties.

4.5.1 Activity against dermatitis

Apigenin aids in the treatment of skin cancer. Psoriasis and eczema can be effectively treated by reducing inflammation. This chemical component reduces dermatitis, which slows down the ageing of the skin (Kiraly et al., 2016).

4.5.2 Mechanism of actions

1. Apigenin inhibits cell division and cell cycle progression while activating AMP-activated protein kinase (AMPK) to treat skin cancer.
2. It can reduce skin malignancies by lowering the production of COX-2, EP1, EP2, and PGE2, increasing terminal differentiation, and inhibiting cell proliferation (Imran et al., 2020).
3. By inhibiting Akt (protein kinase B) and mTOR signalling, apigenin suppresses dermatitis cancer.
4. By demethylating the Nrf2 gene promoter, this phytoconstituent prevents skin cancer.
5. By lowering DNMT epigenetic proteins and HDAC, inhibiting UVB-induced carcinogenesis because of TSP1, enhancing permeability barrier homeostasis, and raising the mRNA level in lipid synthetic enzymes, filaggrin, and lamellar body production, it can aid in preventing the development of skin cancer.
6. Apigenin aids to cure inflammatory diminution such psoriasis and eczema by suppressing the inflammatory cytokines owing to TSP1.
7. It can minimise skin aging by lowering of skin harshness and alleviate the fine cracks and wrinkles due antioxidant activity (Zari et al., 2015).

Table 1. Various plant sources with their activities on dermatitis (Chithra et al.,1998; Christenson et al., 1981; Poltanov et al., 2009; Olmedo et al., 2013; Griere, 1992; Katiyar et al., 2000; Gediya et al., 2011; Callen et al., 2007; Armstrong et al., 1999; Benzie et al., 2011)

Plants Name	Biological Sources	Uses on skin	Herbal Formulations
Aloe-vera	*Aloe barbadensis*, Liliaceae	Anti-Inflammatory	Gel, Scrub
Sandalwood	*Santulum album*, Santalaceae	Anti-Inflammatory	Oil
Amla	*Emblica officinalis*, Phyllanthaceae	Anti -oxidant	Scrub
Rosemary	*Rosmarinus officinalis,*	Anti- ageing	Oil
Papaya	*Carica papaya*, caricaceae	Depigmentation Effects	Face wash
Cucumber	*Cucumis sativus*, Cucurbitaceae	Anti- wrinkle, Anti- oxidant	Sheet masks and Gel
Carrot	*Daucus carota*, apiaceae	Anti- aging	Packs and Lotions
Neem	*Azadirachta indica*, meliaceae	Antiseptic, anti-acne	Toothpaste, soap, shampoo, balms
Oat, Shofan	*Avena sativa*, Poaceae	Treatment of eczema, wounds, irritation, inflammation, erythema, burns, itching, sunburn	Cream
Chamomile, Babuna	*Matricaria chamomilla,* Asteraceae	ointment or cream	ointment or cream

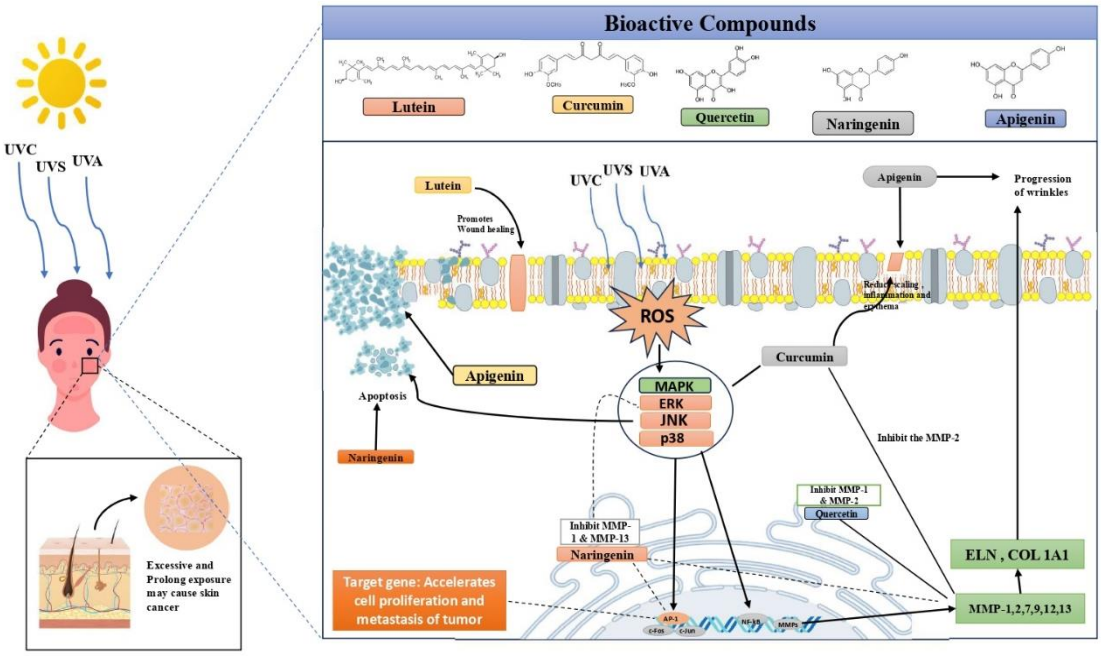

Fig. 2. Mechanism of actions of some phytoconstituents against dermatitis

5. POTENTIAL AND DIFFICULTIES OF NATURAL PRODUCTS IN DIFFERENT DELIVERY SYSTEMS

There are conventional transdermal formulations, such as ointments, creams, and lotions, but they have certain limitations too, like being sticky, not being spreadable, and having stability problems, all of which lead to non-compliance. Transdermal dissemination has advanced to the point where more effective and patient-compliant transparent gels and emulgels have been created. As a result, both the pharmaceutical and cosmetics industries are experiencing an increase in the use of these formulations. The literature claims that by rupturing the lipid bilayer and extending their retention at the site of action, topical formulations with nanoscale particles can improve the permeability of natural substances (DeLouise, 2012). Nowadays, some of the most significant nano-formulations utilised for cutaneous and dermatological applications of phytomedicines are polymeric nano micelles, ethosomes, niosomes, liposomes, lipid nanoparticles, phytosomes, nano emulsions, transferosomes, niosomes, β-cyclodextrin complexes, and phytosomes (Jeevanandam et al., 2016). The low viscosity and spreadability of nano emulsions tend to limit their potential to augment the accessibility of endogenous constituents over simple micellar solutions and to provide greater thermodynamic stability when compared to unstable dispersions such as emulsions and suspensions. Water-in-oil (w/o) or oil-in-water (o/w) nano emulsions with a gelling agent are known as nanoemulgels. In contrast to alternative carriers like solid lipid nanoparticles, liposomes, or microemulsions, nanoemulgels offer a number of benefits, such as improved drug-loading ability, less skin irritation, and enhanced permeability (Huang et al., 2010). There have been several methods employed in the development of nano phytomedicines. The methods include salting out, solvent emulsification-diffusion, complex coacervation, nanoprecipitation, co-precipitation, and self-assembly. Notwithstanding the promising results of nano formulations of natural goods, it is crucial to conduct a comprehensive evaluation of their safety, taking into account any potential toxicity to the phytomedicine or an aspect of the nano system. The negative aspects of natural products in the creation of skin formulations to treat a range of skin conditions could be addressed by any of the ways mentioned (Kalani et al., 2011).

6. CONCLUSION

Herbal remedies are a viable substitute for the attitudes of dermatitis, enabling a safe, natural, and efficient means of reducing symptoms and enhancing skin health. The anti-inflammatory, antioxidant, and antibacterial qualities of the bioactive aspects found in many herbs make

them a desirable choice for the treatment of dermatitis. Significant effectiveness has been proven by herbal formulations in lowering dermatitis-related skin lesions, irritation, and itching. In general, herbal formulations are thought to be safe, well-tolerated, and to have few known adverse effects. Patients frequently favour natural remedies because of their perceived safety and natural origin. Combining herbal formulations with traditional treatments can increase their effectiveness and lessen their negative effects. In order to guarantee uniformity and predictability of results, standardisation of natural remedies is essential. For herbal compositions to be shown safe and effective in treating dermatitis, extensive clinical trials are required. To clarify how herbal bioactive components work in dermatitis, more research is required. For dermatitis, herbal formulations impart a useful adjunct or equivalent to traditional therapies. A trend towards more natural and integrative strategies to skin health may occur as research into the benefits of herbal therapy continues.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The Application of Joint Classroom Discussion Case-Based Learning Teaching Method in the Field of Urology

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This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In teaching poses significant challenges for clinical doctors and medical students. Students frequently struggle to apply theoretical knowledge in real clinical contexts. We explore the joint discussion of flipped classroom in urological surgery theory teaching case-based learning (CBL) applies effects. This study aims to assess the effectiveness of integrating Case-Based Learning (CBL) with a Flipped Classroom (FC) approach in the context of nephrology bedside teaching.
Methods: Select students from International Education College of Hebei North University and Lahore University from June to September 2021.

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Classes 1 to 4 of the 2018 clinical medicine undergraduate students belonging to the hospital were selected as the research subjects. There were 160 students in total, and 80 students were randomly selected as the experimental group. The flipped classroom joint discussion The CBL teaching method is used when teaching lectures. The process prepares teaching cases for teachers, writes courseware, and enables students to study independently before class. practice, classroom discussion-based CBL teaching, and after-class review; 80 students served as the control group, adopting pre-class preview. Teacher's explanation; after-class review; after-class homework traditional teaching model. Examination and questionnaire will be conducted after the urinary system theory lecture. Survey students' satisfaction with this teaching model.

Results and Test Scores of the Experimental Group: The score of (91.52±4.78) was higher than that of the control group (83.76±3.52), and the difference was statistically significant. Economic significance ($P < 0.05$); the score of case analysis questions (25.87±0.54) is higher than the score of the control group (21.46±0.76); the difference was statistically significant ($P < 0.05$); Moreover, the proportion of people with test scores of 80 and 90 or above in the experimental group was also higher than the control group ($P < 0.05$). The experimental group is improving its independent learning ability and strengthening clinical practice. Cultivate clinical thinking ability, improve the ability to analyze and solve clinical problems, and strengthen understanding and memory of knowledge points, strengthening teamwork skills, strengthening teacher-student interaction, The satisfaction of these 7 items of this teaching model is higher than that of the control group ($P < 0.05$); in terms of taking up spare time and increasing their study load, the students in the experimental group. The degree of dissatisfaction was higher than that of the control group ($P < 0.05$).

Conclusion: Joint discussion on flipped classrooms. The theory-based CBL teaching method has significantly improved the quality of teaching and has made a significant contribution to the medical teaching model. The exploration has certain reference value and can guide the reform of medical teaching.

Keywords: Case-based teaching method; flipped classroom; teaching reform; teaching evaluation; medical theory; urology.

1. INTRODUCTION

Medicine is related to human health and the future, and it is a subject that requires lifelong learning. Subject (Yin et al., 2022), thus improving students' interest in learning and enhancing theoretical knowledge and practical skills. Being able to use abilities flexibly, improve independent learning abilities, and have the ability to unite and innovate are qualities that excellent medical students and doctors must possess. However, for a long time, our country's higher medical education mainly adopted a teacher-centred teaching method. Students passively accept knowledge, which is not conducive to the cultivation of comprehensive quality in medical students (Liu and Qi, 2019). The State Council Office on Deepening Medical Education Collaboration and Further Promoting Medical Education Reform," issued by the Public Office and Development Opinions, aims to improve the quality of medical talent training. Therefore, this study adopts the flipped classroom joint discussion method in urology theory teaching. Case-based learning (CBL), explore this effect of a new teaching model and student satisfaction.

2. MATERIALS AND METHODS

2.1 General Information

Select the 2018 clinical medicine majors at Hebei North University and Lahore University from June to September 2021. Classes 1 to 4 of undergraduate students are the research subjects, and they are divided into experimental group and experimental group using random number table method.

Control group, 80 people in each group. There were 38 males and 42 females in the experimental group, with an average age of (20.15±1.21) years old; in the control group, there were 39 males and 41 females, with an average age of (20.23±0.96) years old. Comparison of age, gender, and educational background of the two groups of students. The difference is not statistically significant ($P \leq 0.05$) and is comparable.

2.2 Research Methods

Two groups of teachers and teaching hours (24 hours, 2 times a week, 4 hours each time), performance assessment, and questionnaire methods are all the same. Control group: Preview before class - Teacher's explanation in class - Students' review after class Study: the traditional teaching model of completing problems assigned by the teacher, mainly based on the teacher's explanation. Students mainly listen to lectures. Experimental group: adopt flipped classroom joint discussion CBL teaching method. Student randomly divided into 8 groups of 10 students each, with designated group leaders, and learning activities conducted in groups. 1) Teaching cases: In accordance with the requirements of the talent training plan, teaching syllabus, and teaching objectives, organise teachers to write appropriate cases. The cases should be close to life and clinical to show its reality; 4 to 6 cases are designed for each class, and the cases all have key issues.

Questions, at least 2 to 3 questions, and it should involve the current status and development direction of research related to this disease. Teachers connect the key and difficult points of the course through discussions to broaden students thinking and guide students to learn independently. For example: Male patient, 56 years old, due to I was hospitalised for 1 year with intermittent painless gross haematuria, Hb 105 g/L, B-ultrasound showed liver and gallbladder Write courseware: A 2.0 cm solid mass was seen on the left wall of the bladder in this patient.

What is the clinical diagnosis? What is the first test for this disease? Principles of treatment for this disease: What is it? How do I choose a technique? How should I take medication after surgery? 2) Write courseware: According to the requirements of the teaching syllabus, teachers focus on teaching objectives and combine teaching cases to compile Write courseware for students to preview before class, but the content of the courseware should not be limited to the syllabus. You can add multimedia materials, expand your knowledge, explain things in a simple way, and use various special features to Characteristic icons and pictures display teaching content to students, connect knowledge points, and make the lesson more convenient. The files are more vivid, focused, and rich in content, making it easier for students to preview and understand, improve their preview interest (Xu et al., 2018), and introduce relevant academic cutting-edge content. 3) Students learn independently before class; teachers will provide teaching cases and teaching courseware 2 weeks in advance. Send it to students for preview, requiring students to master key and difficult knowledge and prepare for problems that arise during study. Students first search for literature and read books to solve them. Decision, and then discuss within the group or seek help from teachers or teachers through the class communication group. Help from classmates. Each group can choose student representatives to collect and sort out the questions in the preview. Use this question as a guide as part of your class discussion. 4) Class time Arrangement: In class, teachers teach based on teaching cases and follow the order of questions. Representatives from each group reported case issues using PPT. After the report, each group discussed complementing each other, asking their own questions, and finally finding the correct answer; during this period, the teacher provides guidance and adds additional information to the key or difficult content that has not been explained to the students. To supplement, deepen the impression, and promote the internalisation of knowledge. At the same time, the ideological and political content of the course can be throughout it; it triggers students to think deeply. 5)

After-class review: Complete all after reading the teaching content of this section, the teacher guides the students to review the learning content of this section and Comment and summarise the performance of the class, affirm the students' achievements, and also point out the existing problems. The shortcomings of the teaching model can be identified step by step to improve the effectiveness of the teaching model.

2.3 Observation Indicators

(1) Written test assessment: Objective questions check students' mastery of basic theoretical knowledge; grasp the situation; ask subjective questions evaluate students' clinical thinking ability through case analysis questions, etc. The full score is 100 points; the test question type and score distribution are objective questions with a total of 40 points (including multiple-choice questions, name explanations), and subjective questions total 60 points (including short answer questions, case analysis questions). Perform statistics and analysis on the number of people with high scores and the

number of people who failed in the two groups: The number of people with high scores is the number of people with scores above 80 and above 90; the number of people who failed that is, the number of people below 60 points. The more people in high segments, the better, and vice versa. The larger the number of people in the group, the worse the learning effect within the group.

(2) Questionnaire: Anonymous questionnaire on satisfaction with teaching methods investigation. Content includes: independent learning ability, clinical thinking ability cultivation, analysis Ability to analyse and solve clinical problems, understanding and memory of knowledge points, and teamwork ability; teacher-student interaction; taking up spare time to increase learning burden; recognition of the teaching mode; selecting "yes" or "no" to answer.

2.4 Statistical Methods

Specialists collected research data and used SPSS 19.0 statistical software to conduct the research.

For analysis and processing, the count data is expressed as n (%), and the $\chi 2$ test is performed to measure the data. Material based on (x-±s), perform a t test, and $P < 0.05$ means the difference is statistically significant.

3. RESULTS AND DISCUSSION

3.1 Comparison of Test Scores

After statistics, it was shown that the total score and case analysis question scores of the experimental group were significantly higher. Higher than the control group, the difference was statistically significant ($P < 0.05$), see Table 1. At the same time, the proportion of patients with scores ≥ 80 and < 90 and ≥ 90 in the experimental group was significantly higher than that in the control group. The score <60 was significantly lower than that of the control group, see (Table 2).

3.2 Analysis of Survey Results

After the course, the two groups distributed and recovered 160 questionnaires.

The effective recovery rate of the volume is 100%. The results of the questionnaire showed that compared with the control group, the experimental group in independent learning, clinical thinking ability cultivation, ability to analyse and solve clinical problems, understanding and memory of knowledge points, teamwork ability, teacher-student interaction, and recognition of the teaching mode is better than the control group, and the difference is statistically significant ($P < 0.05$); because the experimental group occupied their spare time and increased their study burden, the degree of dissatisfaction was higher than that of the control group, and the difference between the two groups was statistically significant ($P < 0.05$); see (Table 3).

College students are the main group that masters new technologies and new ideas in society.

High-quality talents cultivated by the state are the main group of people who promote social progress and the ability to learn independently and analyse and solve clinical problems through teamwork. It is the standard for qualified college students in the new era. In the era of network Informaionization, medical education. Education has also kept pace with the times, and a variety of teaching models have emerged. Flipped classroom, FC) subverts teacher teaching, Traditional teaching of students' passive learning in the education model, students focus on self-study, teachers answer questions and solve doubts, and they follow the "student-cantered" approach to teaching philosophy, which leads to the exchange of roles between teachers and students and the diversity of teaching modes. Series of changes. Flipped classroom re-adjusts learning arrangements inside and outside the classroom. The initiative of learning is transferred from teachers to students, cultivating students' independent learning ability. Make learning more active and students more engaged (Wang et al., 2020). Foreign research found that transferring classrooms can better stimulate students' independent learning ability, and students can take charge of their own learning. Rhythm, greater participation, and more authentic learning are currently applied in subjects such as pharmacy (Saba et al., 2019), physiology (Zante et al., 2020), and epidemiology (Shiau et al., 2018). Students first become independent. Learning is the first step in flipping the classroom. In the classroom, teachers and students complete the knowledge transfer process independently with students. The process of further internalisation of knowledge is completed through classroom discussions. However, due to

the limited energy of teachers, they cannot take into account the lack of knowledge. Students who lack the ability to learn independently lead to a decline in teaching quality (Zhao et al., 2021). CBL teaches the study of law originated at Harvard University and revolves around real-life situations with no specific solutions. Cases inspire students to analyse, think and discuss, and apply them around the world in fields such as law, medicine, and business (Ren and Chen, 2019). Compared with traditional teaching, teachers are more when designing motivating characters, lead students to participate in discussions. After the 1990s, CBL teaching methods have begun to be explored in clinical medicine and other fields, and students are no longer actively sued. Students know what to do, and students deepen their knowledge by actively consulting relevant information. Understanding, and transform knowledge into abilities through teamwork and other methods. Competencies are often skills that good doctors must possess (Wang et al., 2020). Types of CBL Teaching Including lecture-based CBL teaching, discussion-based CBL teaching, and contextualised CBL teaching. Discussion-based CBL teaching is a commonly used teaching model. Teachers use relevant Compile or select appropriate cases based on knowledge points, and ask relevant questions based on the cases. Then conduct discussions within and between groups. The teacher mainly guides the discussion direction. This kind of the teaching model is highly motivated, has a strong sense of participation, and can also inspire Students think to master the relevant knowledge points of the disease (Pons and Zheng, 2019). The author will translate. Combining classroom transfer with discussion-based CBL teaching methods, the study concluded: the experimental group. The theoretical test score (91.52±4.78) was higher than that of the control group (83.76±3.52) divided.

The difference was statistically significant ($P < 0.05$). The scores of the experimental group (25.87±0.54) were higher than those of the control group (21.46±0.76), and the difference was statistically significant ($P < 0.05$). In addition, the number of test scores ≥ 80 and < 90 and ≥ 90 in the experimental group was higher than that in the control group, and the difference was statistically significant ($P < 0.05$). < 60 points, ≥ 60 points, and < 70 points, there was no significant difference between the experimental group and the control group ($P > 0.05$). Through self-study, repeated discussion and thinking, and teacher supplementation, students have a deeper understanding of key and difficult knowledge and improve their ability to understand cases, analyse problems, solve clinical problems, and think clinically.

Table 1. Comparison of theoretical assessment scores of two groups of students (points, x - ±s）

Group	Case analysis question scores	Overall score
Experimental Control group (n=80)	25.87±0.54	91.52±4.78
Experimental Control group (n=80)	21.46±0.76	83.76±3.52
t value	42.308	11.692
P value	＜ 0.05	＜ 0.05

Table 2. Comparison of the distribution of students' theoretical test scores between the two groups [Name (%)]

Constituencies	< 60 points	≥ 60 points and < 70 points	≥ 70 points and < 80 points	≥ 80 and < 90 points	≥ 90 points
Experimental group (n=80)	0	14（17.50）	22（27.50）	29（36.25）	15（18.75）
Control group (n=80)	5（6.25）	24（30.00）	34（42.50）	12（15.00）	5（6.25）
χ2 value	_	3.451	3.956	9.477	5.714
General value	＞ 0.05	＞ 0.05	＜ 0.05	＜ 0.05	＜ 0.05

Table 3. Comparison of students' satisfaction with teaching [Name (%)]

Constituencies	Improve self-directed learning	Strengthen the clinic Thinkingskills training	Improve analysis and resolution Ability to do clinical problems	Strengthen theunderstanding and memorization of knowledge points	Strengthen the team Ability to collaborate	Strengthen teachers and students interaction	Acknowledging the teaching mode	occupies spare time, Increase the study load
Experimental group (n=80)	72（90.00）	75（93.75）	78（97.50））	76（95.00）	75（93.75）	74（92.50）	76（95.00）	57（71.25）
Control group (n=80)	56（70.00）	61（76.25）	60（75.00）	58（72.50）	60（75.00）	59（73.75）	60（75.00）	40（50.00）
χ2 value	10.000	9.608	17.075	14.879	10.667	10.025	12.549	7.567
General value	＜ 0.05	＜ 0.05	＜ 0.05	＜ 0.05	＜ 0.05	＜ 0.05	＜ 0.05	＜ 0.05

More flexibility in the application of knowledge. In the teaching satisfaction survey, the dissatisfaction of the students in the experimental group was higher than that of the control group in terms of occupying spare time and increasing the learning burden, which indicated that the students' self-directed learning ability was not good and needed to be improved, which is also a common weakness of college students in China at present, which is not unrelated to the previous "cramming" teaching (Fang et al., 2018), and the teaching reform should not be abandoned because of the dissatisfaction caused by this aspect. With the extensive and in-depth advancement of education reform, when self-learning ability becomes a habit of college students, this dissatisfaction will gradually decrease (Wang et al., 2021). The combination of the flipped classroom and the discussion-based CBL teaching method can organically combine their advantages and improve the teaching effect more significantly, so that students' comprehensive ability can be comprehensively improved (Liu et al., 2019).

In the process of CBL teaching, more attention is paid to two-way communication, and the process of discussion is anytime and anywhere, which requires teachers to constantly think, carefully plan, and summarise, which puts forward higher requirements for teachers. Teachers need to prepare cases in advance, which should be objective and vivid, and the results should be complex and varied. Teachers should correctly guide students' problems and discussions in teaching, change the teaching form, brainstorm ideas, and make timely adjustments to meet the needs of teaching (Ding et al., 2021).

4. CONCLUSION

The results of this study suggest that the application of the flipped classroom and discussion-based CBL teaching method in the study of urology theory is significantly improved compared with the traditional teaching method in improving teaching effectiveness and student satisfaction. The flipped classroom joint discussion CBL teaching method is worthy of further application and exploration in clinical teaching.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It Is Not Applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Development and Evaluation of Polyherbal Dispersible Tablets Using Aqueous Leaf Extracts

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The increasing demand for natural and herbal medicines has prompted the exploration of polyherbal formulations as viable therapeutic options. Dispersible tablets offer advantages such as ease of administration and rapid onset of action. The objective of this study was to develop and evaluate polyherbal dispersible tablets incorporating aqueous leaf extracts of *Camellia sinensis*, turmeric, grape seed, oregano, and *Salvia officinalis*. Polyherbal dispersible tablets were formulated and evaluated for their physicochemical properties, disintegration, and stability. Various extracts, including those from *Camellia sinensis*, turmeric, grape seed, oregano, and *Salvia officinalis*, were used. Micromeritic properties, such as angle of repose, bulk density, and Hausner ratio, were assessed. The results revealed that the prepared polyhedral dispersible tablets were non-sticky and looked high-quality. The maximum weight variation obtained was 2.50%, which falls within the acceptable weight variation range, i.e., ±5%, hence passing the weight variation test.

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The hardness of prepared tablets was in the range of 2.94 to 3.02 kg/cm^2, which falls within the limit of not < 3.0 kg/cm^2. All the tablets showed a friability value at most 0.90%, which is less than the ideal limit, i.e., 1%. Formulation AP4 exhibited superior characteristics, with rapid disintegration time and stable drug release profile. Stability studies further validated the formulation's consistency under varying storage conditions. Preliminary phytochemical screening of the individual drugs and polyherbal formulation confirmed the presence of phytoconstituents such as flavonoids, alkaloids, carbohydrates, gums & mucilage, fats & fixed oils, steroids, glycosides, phenols, saponins but no volatile oils. The findings underscore the potential of polyherbal dispersible tablets as efficient delivery systems for medicinal herbs. Carr's index and Hauser's ratio showed that the powder mixtures possess good flow properties. AP1 to AP9 were determined for the uniformity in weight, hardness, drug content and friability, which complied with the official requirements and the official limits mentioned in IP 2010.

Keywords: Polyherbal formulation; dispersible tablets; micromeritic properties; stability studies; drug release profile.

1. INTRODUCTION

"At present, most of the present-day medicines have plant origin, as it has been implicated that modern medicine has evolved from folk medicine and traditional systems on the basis of pharmaceutical and chemical screenings carried out" (Mathew & Babu, 2011; Pandey et al., 2013). Polyherbal plant extracts, which usually comprise of two or more plant parts, often contain a wide array of key phytoactive constituents relevant in attaining greater therapeutic efficacy" (Idu et al., 2021). "The increasing demand for natural and herbal medicines has prompted the exploration of polyherbal formulations as viable therapeutic options" (Agnihotri & Singh, 2014). "Dispersible tablets offer advantages such as ease of administration and rapid onset of action" (Sharma & Leel, 2022; Kapoor & Singla, 2015). The objective of this study was to develop and evaluate polyherbal dispersible tablets incorporating aqueous leaf extracts of *Camellia sinensis*, turmeric, grape seed, oregano, and *Salvia officinalis*. "The study focused on optimizing tablet formulations and assessing their physicochemical properties, disintegration, and stability" (Nagar et al., 2011). The identification of structures with unique biodynamic effects can also lead to an innovative chemical entity trail for drug development" (Parasuraman et al., 2014; Parfati et al., 2018). "The scope of Reverse Pharmacology is to understand the mechanisms of action at diverse stages of biological organization and to make optimal safety, effectiveness and acceptability of the leads in natural products, based on relevant science. There are two discrete forms of research on medicinal plants. In the first segment, the choice of plant is mainly based on their genuine use and reputation in the Indian traditional system of medicine, although in second stage, more extensive base, in which screening of a large number of natural products for biological activity is commenced, irrespective of the circumstance whether these plants are being used by the traditional system of medicine or not. Herbal medicines include herbs, herbal materials, herbal formulations and finished herbal products" (Saggar et al., 2022). Herbal medicine has perpetually represented a crucial element of primary healthcare (Awasthi et al., 2014; Gurley, 2012). Approximately 80% of the global population is estimated to use herbal medicinal products for their therapeutic benefits (Balkrishna et al., 2024). "Herbs include crude plant material, such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered (Rajab, 2018; Rawat & Vashistha, 2011; Srivastava et al., 2012). Herbal materials include, in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs" (Chaudhary & Singh, 2011). "In several countries, these materials may be treated by various local processes, such as steaming, roasting or stir-baking with honey, alcoholic beverages or other materials" (Garg et al., 2012). "Herbal preparations are the basis for finished herbal products and may comprise powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials" (Qadir & Raja, 2021). They are formed by extraction, fractionation, purification, concentration, or other physical or biological processes (Kulkarni, 2009). They also include preparations prepared by steeping or heating herbal ingredients in alcoholic beverages and/or honey or in other materials. Finished herbal products consist of herbal preparations made from one or more herbs. If

more than one herb is used, the term —mixture herbal product can likewise be applied. Finished herbal products and mixture herbal products may contain excipients in addition to the active ingredients.

2. MATERIALS AND METHODS

2.1 Materials

Fresh leaves of *Camellia sinensis*, turmeric rhizomes, grape seeds, oregano leaves, and *Salvia officinalis* were sourced and authenticated. All reagents and chemicals used were of analytical grade.

2.2 Extraction Procedure

The plant materials were dried at 37° C for six days, powdered, and extracted with methanol for 48 hours. Extracts were concentrated using a vacuum evaporator at 50° C, yielding viscous masses stored at 4° C.

2.3 Preparation of Polyherbal Dispersible Tablets

Formulations AP1 to AP9 were prepared using direct compression. Ingredients included polyherbal extracts (250 mg), β-cyclodextrin, microcrystalline cellulose, sodium saccharin, magnesium stearate, and talc. Powdered ingredients were mixed via geometric dilution, sieved through a 120-mesh sieve, and compressed using a rotary tablet compression machine. Formulation and characterization of tablets Polyherbal dispersible tablets were compressed each of 550 mg weight on a 10-station Mini Press-I rotary tablet compression machine fitted with a 12 mm punch size. No tablet manufacturing defects like capping, lamination, and chipping were observed.

2.3.1 Procedure

1. A mixture of powdered herbs of each 250 mg, weighted separately to tablets.
2. After weighing, the powder herbs were pulverized properly using a mortar pestle.
3. After uniform mixing of all the particles, sieving was performed by using sieve No. 85.
4. After that, the powder material was taken for compression. By an automatic tablet compression machine, 25 tablets were compressed. This is how poly-herbal tablets were prepared.
5. All formulations (AP1-AP9) were subjected to evaluation of characteristic parameters like size, shape, colour, and appearance. The colour and shape of all formulations were observed organ optically and found to be similar.

2.4 Evaluation Parameters

Micromeritic Properties:

- Bulk density
- Tapped density
- Compressibility (Carr's Index)
- Hausner ratio
- Angle of repose

Tablet Evaluation:

- Weight variation
- Hardness
- Friability
- Disintegration time
- In vitro dispersion time

Stability Studies: Formulations were subjected to accelerated stability testing under varying temperature and humidity conditions for three months, as per ICH guidelines.

Dissolution Studies: In vitro drug release was evaluated using a USP dissolution apparatus. Absorbance was measured at 221 nm to calculate cumulative drug release.

Table 1. Preparation of polyherbal dispersible tablets

Ingredients	Formulation Code								
	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	AP9
AEP (in mg)	250	250	250	250	250	250	250	250	250
B Cyclodextrin	215	220	225	230	235	210	200	200	200
MCC	65	60	55	50	45	60	65	60	65
Sod. Saccharin	10	10	10	10	10	10	10	10	10
Mg. Stearate	05	05	05	05	05	15	20	25	20
Talc	05	05	05	05	05	05	05	05	05
Total	550	550	550	550	550	550	550	550	550

3. RESULTS AND DISCUSSION

Characterization of powder the primary characterization of powder and micrometric properties of formulations containing Polyherbal aqueous leaf extracts powder used are mentioned in Table 1.

The prepared polyhedral dispersible tablets were non-sticky and looked high-quality. The diameter and thickness of tablets was determined using 20 tablets of a single formulation via digital venire scale during the physical study because it permits accurate measurements and provides exact information about variations between tablets of each formulation.

Table 2. Micromeritic parameters of polyherbal aqueous root extracts powder (AEP)

Parameters	Extract (AEP)
Bulk Density (GM/ML)	0.42±0.04
Tapped Density (GM/ML)	0.59±0.08
% Compressibility	20.64
Hausner Ratio	1.39±0.14
Angle of Repose (°)	26.48±1.02

Table 3. Physical Properties of the tablet

Parameter	Result
Colour	Dark Buff-Brown
Shape	Round, Biconvex
Odor	Characteristic odour
Taste	Pleasant taste

The maximum weight variation obtained was 2.50%, which falls within the acceptable weight variation range, i.e., ±5%, hence passing the weight variation test. The hardness of prepared tablets was in the range of 2.94 to 3.02 kg/cm^2, which falls within the limit of not < 3.0 kg/cm^2. All the tablets showed a friability value at most 0.90%, which is less than the ideal limit, i.e., 1%. The disintegration apparatus used for the study was determined using USP (Electro lab-ED2 SAPO). It contains two basket rack assemblies. Each basket rack assembly comprises six glass tubes that are 3 inches long, open at the top and held against ten mesh screens at the bottom. Each tablet was placed in each basket tube, and the basket rack was dipped in a 1-L beaker of distilled water. The dispersion time of polyherbal dispersible tablets was observed by placing two tablets in 100 ml of water in a beaker and gently stirring until dispersed completely. We obtained a smooth dispersion by passing through a sieve screen with a nominal mesh aperture.

3.1 *In vitro* Dissolution Study

The dissolution profile of a polyherbal tablet was evaluated by utilizing the USP dissolution equipment II with 900 ml of 0.1 PBS at 37±0.5 degrees Celsius and a stirring rate of 100 revolutions per minute. The absorbance at a wavelength of 221 nm was measured with the assistance of a UV spectrophotometer after various samples totaling 5 ml were removed and replaced with simulated fluid of the same amount at 1, 2, 4, and 8 hours, respectively. The samples were then filtered through Whatman filter paper before the absorbance was measured.

3.2 Macroscopical Evaluation

The macroscopic evaluation was carried out to assess the color, odor, taste, shape, and texture of the individual drugs, and the polyherbal formulation was observed and recorded.

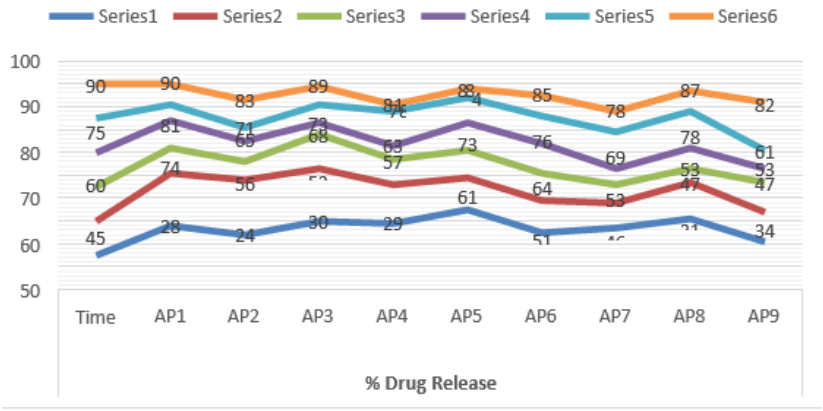

Fig. 1. Drug release profile

Table 4. Characterization of polyhedral dispersible tablets

Formulation Code	Average weight (mg)	Weight variation (%)	Content uniformity (%)	Hardness (kg/cm)	Friability (%)	Disintegration time (Min)	Dispersing time (Min)
AP1	560.80±10.2	1.93	099.85	2.94±0.13	0.90	02.18±0.51	2.50±0.6
AP2	565.31±8.11	2.71	103.10	3.00±0.12	0.86	02.00±0.45	3.00±0.7
AP3	563.28±7.78	2.36	105.05	2.99±0.14	0.79	01.10±0.10	2.00±0.4
AP4	562.40±8.01	2.30	101.25	2.91±0.09	0.82	01.45±0.28	2.30±0.6
AP5	563.15±9.38	2.34	102.38	2.98±0.13	0.90	02.08±0.62	3.18±0.8
AP6	564.13±8.78	2.50	099.68	2.97±0.11	0.88	01.50±0.58	3.00±0.8
AP7	563.18±8.07	2.34	102.00	3.02±0.18	0.78	02.06±0.70	2.55±0.7
AP8	558.34±7.68	1.22	098.96	2.96±0.16	0.85	02.15±0.55	3.25±0.8
AP9	563.63±7.40	2.42	101.80	2.95±0.12	0.80	01.55±0.60	2.24±0.5

Table 5. Dissolution profile of a polyherbal tablet

TIME (MIN)		AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	AP9
15		28	24	30	29	35	25	27	31	21
30	Percentage	51	48	53	46	49	39	38	47	34
45	Released (%)	62	56	68	57	61	51	46	53	47
60		74	65	73	63	73	64	53	62	53
75		81	71	81	78	84	76	69	78	61
90		90	83	89	81	88	85	78	87	82

Table 6. Stability studies of AP1 formulation

% Drug content at different storage conditions			
Time duration	**25 °C and 60 % RH**	**30 °C and 65 % RH**	**40 °C and 75 % RH**
30 Days	98.3	98.5	99.3
60 Days	99.5	99.3	98.3
90 Days	99.2	99.1	97.2

Table 7. Preliminary phytochemical screening

Test of Constituents	CS Ext.	TUR. Ext.	GS. Ext.	OR. Ext.	SO. Ext.
Flavonoids	√	#	√	#	√
Alkaloids	√	#	#	#	#
Carbohydrate	√	√	#	√	#
Gum/Mucilate	#	#	√	√	√
Protein/ Amino Acid	#	√	#	√	#
Fats	√	#	#	#	#
Steroids	√	√	√	#	√
Glycosides	√	#	√	#	#
Phenol	√	#	√	√	√
Saponin	√	√	#	#	#
Volatile Oil	#	√	√	√	√

√: Present, #: Very little or Absent

3.3 Physicochemical Analysis

"Physicochemical analysis of individual ingredients and PHF was studied and represented with standard deviation. In physicochemical evaluation, such as total ash, water-soluble ash, acid insoluble ash, water-soluble extractive value, ethanol-soluble extractive value, loss on drying, and pH were evaluated. The ash values demonstrate the presence of inorganic salts present in the drug. The extractive values (water and ethanol soluble extractive value) were resolved. The data gathered from this evaluation was helpful for standardization and obtaining the quality standards for a crude drug as well as for PHF formulations" (Kapoor and Singla, 2015). Determination of these physiochemical constants was according to systems referred to as per WHO guidelines.

3.4 Preliminary Phytochemical Screening

"Preliminary phytochemical screening of the individual drugs and polyherbal formulation confirmed the presence of phytoconstituents such as flavonoids, alkaloids, carbohydrates, gums and mucilage, fats and fixed oils, steroids, glycosides, phenols, saponins but no volatile oils" (Nagar et al., 2011).

4. CONCLUSION

The results from the angle of repose, Carr's index and Hauser's ratio showed that the powder mixtures possess good flow properties. AP1 to AP9 were determined for the uniformity in weight, hardness, drug content and friability, which have complied with the official requirements and the official limits mentioned in IP 2010. The AP3 showed suitable disintegration properties and in vitro dispersion time compared to other formulations. The polyherbal extract and the excipients utilized in the dispersible tablet do not appear to have any chemical interactions,

according to the FTIR spectroscopy. The drug's stable peaks stayed the same in the mixtures, displaying distinctive functional groups with a variety of therapeutic qualities, such as the alkynes group, aliphatic amines, alkyl halides, an aromatic group, alkanes, alcohols, and the ester. After being stored for three months, the AP 3 was found to be repeatable and retained for stability investigations.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Knowledge and Practices of Dietary Supplement Use in Type 2 Diabetes Management: A Study in Two Hospitals in Guyana

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This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The increasing prevalence of type 2 diabetes (T2D) globally necessitates exploration of complementary therapies, including dietary supplements, alongside standard medical care. However, the efficacy and safety of many supplements remain unclear, emphasising the need for robust research.

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Objective: To assess the knowledge and practices regarding the use of dietary supplements among T2D patients in two Guyanese hospitals: Georgetown Public Hospital Corporation (GPHC) and Suddie Hospital (SH).
Methods: A cross-sectional, quantitative survey using a structured interview questionnaire was administered to a stratified random sample of 115 patients with type 2 diabetes (T2D) (46 from GPHC and 69 from SH). The data were analysed using SPSS version 20.0.
Results: Over half (67%) of participants used dietary supplements, with multivitamins being the most common (63.5%). A significant association was observed between hospital type and dietary supplement prescribing ($\chi^2(1) = 8.22$, $p = .004$), as well as knowledge of dietary supplements ($\chi^2(2) = 11.98$, $p = .003$), indicating significant differences between GPHC and SH. A substantial knowledge gap existed regarding the safety and efficacy of supplements, with a high proportion of participants incorrectly believing that over-the-counter supplements are always safe.
Conclusion: Widespread use of dietary supplements among T2D patients highlights the need for standardised prescribing guidelines and improved patient education to ensure safe and effective use. Further research should explore the reasons behind the observed disparities in prescribing and knowledge levels between hospitals.

Keywords: Dietary supplements; type 2 diabetes; patient knowledge; healthcare practices; Guyana.

1. INTRODUCTION

Managing Type 2 diabetes is a lifelong struggle. Most people must follow a proper diet, make lifestyle changes, and take medications. However, in addition to these conventional treatments, many people also use natural remedies such as bitter melon, ginseng, and aloe vera to help control their blood sugar (Mospan, 2018). Some people believe that dietary supplements can help manage diabetes, which may be why many individuals are starting to use them.

A 2021 report prepared by the Pan American Health Organization (PAHO) for Latin America and the Caribbean described diabetes as a significant public health problem in that region and worldwide (PAHO, 2025). "The citizens of Guyana are severely impacted by Type 2 diabetes, partly as a consequence of national ethnic diversity with Indo-Guyanese (43%), Afro-Guyanese (30%), and Amerindian (9%) – all risk groups" (Guyana Diabetes Care Project, WDF14-862). In 2013, it was estimated that 14% of the then-adult population (20–79 years) was afflicted with diabetes (Guyana Diabetes Care Project, WDF14-862). Dietary supplements are not intended to treat, diagnose, prevent, or cure diseases (Office of the Commissioner, 2022); they are becoming widespread for diverse non-conventional treatments, such as general health boosts, symptom reductions, and specific disease prevention (Mahdavi-Roshan et al., 2021).

A dietary supplement is defined as a product that contains a vitamin, a mineral, a herb or other plant product, an amino acid, or a dietary substance that supplements the diet by increasing total intake (Wang et al., 2019; Zimmet et al., 2005). There is limited literature in this area of study. While few studies have been conducted on the use of dietary supplements, herbs, and traditional medicine for Type 2 diabetes, for example, Jagessar & Kingston, (2015) and Boston et al., (2019), there is no study of this kind conducted in Guyana to the researchers' knowledge.

Use of Dietary Supplements to Manage Type 2 Diabetes: In the U.S., about 22% to 67% of people with diabetes take some supplement. People with diabetes are more likely to use supplements than those without, and many prefer natural remedies over meds from the doctor (Mospan, 2018). Additionally, it seems that those diagnosed with Type 2 Diabetes tend to use more supplements than those with Type 1. Mospan, (2018) even pointed out that some individuals who undertook certain supplements had lower levels of HbA1c, which reflected better control of blood glucose levels.

Given the above, it is essential to remember that supplements should not be viewed as treatment drugs (Center for Food Safety and Applied, 2022). Unlike medications, which must undergo thorough scrutiny and are validated before being dispensed, supplements are categorised as food and thus do not have to meet the same level of safety and effectiveness requirements. Thus, according to Mospan, (2018), it is prudent that patients be warned about the possible risks and any negative incidents they may face.

Besides multivitamins, some natural compounds are being studied for diabetes, such as resveratrol. Huang et al., (2020) stated that Type 2 diabetes management lowers blood sugar levels, improves insulin utilisation, and protects pancreatic cells that produce insulin. Their research suggests Resveratrol may help with all of this.

Resveratrol is produced by plants in response to stress, and it's found in fruits, vegetables, grains, and beverages such as tea, coffee, and wine (Huang et al., 2020). Some trials tested Resveratrol on diabetics. In clinical trials, oral administration of a 5 mg-5 g single-daily dose of RES for 12 months reduced blood glucose and improved insulin sensitivity in diabetic patients. Huang et al., (2020) noted that clinical trial results suggested that activating specific protein pathways, such as AMP-dependent protein kinase (AMPK) and sirtuin (SIRT), can restore abnormal levels of insulin, insulin-like growth factors (IGFs), and blood glucose.

Frequently Prescribed Supplements for Type 2 Diabetics: The standard treatment for Type 2 diabetes is evidence-based medicines, such as metformin, which health care providers prescribe for such patients (Centers for Disease Control and Prevention, 2016). Studies by Suksomboon et al., (2016) and Gothai et al., (2016) suggested alternative treatments, such as dietary supplements, that effectively manage type 2 diabetes and its complications.

Vitamins are important because they act as antioxidants and may help people with chronic illnesses. Balbi et al., (2018) stated that oxidative stress plays a significant role in causing diseases like diabetes. It lowers the body's antioxidant defences and increases damage. Ceriello et al., (2016) and Kositsawat & Freeman, (2011) also noted that low levels of antioxidants may lead to increased diabetes complications. The most studied vitamins were B, C, D, and E. Vitamins C, D, and E were found to have notable antioxidant activity by blocking free radicals.

Another mineral, chromium, is an essential nutrient involved in normal carbohydrate and lipid metabolism. The chromium requirement increases with the progression of glucose intolerance and diabetes (Anderson et al., 1997). In a double-blind, placebo-controlled study by Anderson et al., (1997), 180 individuals with Type 2 diabetes were divided into three groups and received two different dosages of chromium or a placebo. The findings showed that supplementary chromium at various dosages statistically and clinically meaningfully influences insulin and glucose variables in people with Type 2 diabetes. One such variable is the HbA1c, which was lowered by chromium supplementation. It is worth noting that chromium picolinate is a suitable form of chromium, and its use is more efficient than some other forms of chromium (Anderson et al., 1999). Additionally, Yang et al., (2023) suggest that zinc supplementation may be a viable adjunct therapy for improving glycemic control and reducing insulin resistance in overweight and obese individuals, particularly those with higher BMIs or pre-existing diabetes. However, further large-scale, randomised controlled trials (RCTs) are needed to confirm these findings and determine the optimal zinc dosage, taking into account the potential for adverse effects associated with excessive zinc intake.

Prescribing Dietary Supplements: Doctors typically follow treatment guidelines when prescribing medication for diabetes; however, how they prescribe can vary. The American Diabetes Association (ADA, 2019) has established guidelines, known as Standards of Medical Care in Diabetes, that outline best practices for caring for individuals with diabetes. They also recommend medical nutrition therapy (MNT), which involves managing diabetes through a combination of diet and medication.

The ADA states that there is no substantial evidence to support the use of vitamins, minerals such as chromium and vitamin D, or herbs and spices like cinnamon and aloe vera in helping to control blood sugar levels in individuals without a deficiency. So, supplements are not usually recommended (ADA, 2019). However, it is still important for people with diabetes to get the nutrients they need, and there are concerns about whether it is safe to take supplements long-term.

Nonetheless, some other studies claim that nutrients such as chromium, magnesium, and vanadium could assist with diabetes (Wang et al., 2019). Wang et al., (2019) noted that several compounds of chromium, including chromium chloride, chromic picolinate, and those ligated with biotin, can reduce HbA1c and fasting glucose levels. Additionally, biotin and chromium, when combined, facilitate glucose absorption by the body more effectively than other forms. Plus, magnesium supplements may improve blood sugar, blood pressure, and insulin sensitivity,

according to Guerrero-Romero et al., (2004), as cited by Wang et al., (2019).

Knowledge of the Purpose of Dietary Supplement Use by People with Type 2 Diabetes: Generally, supplements are prescribed to patients to improve or maintain their health, rectify a nutritional shortfall, or treat a specific health issue. Many people believe that dietary supplements are safer and potentially more effective than medications in treating diabetes. Despite insufficient evidence suggesting that supplements heal or contribute to overall wellness globally, sales have exceeded $ 100 billion (Hannon et al., 2020). They found that most supplements for diabetes had insufficient evidence to support their use. Diet and lifestyle changes, as well as pharmaceutical therapies, are the mainstays of diabetes management. However, consumers should be assured that producers must verify that their products are safe and satisfy specific quality criteria.

The United States recommends that supplement manufacturers refrain from claiming that their products prevent or treat any ailment. According to the Centre for Food Safety and Applied Nutrition, if there is substantiation of scientific data for a supplement delivering a possible health impact, companies are authorised to market their supplements with notices such as "Structure/Function'‘ and " helps maintain healthy joints,". However, the label must state that the Food and Drug Administration "has not reviewed the claim" and that the dietary supplement product is not meant to "diagnose, treat, cure, or prevent any illness," because only a medicine may lawfully make such a claim (Centre for Food Safety and Applied Nutrition).

There has been an increase in the use and sales of dietary supplements. In Guyana, dietary supplements are readily available and accessible nationwide at local markets, herbal stores, and pharmacies. This does not necessarily indicate that the knowledge and benefits of these dietary supplements are increasing or even known by Type 2 diabetics.

Against this backdrop, the researchers were interested in the knowledge and practice of supplement use in Guyana. This study aimed to determine the percentage of Type 2 diabetic patients from the diabetic clinics at Georgetown Public Hospital Corporation (GPHC) and Suddie Hospital (SH) who used dietary supplements and their awareness of using them to manage their condition.

2. METHODS

This study employed a cross-sectional quantitative survey design to investigate knowledge and practices regarding dietary supplement use among Type 2 diabetes mellitus (T2DM) patients. The study was conducted at two hospitals in Guyana: the Georgetown Public Hospital Corporation (GPHC) and Suddie Hospital (SH), representing Regions 4 and 2, respectively, and potentially differing patient demographics.

Variables:

Independent variables-

- Age, gender, marital status, employment status, ethnicity, educational background, and Dependent variables
- Dietary supplements, source of information

Population: The sample for this study was drawn from the Guyana Public Hospital Corporation (GPHC) and Suddie Hospital (SH). The sample at GPH consisted of 520 participants, while the sample at SH comprised approximately 500 participants, totalling 1,020 participants.

Population and Sampling Frame: The study population consisted of T2DM patients attending diabetic clinics at GPHC (n = 520) and SH (n = 500), totalling 1,020 patients. A stratified random sampling technique was initially planned, with strata defined by the two clinics. The proportional allocation of the sample size was based on the relative sizes of the clinic populations, with 143 participants from GPHC and 137 from SH, as calculated using an online tool (SurveyMonkey) with a 95% confidence level and a 5% margin of error.

Sample Size Adjustments and Limitations: Due to logistical constraints during the four-week data collection period, the study deviated from the planned stratified random sampling approach. Instead, a convenience sampling method was employed, resulting in a final analysed sample of 115 participants (46 from GPHC and 69 from SH). Nine questionnaires from GPHC were excluded due to significant missing data (more than 66%).

The study achieved a sample of 115. As countermeasures to the challenges presented by

this sample and considering the post-hoc power analysis, non-parametric Pearson Chi-squared tests were conducted to assess the significance of the association. A post-hoc power analysis in SPSS measured the study's statistical power relative to the remaining sample size of 115. With a sample size of 115, an assumed effect size of Cohen's d = 0.5, an alpha level of 0.05, and a two-tailed test, the power calculated for this study was 0.72 (72%). Therefore, the study had a 72% chance of detecting a significant effect if one exists. This means the study had moderate statistical power.

Data Collection Instrument: A structured interview questionnaire served as the data collection instrument (see Appendix). The questionnaire included items to assess:

- **Independent variables-** Age, gender, marital status, employment status, ethnicity, educational background, duration of diabetes diagnosis, diabetes treatment regimen (including insulin use and type, oral medications, diet plan adherence).
- **Dependent variables-** Dietary supplement use (type, frequency, duration), source of information regarding supplement use, self-reported knowledge about supplement safety and efficacy.

Inclusion/exclusion criteria: Inclusion criteria-

- All subjects were 18 years and older
- All subjects were patients of the GPHC and SH Diabetic clinics.
- All subjects were Type 2 diabetics

Exclusion criteria-

- Type 2 diabetics who were newly diagnosed
- Type 2 diabetics who were on treatment for a nutritional condition or disorder
- Pregnant women with type 2 diabetes

3. RESULTS

In this study, 115 participants were included, comprising 69 from Suddie Hospital (SH) and 46 from Georgetown Public Hospital Corporation (GPHC). The majority of participants from both hospitals were female, constituting around 66% of each cohort. Examining the participants' ethnicity, most were of East Indian origin, accounting for approximately 85.5 percent of the sample from that location. In comparison, GPHC had a more diverse ethnicity. About one-third (32.6%) identified as Afro-Guyanese, 28.3% were of East Indian descent, 15.2% were Amerindian, and 23.9% reported mixed heritage. When examining age, most patients at SH were older, with nearly half (43.5%) being 65 years or older. On the other hand, at GPHC, the largest group was between 45 and 54 years old, comprising 39.1% of the population. Not many participants from either hospital fell within the 25-34 age range, making it the smallest age group.

Education levels were also different across the two hospitals. At Suddie, most people had only attended primary or secondary school, and a few had not completed primary school. Approximately 87% of that group did not pursue education beyond the secondary level. Meanwhile, at GPHC, most participants had at least a secondary education, and some even had tertiary-level qualifications.

Marital status showed some interesting differences. Most people at SH were married (59.4%), while a significant number were widows or widowers (26.1%). At GPHC, the most prominent groups were married (37%) and single (30.4%). The two groups varied significantly in terms of their employment status. At Suddie Hospital, a substantial 81.2% of participants were unemployed, which corresponded with the older age demographics of the majority of participants. In comparison, participants at GPHC had a much more balanced distribution, with 41.3% employed and 45.7% unemployed. These demographic characteristics are described in more detail in Table 1.

Many participants from Georgetown Public Hospital Corporation (GPHC) and Suddie Hospital (SH) reported a family history of diabetes. 71.7% of those from GPHC and 82.6% from SH said that relatives, such as parents or grandparents, also had the disease. These relatives included parents, siblings, and grandparents, showing that diabetes is often a family issue passed down through generations.

The number of affected relatives varied among those with a family history. At GPHC, most people (60.6%) said they had just one family member with diabetes, while about 30.3% had two relatives, and a small group (6.5%) had three or more family members living with the disease. Over at SH, 42.9% had one diabetic relative, 25% had two, and a more significant portion (32.1%) had three or more.

All the participants in this study were diagnosed with Type 2 diabetes, and the amount of time they had been living with it ranged widely — some had been recently diagnosed, while others had been managing it for more than 20 years. Most of them had received advice or education from health workers on how to care for themselves, though this was more common at SH (84.1%) than at GPHC (65.2%).

Table 1. Demographics characteristics of the studied population

Demographic Factors	SH (n = 69)		GPHC (n = 46)	
	Frequency	%	Frequency	%
Age groups (years)				
25 – 34	1	1.4	1	2.2
35 – 44	*5*	*7.2*	*11*	*23.9*
45 – 54	13	18.8	18	39.1
55 – 64	20	29.0	8	17.4
65 or more	30	43.5	8	17.4
Gender				
Female	46	66.7	31	67.4
Male	23	33.3	14	30.4
Prefer not to answer	0	0.0	1	2.2
Ethnicity				
Amerindian	1	1.4	7	15.2
East Indian	59	85.5	13	28.3
African	2	2.9	15	32.6
Mixed/Others	7	10.1	11	23.9
Education				
No formal schooling	4	5.8	0	0.00
Primary	30	43.5	4	8.7
Secondary	30	43.5	30	65.2
Tertiary	5	7.2	12	26.1
Marital Status				
Single	8	11.6	14	30.4
Married	41	59.4	17	37.0
Divorced	2	2.9	9	19.6
Widowed	18	26.1	3	6.5
Prefer not to answer	0	0.00	3	6.5
Employment Status				
Employed	13	18.8	19	41.3
Unemployed	56	81.2	21	45.7
Prefer not to answer	0	0.00	6	13.0

Table 2. Time diagnosed as diabetic and treatment

Factors	SH (n = 69)		GPHC (n = 46)	
	Frequency	%	Frequency	%
Time diagnosed (years)				
1 -3	15	21.7	12	26.1
4 – 7	10	14.5	21	45.7
8 – 12	13	18.8	9	19.6
13 – 20	11	15.9	2	4.3
> 20	20	29.0	2	4.3
Ever instructed on diabetes care				
Yes	58	84.1	30	65.2
No	11	15.9	14	30.4
Prefer not to answer	0	0.0	2	4.3
Treatment				
Diet (only)	2	2.9	1	2.2
Insulin	5	7.2	3	6.5

Factors	SH (n = 69)		GPHC (n = 46)	
	Frequency	%	Frequency	%
Oral Medications	41	59.4	9	19.6
Insulin & Oral medications	1	1.4	1	2.2
Diet & oral meds	17	26.6	30	65.2
Diet/ insulin/ oral medications	1	1.4	1	2.2
Insulin and diet	3	4.3	2	4.3

Everyone was using some form of treatment, whether it was insulin, tablets, dietary changes, or a combination of these. The most common medications were metformin, gliclazide, and glyburide. However, some people using insulin did not know the name of their medication, and about 70% were not sure of the dosage they were taking. More details about how long they had diabetes and what treatments they were using can be found in Table 2.

The study showed that many people with Type 2 diabetes are using dietary supplements. At Georgetown Public Hospital Corporation (GPHC), approximately 76.1% of participants reported taking supplements; at Suddie Hospital (SH), 60.9% reported the same. These results align with those of other researchers, such as Mospan (2018) and Mahdavi-Roshan et al. (2021), who have found that more people with chronic illnesses, including diabetes, are turning to dietary supplements to help manage their symptoms and potentially avoid complications.

Regarding the types of supplements used, more than half of the participants from Suddie Hospital (58%) reported using herbal remedies. In comparison, just over half of those from GPHC (56.5%) reported taking vitamins. Interestingly, many people from Suddie make their herbal remedies using plants, highlighting how deeply rooted traditional medicine is in Guyanese culture. Boston et al. (2018) also noted that herbal medicine is widely used throughout the country.

However, many people are not receiving professional advice on which supplements to take. Only a few participants — 5.8% at SH and 6.5% at GPHC — reported that a pharmacist had provided them with advice. Instead, most of them figure it out independently or consult local herbalists; 58% of those from SH reported relying on their knowledge or what an herbalist tells them. This can be risky because, as Halat & Dennehy, (2003) pointed out, people may not know the correct dosage or how to mix substances safely.

There were noticeable differences in how patients from the two hospitals managed their diets. At GPHC, over half (59%) of participants reported sticking to a diet plan to help control their diabetes. However, at Suddie Hospital, most people (78%) admitted they did not follow any specific plan. Instead, they avoid consuming excessive amounts of sweet foods and simple carbohydrates. Even though that might help a little, following a proper diet is not the same. The American Diabetes Association's Standards of Medical Care emphasize that following a structured diet is an essential part of managing diabetes, taking medication, and making lifestyle changes.

Prescribing of Dietary Supplements by a Healthcare Provider/ Doctor: The Table 3 shows that 67.8% of participants from both hospitals reported that their doctors prescribed dietary supplements, suggesting that supplement prescribing is a common practice. However, upon closer examination of GPHC, there is only a slight difference — about 4.3% — between those who received a supplement prescription and those who did not. This means that nearly half of the patients at GPHC were not prescribed supplements.

Breaking it down, about half (50%) of GPHC participants said their doctors prescribed supplements, compared to 79.7% at Suddie Hospital. At SH, three people were specifically given B complex for neuropathy, but interestingly, 20 participants admitted they had no idea why they were taking the supplements. This contradicts the American Diabetes Association's Standards of Medical Care (ADASMC), which recommend that patients be properly informed about any treatment they receive. Table 4 shows that those who did not get prescriptions often turned to other sources for supplements.

The Table 4 shows that 51.4% (11 – SH; 7 – GPHC) of subjects who obtained supplements from sources other than healthcare providers bought them from pharmacies. It is worth noting that 31.4% of the total samples did not respond to this item.

Table 3. Prescribing of dietary supplements by healthcare provider/doctor

	GPHC n=46		SH n=69		GRAND TOTAL n=115	
Healthcare providers/doctors prescribe dietary supplements	**No.**	**%**	**No.**	**%**	**No.**	**%**
Yes	23	50	55	79.7	78	67.8
No	21	45.7	14	20.3	35	30.4
No response	2	4.3	0	0	2	1.7

Table 4. Sources of dietary supplements

	SH (n= 14)	GPHC (n=21)
Source of dietary supplement	**No.**	**No.**
Purchase from pharmacy	7	11
Purchase online	0	2
Other	2	2
No response	5	6

Dietary Supplement Usage Frequency: The chart shows that multivitamins were the most used supplement, with 63.5% of participants (34 from SH and 39 from GPHC) using them regularly. Of these, 36.2% at SH and 41.3% at GPHC said they "always" use them. Vitamin C was the second most prevalent (39.1%), followed by bitter melon (28.7%), which is traditionally used for diabetes, as noted by Mospan (2018). Most SH participants (95%) reported using additional supplements, such as B complex, One-A-Day Women, and turmeric, with B complex being the most commonly used. In contrast, only 8.7% of GPHC participants mentioned extra supplements.

Overall, 90% of participants had no side effects. Out of all participants, only 10% reported side effects. At Suddie Hospital, some people got rashes from herbal remedies, while a few at GPHC said they felt sick to their stomach or vomited. Interestingly, the people at Suddie who had rashes never reported them to their doctors, despite Mospan (2018) recommending that they inform healthcare providers about these issues.

Additionally, 7% of all participants reported never using supplements, primarily due to financial constraints, uncertainty about their safety, or a lack of perceived need.

Knowledge of Type 2 Diabetic Patients about their Supplements: The Table 5 reveals that subjects at both hospitals accessed information about dietary supplements from a total of nine different sources. The top three sources were family (9.6%, SH: 9; GPHC: 2), physicians (7%, SH: 4; GPHC: 4), and fellow patients (7%, SH: 7; GPHC: 1). At SH, 34.8% (24 subjects) relied on multiple sources for supplement information, while 10.1% (7 subjects) did not seek information. For GPHC, 67.4% (31 subjects) indicated multiple sources, while 10.9% did not respond.

Table 5. Sources of Information on Dietary Supplements

	GPHC N= 46		SH N=69	
Information sources	**No.**	**%**	**No.**	**%**
Internet	1	2.2	4	5.8
Family	2	4.3	9	13.0
Print media	-	-	3	4.3
Pharmacist	1	2.2	2	2.9
Fellow patient	1	2.2	7	10.1
Physician	42.	8.7	4	5.8
Television	1	2.2	1	1.4
Other	-	-	6	8.7
Friend	-	-	2	2.9
Total	10 (21.7%)		38 (55.1%)	

Table 6. Knowledge Type 2 Diabetics at GPHC have of their supplements. (n=49).

Statements	Agree		Disagree		Not sure	
	No.	%	No.	%	No.	%
FDA requires that dietary supplements be proven to be safe and effective before they are marketed.	39	84.8	1	2.2	5	10.9
Regular use of dietary supplements prevents Type 2 diabetes	13	28.3	11	24.0	21	45.7
Dietary supplements are sold over the counter, so they are entirely safe to take	29	63.0	6	13.0	10	21.7
The number of supplements you get from food is enough for your health needs	28	60.9	3	6.5	14	30.4

Table 7. Knowledge Type 2 diabetics at SH have of their supplements. (n=69)

Statements	Agree		Disagree		Not sure	
	No.	%	No.	%	No.	%
FDA requires that dietary supplements be proven to be safe and effective before they are marketed.	55	79.7	4	5.8	10	14.5
Regular use of dietary supplements prevents Type 2 diabetes	25	36.2	32	46.4	12	17.4
Dietary supplements are sold over the counter, so they are entirely safe to take	35	50.7	22	31.9	12	17.4
The number of supplements you get from food is enough for your health needs	20	29.0	41	59.4	8	11.6

Overall, the percentages of subjects seeking information from any source were nearly equal: 89.9% from SH and 89.1% from GPHC, indicating a strong interest in understanding dietary supplements. This finding aligns with Mospan (2018) in the literature review. Additionally, data was collected on patients' knowledge of dietary supplements for Type 2 diabetes, with responses categorised as 'agree,' 'disagree,' or 'not sure.' The results for these items were grouped and summarised with corresponding percentages in Tables 6 and 7.

The results show that most people from both hospitals answered item 20 correctly — 84% at GPHC and approximately 80% at Suddie — which aligns with Mospan's (2018) findings. However, when it came to other questions, like items 21, 22, and 23, there were apparent gaps in what patients knew about dietary supplements. For example, in item 21, fewer than half of the total group disagreed with a false statement, indicating that many people were unsure.

Even more worrying, on item 22, many participants — 63% at GPHC and just over half at Suddie — agreed with a statement that was not true. This suggests that many people believe over-the-counter supplements are always safe, which is not the case and is something Mospan (2018) has warned about. Although pharmacists and doctors should be educating patients about supplements, this does not always happen in Guyana. As a result, patients may be using supplements without fully understanding the risks. Item 23 highlighted this problem too. More people at Suddie — around 59% recognised a false statement — but at GPHC, only about 6.5% got it right, which is a significant difference.

It is surprising that, despite more people at GPHC having higher education — 26.1% attended university compared to just 7.2% at Suddie — this did not seem to help them understand supplements better. Overall, most people did not seem to have enough information to make good choices about using supplements for their diabetes. Many believed that store-bought supplements are entirely safe, which contradicts the findings of Mospan (2018) and Cefalu et al., (2011). Additionally, while many GPHC patients believed supplements were sufficient to meet their needs, most people from Suddie disagreed.

Statistical Results: Three chi-square tests examined associations between hospital type (GPHC vs. Suddie Hospital) and (a) dietary supplement use, (b) dietary supplement prescribing, and (c) knowledge of dietary

Table 8. Chi-Square test of dietary supplement use by hospital

Hospital	Observed Frequency (Yes)	Observed Frequency (No)	Expected Frequency (Yes)	Expected Frequency (No)
GPHC	35	11	30.8	15.2
Suddie Hospital	42	27	46.2	22.8
Total	**77**	**38**		
χ^2 (1) = 2.24, p = .134				

Table 9. Chi-Square test of dietary supplement prescribing by hospital

Hospital	Observed Frequency (Yes)	Observed Frequency (No)	Expected Frequency (Yes)	Expected Frequency (No)
GPHC	23	21	30.37	13.63
Suddie Hospital	55	14	47.63	21.37
Total	**78**	**35**		
χ^2 (1) = 8.22, p = .004				

Table 10. Chi-Square test of knowledge of dietary supplements by hospital

Hospital	Observed Frequency (Agree)	Observed Frequency (Disagree)	Observed Frequency (Not Sure)	Expected Frequency (Agree)	Expected Frequency (Disagree)	Expected Frequency (Not Sure)
GPHC	13	11	21	15.00	16.97	13.03
Suddie Hospital	25	32	12	23.00	26.03	19.97
Total	**38**	**43**	**33**			
χ^2 (2) = 11.98, p = .003						

supplements. Regarding dietary supplement use, no significant association was found between hospital type and the likelihood of use, $\chi^2(1) = 2.24$, $p = .134$. However, a significant association emerged between hospital type and dietary supplement prescribing, $\chi^2(1) = 8.22$, $p = .004$, indicating that hospitals differed significantly in their prescribing practices. Finally, a significant association was also observed between hospital type and knowledge of dietary supplements, $\chi^2(2) = 11.98$, $p = .003$. This suggests that patients at the two hospitals differ significantly in their knowledge of dietary supplements.

Further investigation might explore the nature of these associations, mainly why differences exist in prescribing practices and knowledge levels between the two hospitals.

4. DISCUSSION

This study investigated the prevalence and patterns of dietary supplement use among patients with type 2 diabetes at two healthcare facilities in Guyana: Suddie Hospital (SH) and the Georgetown Public Hospital Corporation (GPHC). The findings show that supplementary use and knowledge at the two facilities are impacted by significant differences in the demographic characteristics of their patient populations.

Demographic Disparities and Supplement Use: There were apparent differences in the demographic compositions of the SH and GPHC patient populations. SH patients were older, with 43.5% being 65 years or older. They were predominantly East Indian (85.5%), less educated (87% had only a primary and/or secondary education), more likely to be unemployed (81.2%), and had a higher proportion of widows (26.1%). In contrast, GPHC patients exhibited greater ethnic diversity, attained higher levels of secondary and tertiary education, and had more balanced employment figures. These demographic differences were likely to affect the patterns of supplement usage due to varying information, financial resources, and cultural healthcare frameworks.

The high prevalence of herbal supplement use at SH (58%), which is often prepared from raw herbal ingredients, highlights the traditional medical practices in Guyana (Boston et al., 2018). Yedjou et al., (2023) substantiate the role of patient education in diabetes management

while also stressing the importance of medicinal plants and vitamins in enhancing glycemic control. They discussed the diabetes prevention effects of certain supplements, including Allium sativum (garlic), Momordica charantia (bitter melon), and vitamins C, D, and E, which corroborate the original study's findings on the use of dietary supplements. For example, 76.1% of participants from GPHC and 60.9% of participants from SH reported using dietary supplements, particularly vitamins and herbal supplements.

Shared Risk Factors and Treatment Similarities: Despite demographic differences, a high percentage of patients from both SH (82.6%) and GPHC (71.7%) reported a family history of diabetes, indicating a shared genetic predisposition. Both facilities' patients received diabetes education from healthcare professionals, although at differing rates (84.1% in SH vs. 65.2% in GPHC), and utilised similar treatments, including insulin, oral medications (such as metformin, gliclazide, and glyburide), and dietary management. Notably, a concerning 70% of insulin users across both facilities were unsure of their dosage, highlighting a significant gap in patient education and reinforcing the need for improved patient-provider communication regarding medication management (Mahdavi-Roshan et al., 2021).

Dietary Supplement Use and Knowledge Gaps: The study revealed a high prevalence of dietary supplement use in both groups (60.9% in SH, 76.1% in GPHC), consistent with the observations of Mospan (2018) and Mahdavi-Roshan et al. (2021) regarding the increasing trend of supplement use for disease management. Additionally, there is concern over the sources and methods of supplement usage. A significant proportion of SH patients relied on self-knowledge and local herbalist recommendations (58%), raising concerns about the appropriateness of dosing and preparation (Halat & Dennehy, 2003).

Only a handful of participants were provided professional counsel on the use of supplements, specifically 5.8% at SH and 6.5% at GPHC. The absence of guidance is problematic due to the misuse and side effects that may arise from it. Blahova et al. (2021) support this concern by remarking that natural therapeutic products such as polyphenols and flavonoids, which stand to offer significant advantages, require more in-depth analysis for their effectiveness and safety. They call for pre-determined and professionally controlled dosing to ensure risk mitigation.

While a substantial number of patients at both sites (67.8% overall) reported receiving dietary supplement prescriptions from healthcare providers, this practice was significantly less common at GPHC (50%). This discrepancy, as highlighted by the chi-square test (χ^2 (1) = 8.22, p= .004), warrants further investigation into the prescribing practices at each facility. A significant finding was the difference in patient knowledge regarding dietary supplements, particularly in terms of safety and efficacy (χ^2 (2) = 11.98, p =.003). Although both groups demonstrated a high degree of awareness of FDA regulations, a sizable portion incorrectly perceived over-the-counter supplements as entirely safe (63% GPHC, 50.7% SH), aligning with Mospan's (2018) concerns about the misperception of supplement safety.

This highlights the importance of public health educational campaigns that aim to address gaps in understanding supplement policy and its associated risks (Mospan, 2018; Cefalu et al., 2011). The difference in knowledge levels among hospitals noted even after patient education at GPHC was presumably due to their higher level of education, suggesting that there is more to the patient’s understanding than Plain Education. That invites consideration of specific strategies appropriate to the unique population of patients attending each hospital. Based on these findings, it can be concluded that unique hospital policies and the level of patient education have a significant impact on the management of diabetes. Blahova et al. (2021) confirm this with their argument, which supports the coexistence of drugs and natural therapies aimed at improving treatment outcomes. They highlight the benefits of combining metformin with natural products, such as resveratrol and curcumin, to achieve better control of insulin and blood sugar levels.

Clinical Implications and Future Research: The result of this study is significant for the management of diabetes within the context of Guyana. The rampant use of dietary supplements, frequently obtained from non-medical sources, poses a possible danger from negative interactions with other prescribed therapies, warranting close supervision. The lack of sufficient knowledge regarding the safety and usefulness of the supplements for the patient underscores the accountability of such patients in the education that is aimed at reducing these risks. Further studies should aim to identify the

knowledge gaps regarding the prescription of supplements at the two facilities.

Patient and healthcare provider perceptions of prescribing practices, along with the cultural influences on supplement selection and use, could be better understood through in-depth qualitative studies. Understanding the effectiveness of different health education approaches in modifying the patient's knowledge and behaviour would also be very useful. Most importantly, understanding the specific types of supplements taken and their possible interactions with prescribed medicines needs to be studied systematically. There is also a need to conduct further research to identify significant knowledge gaps between the two hospitals and to make improvements in terms of generalizability.

5. CONCLUSION

This study reveals a notable variation in the patterns and levels of knowledge regarding dietary supplements between Suddie Hospital (SH) and the Georgetown Public Hospital Corporation (GPHC). Specifically, the World Health Organization has recognised a high prevalence of supplement use among Guyanese type 2 diabetics. Exacerbating the issue, several participants, particularly at SH, contravened professional guidelines by disregarding recommended guidelines for supplements. This highlights the potential for harm caused by reliance on non-professional health sources. Despite high educational levels, the significant difference in knowledge among patients from the two hospitals regarding the safety and efficacy of dietary supplements underscores the overwhelming need for change to address these knowledge gaps. These findings underscore the crucial need for enhanced patient education, standardised prescribing practices, and additional research to comprehensively understand the intricate interplay of cultural factors, healthcare access, and supplement use in managing type 2 diabetes within the Guyanese context. Future qualitative studies could provide valuable insights into patients and provide perspectives to inform more effective interventions.

6. RECOMMENDATIONS

1. **University of Guyana:** Update the Medicine and Pharmacy curricula to emphasise practical application and patient-centred care for the management of type 2 diabetes.
2. Ministry of Public Health: Develop policies to integrate dietary supplements into diabetes treatment and establish a research unit to investigate their efficacy. Disseminate findings through accessible media at health facilities.
3. **Hospitals/clinics:** Incorporate diabetes education sessions into clinic schedules, ensure patients understand their medication prescriptions, actively follow up on patients who are absent, and encourage (or arrange for) in-person medication refills for better monitoring and counselling.
4. **Pharmacies:** Enhance pharmacists' knowledge of dietary supplements and ensure facilities provide quality care for individuals with type 2 diabetes.
5. **Type 2 diabetics:** Actively learn about relevant and effective dietary supplements, understand potential interactions with medications, and consult healthcare professionals before selecting supplements.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX

Dietary Supplement Questionnaire To be completed by respondents.

Please select your desired answer by placing a tick (√) where necessary. Please note that the information gathered will be treated confidentially.

1. Diabetic Clinic

   ☐ GPHC ☐ Suddie Hospital

2. Gender

   ☐ Male ☐ Female ☐ Other

3. Marital Status

   ☐ Single ☐ Married ☐ Divorced ☐ Widowed

4. Employment status

   ☐ Employed ☐ Unemployed

5. Age (yrs.)

   ☐ 18-24 ☐ 25-34 ☐ 35-44 ☐ 45-54
   ☐ 55-64 ☐ 65+

6. Ethnicity

   ☐ Amerindian ☐ East Indian ☐ Asian ☐ African
   ☐ European ☐ Mixed

7. Educational background

   ☐ Did not attend school ☐ Primary
   ☐ Secondary ☐ Tertiary

8. Has anyone in your family been diagnosed with diabetes? ☐Yes ☐No

   If yes, please tick all that apply:

   ☐ Father ☐ Mother ☐ Brother
   ☐ Sister ☐ Grandparents ☐ uncle/aunt

9. How long have you been diagnosed with Diabetes?

   ☐ 1-3years ☐ 4-7 years ☐ 8-12 years
   ☐ 13-20 years ☐ 20+ years

10. Have you ever been instructed on diabetes care? ☐ Yes ☐ No

Where and by whom? _____

11. What is your current treatment for diabetes? (Check all that apply)

☐ Diet
☐ Insulin: name _____ Dosage _____units
☐ Oral Medications:
☐ Metformin ☐ Gliclazide MR ☐ Glyburide/ Glibenclamide
☐ Gliclazide ☐ Metformin & Gliclazide (combined)

Other (please state): _____

12. Do you take any dietary supplements to manage your blood sugar levels?
☐ Yes ☐ No

13. If yes, please select which one (s)

☐ Vitamins
☐ Herbal supplements
☐ Other_____

14. Are your instructions for use of supplements directed by?

☐ Prescription ☐ Pharmacist ☐ Label

Dietary Supplement	Always	Often	Sometimes	Rarely	Never
Multivitamin					
Vitamin B12					
Vitamin C					
Alpha Lipoic Acid (ALA)					
Biotin					
Zinc					
Magnesium					
Ginseng					
Capsiacin					
Bitter melon/ Carilla					
Resveratrol					
Cinnamon					
Vitamin D					
Vitamin E					
Chromium Picolinate					
Chromium					
Aloe Vera					
Other:					

14. Using the table above, please rate your use of the supplements listed within the last 30 days.

15. Did you experience any side effects from any of the dietary supplements?

☐ Yes ☐ No

If yes, please state supplement name and the side effect below

Supplement_____Side effect _____
Supplement_____Side effect _____
Supplement_____Side effect _____

16. If you have never used any dietary supplements for diabetes, please select all the reasons that apply. If you have used any of the supplements listed above, proceed to the next question.

- ☐ Never heard of it/ don't know much about it
- ☐ Never thought about it
- ☐ No reason
- ☐ Don't need it
- ☐ Don/t believe in it/ it doesn't work
- ☐ It costs too much
- ☐ It is not safe to use
- ☐ A health care provider told me not to use it
- ☐ Medical science has not shown that it works
- ☐ Other ___

17. Do you have a diet plan to help manage your condition? ☐ Yes ☐ No

18. Does your healthcare provider/ doctor prescribe dietary supplements for you?

☐ Yes ☐ No

If no, how do you access dietary supplements?

☐ Purchase from a pharmacy ☐ Purchase online

☐ Sent / provided by relatives

Other (please state): _____

19. Where do you get information about dietary supplements from? Please check all that apply.

☐ Internet (Websites, Facebook)		☐ Pharmacist
☐ Physician (Doctor)	☐ Family	☐ Fellow patient
☐ Television	☐ Physio/Massage Therapist	☐ Health Food Store
☐ Print Media (magazines, books, newspapers)	☐ Coach Dietitian	
☐ Nutritionist	☐ Product Labels	☐ Workshops/Classes
☐ Friend	☐ Other_____	

20. The Food and Drug Administration requires that dietary supplements be proven to be safe and effective before they are marketed.

☐ True ☐ False ☐ Don't know

21. Regular use of dietary supplements prevents Type 2 diabetes.

☐ Agree ☐ Disagree ☐ Not sure

22. Dietary supplements are sold over the counter, so they are entirely safe to take.

☐ Agree ☐ Disagree ☐ Not sure

23. The number of supplements you get from food is enough for your health needs.

☐ Agree ☐ Disagree ☐ Not sure

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Anti-anemic Activity of Aqueous Root Bark Extract of *Morinda lucida* Benth against Phenylhydrazine-induced Hemolytic Anemia in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author KKAM designed the study, was involved in the conduct of the study and drafted the manuscript. Author KKI contributed to the drafting of the manuscript and to the statistical analysis of the data. Author MACS performed the experiment and statistical analysis. Authors SD and NJD contributed to drafting the manuscript. Author DAJ coordinated the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Evaluate the anti-anemic potential of aqueous root bark extract of *Morinda lucida* against induced hemolytic anemia in Wistar rats in addition to its total phenol content and its acute toxicity.
Study Design: Experimental Design.
Place and Duration of Study: Laboratory of Biology and Health, Training and Research Unit Biosciences, Félix Houphouët-Boigny University of Abidjan, June 2024 to September 2024.
Methodology: Total phenol content of aqueous root bark extract of *Morinda lucida* was determined using Folin-Ciocalteu reagent. The acute toxicity test was performed according to OECD guideline no. 425. Anemia was induced in Wistar rats by intraperitoneal administration of phenylhydrazine at 40 mg/Kg bw for two days. The rats were orally treated during 21 days with 100 and 200 mg/kg bw of *Morinda lucida* extract and Ranferon® (anti-anemic drug) at 50 mg/kg bw. Blood was collected from all rats before and after induction of anemia, and then weekly during the treatment period to monitor changes in hematological parameters such as hemoglobin level, red blood cell count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration.
Results: The results show that the aqueous root bark extract of *Morinda lucida* contains phenolic compounds, estimated at 0.169 ± 0.015 mg gallic acid equivalent/g extract, and has low toxicity. In anti-anemic test, phenylhydrazine caused a significant reduction in hemoglobin level in rats, from 13.33 to 8.33 g/dL, with a disturbance in the other hematological parameters. After 15 days of treatment, hemoglobin levels significantly increased in rats treated with *M. lucida* extract at 100 mg/kg (13.27±0.26 g/dL) and Ranferon® (12.93±0.145 g/dL), comparable to normal controls (13.17±0.03 g/dL). There were also improvements in the other hematological parameters after two to three weeks treatment.
Conclusion: These results show that *Morinda lucida* is effective in repairing phenylhydrazine-induced erythrocyte damage and highlight its anti-anemic potential.

Keywords: Anemia; phenylhydrazine; Morinda lucida; anti-anemic activity.

1. INTRODUCTION

Anemia is a condition characterized by an abnormal drop in the number of red blood cells or the hemoglobin concentration within them. It is a serious global public health problem that particularly affects young children, menstruating adolescent girls and women, and pregnant and postpartum women (WHO, 2023). According to WHO (2023), 40% of children 6-59 months of age, 37% of pregnant women, and 30% of women 15-49 years of age worldwide are anemic. Several factors contribute to the development of anemia: nutrient deficiencies through inadequate diets or inadequate absorption of nutrients, infections (e.g. malaria, parasitic infections, tuberculosis, HIV), inflammation, chronic diseases, gynecological and obstetric conditions, and inherited red blood cell disorders. The most common nutritional cause of anemia is iron deficiency, although deficiencies in folate, vitamin B12 and vitamin A are also important causes (WHO, 2023).

There are many types of anemia such as iron-deficiency anemia, pernicious anemia, aplastic anemia, sickle cell anemia and hemolytic anemia due to malaria infection. Among these, iron-deficiency anemia and hemolytic anemia are the most common. Treatment varies depending on the type of anemia. It may include a supply of iron, vitamin B12 or vitamin B9 orally, treatment with immunosuppressors or corticosteroids, erythropoietin injections, blood transfusion or even bone marrow transplantation (Movaffaghi et al., 2006). Dietary changes, iron supplementation and blood transfusions are commonly used to treat anemia. Iron supplementation has many disadvantages, as the body has few mechanisms for eliminating this trace element, so it accumulates easily and can lead to serious health complications, such as certain neurogenic disorders or cancer (Saha et al., 2018). Meanwhile, blood transfusions often present risks of infection and incompatibility (Pelletier, 2018; Ainley and Hewitt, 2018). Given the undesirable side-effects of available therapies and especially their high cost, attention should be focused on the use of medicinal plants for the treatment of anemia.

Morinda lucida Benth (Rubiaceae) is a medicinal plant whose different parts are used in traditional medicine to treat various diseases in Africa. For

example, preparations based on the leaves of this plant were used against fever, to treat malaria and jaundice, as well as to treat sickle cell disease. The stem barks are used to make bitters, treat malaria and jaundice, and treat hypertension. In addition, the roots are used to treat itching and ringworm and as an antihypertensive (Adewole et al., 2021).

In experimental studies, phenylhydrazine (PHZ) has been used to induce hemolytic anemia in animal models. Its auto-oxidation produces reactive oxygen species and PHZ-derived radicals, which can lead to a number of harmful cellular reactions, including hemolytic anemia (Sung et al., 2013). On the other hand, phenolic compounds can prevent and repair free radical/reactive oxygen species-induced cellular oxidative damage (Shen et al., 2022). They show considerable ability to combat and protect against the effects of oxidative stress. Phenolic compounds have been shown to act as active antioxidants even at low concentrations, although most evidence regarding antioxidant capacity comes from *in vitro* studies (Kruk et al., 2022). Toxicity studies on medicinal plants, even if they are effective against pathologies, are also essential for the assessment of their potential adverse effects and to ensure their safe use. Thus, this study was conducted to investigate the effect of aqueous root bark extract of *Morinda lucida* against phenylhydrazine-induced anemia in Wistar rats in addition to its total phenol content and its acute toxicity.

2. MATERIAL AND METHODS

2.1 Plant Material

Root barks of *Morinda lucida* Benth (Rubiaceae) constituted the plant material. Roots of this plant were collected in the region of N'douci, Southern Côte d'Ivoire. Samples were sent to the National Floristic Center, Félix Houphouët-Boigny University of Abidjan, and were authenticated by comparison with specimens registered under number UCJ019084.

2.2 Animal Material

White *Mus musculus* mice, aged 8 weeks and weighing between 15 and 22 g, were used for the acute toxicity study. Wistar albino rats of the species *Rattus norvegicus*, aged 3 to 4 months and weighing between 150 and 200 g, were used to assess anemic activity. These animals came from the animal house of the Normal High School of Abidjan where they were bred. They were kept at room temperature with 12 h of light during the day and 12 h of darkness in the night, fed pellets and had free access to water. All experimental procedures have been examined and approved by the Ethical Committee of Health Sciences, Félix Houphouët-Boigny University of Abidjan.

2.3 Extract Preparation

Root barks of *Morinda lucida* previously harvested were shade at room temperature for 3 weeks and dried samples were later grounded to a powder using a grinder. One hundred (100) grams of plant powder were shaken in 1 L of distilled water and the mixture was homogenized for 5 min using an electronic mixer. The homogenate was then successively filtered twice on cotton and once on Whatman filter paper (3 mm) (Zirihi et al., 2003). This operation (homogenization and filtration) was repeated 3 times and the filtrates obtained were concentrated to dryness under reduced pressure at 30°C using a rotary evaporator (BÜCHI). The resulting extract constituted the aqueous root bark extract of *Morinda lucida*, which was stored at 4° C for subsequent analysis.

2.4 Determination of Total Phenol Content

The Folin-Ciocalteu reagent was used to determine the total phenol content of the aqueous root bark extract of *Morinda lucida*. For this purpose, 5 mL of Folin-Ciocalteu reagent (1:10 with distilled water) and 4 mL of sodium carbonate (1 M) were added to 0.5 mL of plant extract (0.1 g/mL). The reaction mixture was shaken, incubated at room temperature for 15 min and the absorbance measured at 765 nm. As a standard, gallic acid was used at concentrations ranging from 0 to 250 mg/L in methanol/water (50:50, v/v). The total phenol content was expressed as mg of gallic acid equivalents (GAE) per gram of extract using gallic acid standard curve. All measurements were repeated three times (Mc Donald et al., 2001).

2.5 Acute Oral Toxicity

The acute oral toxicity study of the aqueous root bark extract of *Morinda lucida* was performed according to the Organization for Economic Co-operation and Development (OECD) guideline no. 425 for the testing of chemicals (OECD, 2001). Two groups of 3 mice were formed. The

first group (control group) received distilled water, while the second group was treated with a single dose of 2000 mg/kg b.w. of aqueous root bark extract of *M. lucida*. The animals were fasted overnight before administration of the extract. After treatment, the animals were observed regularly for 24 h, with particular attention paid to the first 4 h, and then daily for 14 days. Observations focused on the following symptoms: agitation, convulsion, torsion, diarrhea and mobility.

2.6 Anti-anemic Activity

The anti-anemic activity of aqueous root bark extract of *Morinda lucida* was evaluated in rats according to the method of Ryu and Yook (2001) with few modifications. The study involved determining the hematological parameters of rats before and after induction of anemia.

2.6.1 Induction of anemia

Anemia was induced in rats by intraperitoneal (ip) injection of phenylhydrazine at a dose of 40 mg/kg bw per day (Naughton et al., 1989) for two consecutive days (D0 and D1). The phenylhydrazine was first dissolved in dimethyl sulfoxide (DMSO) diluted 1:10 in distilled water. On day 3 (D2), rats with a hemoglobin concentration of less than 9 g/dL were considered anemic and selected for further study.

2.6.2 Treatment and monitoring of animals

Twenty-five (25) rats were divided into 5 groups of 5 rats each. Group 1, which had previously received 1:10 diluted DMSO intraperitoneally, consisted of non-anemic rats, whereas groups 2, 3, 4 and 5 consisted of anemic rats. These different groups of animals were treated daily for 21 days (D2 to D22) as follows:

- Groups 1 (normal control) and 2 (anemic control) received orally distilled water daily.
- Group 3 (reference control) was treated orally with the standard anti-anemic product (Ranferon®) at 50 mg/kg bw.
- Groups 4 and 5 were treated orally with the aqueous root bark extract of *Morinda lucida* at doses of 100 and 200 mg/kg bw respectively.

During the experiment, blood samples were collected by eye puncture after the rats were anaesthetized. Blood was taken before induction of anemia (D0) and then at D2, D8, D15 and D22 and collected separately in EDTA tubes for determination of hematological parameters.

2.6.3 Determination of hematological parameters

Hematological parameters were determined using an automated hematological analyzer (URIT-3000 Plus, China) according to the manufacturer's instructions. The parameters determined were hemoglobin level, red blood cell (RBC) count, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

2.7 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 9.0 (Microsoft USA) software. Data were subjected to one-way analysis of variance (ANOVA) followed by Dunett's multiple comparison test (post-test). The difference between means was considered significant at a *P*-value of less than 5% ($P < .05$).

3. RESULTS

3.1 Total Phenol Content

The total phenol content of aqueous root bark extract of *Morinda lucida* was expressed as mg gallic acid equivalent per g of extract (mg GAE/g extract). The values were determined using the standard curve for gallic acid, defined as $y = 0.005903x$, $R^2 = 0.9780$. The total phenol content of this extract was estimated at 0.169 ± 0.015 mg GAE/g extract.

3.2 Acute Toxicity

Oral administration of aqueous root bark extract of *Morinda lucida* at a single dose of 2000 mg/kg b.w. did not cause any deaths in mice after 24 h and 14 days. No signs of agitation, convulsion, torsion, diarrhea or reduced mobility were observed in these animals during this period. These results indicate that the lethal dose 50 (LD_{50}) of aqueous root bark extract of *Morinda lucida* is greater than 2000 mg/kg b.w.

3.3 Anti-anemic Activity

Six hematological parameters were determined during the induction of anemia and its treatment with the aqueous root bark extract of *Morinda*

lucida and the standard anti-anemia drug. These parameters are hemoglobin level, red blood cell (RBC) count, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin content (MCHC).

3.3.1 Effect of *Morinda lucida* extract on hemoglobin level

The effect of aqueous root bark extract of *Morinda lucida* and Ranferon® on hemoglobin level in anemic rats is shown in Table 1. The results show that Phenylhydrazine caused a significant ($P < .0001$) reduction in hemoglobin level on day 2 (D2) in intoxicated rats compared to that in normal control rats (13.33 ± 0.15 g/dL). The hemoglobin level in these rats, ranging from 13.30 ± 0.4 to 13.70 ± 0.21 g/dL before induction of anemia (D0), decreased to values ranging from 8.33 ± 0.42 to 9.53 ± 0.09 g/dL at D2.

Daily administration of *M. lucida* extract and Ranferon® to anemic rats for 7 days significantly increased hemoglobin level (D8) in all treated groups compared to anemic control rats (9.5 ± 0.32 g/dL). In rats treated with aqueous extract of *M. lucida*, hemoglobin level increased significantly ($P < .001$ and $P < .05$), from 9.53 ± 0.09 to 11.27± 0.34 g/dL and from 8.48 ± 0.73 to 11.26 ± 0.021 g/dL, respectively at doses of 100 and 200 mg/kg b.w. With the reference drug (Ranferon®), hemoglobin level increased significantly ($P < .0001$) from 9.17 ± 0.23 to 11.77 ± 0.34 g/dL compared to anemic control rats. After two weeks of treatment (D15), the hemoglobin level of rats treated with Ranferon® (12.93 ± 0.145 g/dL) and those treated with the aqueous extract of *M. lucida* at 100 mg/kg b.w. (13.27 ± 0.26 g/dL) were restored to the level of normal control rats (13.17 ± 0.03 g/dL). The aqueous root bark extract of *M. lucida* at 200 mg/kg b.w. also restored hemoglobin level at the end of treatment (D22).

3.3.2 Effect of *Morinda lucida* extract on red blood cell count

The changes in red blood cell count during treatment of anemic rats with aqueous root bark extract of *Morinda lucida* and Ranferon® are shown in Table 1. A significant ($P < .0001$) decrease in red blood cell count was observed in all phenylhydrazine-intoxicated rats compared with normal control rats (8.35 ± 0.04×10^{12}/L) (D2). The red blood cell count, estimated from 7.94 ± 0.37×10^{12}/L to 8.44 ± 0.69×10^{12}/L at D0, decreased to values ranging from 3.19 ± 0.17×10^{12}/L to 4.3 ± 0.23×10^{12}/L at D2.

After one week of treatment (D8), a significant increase ($P < .05$) in red blood cell count was observed in rats treated with the aqueous extract of *Morinda lucida* at 100 mg/kg bw and in those treated with Ranferon® compared with that of anemic control rats (4.57 ± 0.09×10^{12}/L). Only the extract at 200 mg/kg b.w. did not cause any significant variation. At the second week of treatment (D15), the red blood cell count increased very significantly ($P < .0001$) in all

Table 1. Effect of aqueous root bark extract of *Morinda lucida* and Ranferon® on hemoglobin level and red blood cell count in anemic rats

Parameters	Groups	D0	D2	D8	D15	D22
Hemoglobin level (g/dL)	Normal	13.37±0.09	13.33±0.15	13.10±0.12****	13.17±0.03****	13.30±0.15***
	Anemic	13.50±0.4^{ns}	8.83±0.42$^{####}$	9.50±0.32	10.90±0.21	11.43±0.07
	Ranferon®	13.30±0.4^{ns}	9.17±0.23$^{####}$	11.77±0.15****	12.93±0.15****	13.43±0.34***
	AEMl (100 mg/kg b.w.)	13.40±0.23^{ns}	9.53±0.09$^{####}$	11.27±0.34***	13.27±0.26****	13.57±0.23****
	AEMl (200 mg/kg b.w.)	13.70±0.21^{ns}	8.48±0.73$^{####}$	10.42±0.27*	12.20±0.12***	12.77±0.15**
Red blood cell count (x10^{12}/L)	Normal	8.31±0.06	8.35±0.04	8.35±0.03****	8.36±0.03****	8.31±0.09****
	Anemic	8.35±0.24^{ns}	4.30±0.23$^{####}$	4.57±0.09	5.51±0.37	7.07±0.18
	Ranferon®	8.44±0.69^{ns}	3.89±0.12$^{####}$	5.57±0.29*	7.92±0.35****	8.19±0.09****
	AEMl (100 mg/kg b.w.)	7.94±0.37^{ns}	3.19±0.17$^{####}$	5.72±0.27*	8.18±0.1****	8.60±0.06****
	AEMl (200 mg/kg b.w.)	8.12±0.19^{ns}	3.68±0.36$^{####}$	5.13±0.44^{ns}	8.03±0.03****	8.25±0.08****

*AEMl: aqueous extract of Morinda lucida. Values are expressed as mean ± SEM, with n=5. Symbols (# and *) indicate statistical significance. For each parameter, comparisons were made by column. Values of normal control group were compared with those of other groups from D0 to D3. Values of anemic control group were compared with those of other groups from D8 to D22. $^{####}P < .0001$: significant difference between normal control group and other groups. *P < .05; **P < .01; ***P < .001; ****P < .0001: significant difference between anemic control group and other groups*

groups of rats treated with plant extract and Ranferon® compared with anemic control rats (5.52 ± 0.37×10^{12}/L). The RBC count in rats treated with *M. lucida* extract at 100 mg/kg bw (8.18 ± 0.1×10^{12}/L) was comparable to that in normal control rats (8.36 ± 0.3×10^{12}/L) and significantly higher than that in rats treated with Ranferon® (7.92 ± 0.35×10^{12}/L). After three weeks of treatment (D22), the RBC count of all groups of treated rats were reduced to those of normal control rats (8.36 ± 0.3×10^{12}/L). The values recorded were 8.60 ± 0.06×10^{12}/L; 8.25 ± 0.08×10^{12}/L and 8.19 ± 0.09×10^{12}/L in rats treated with aqueous root bark extract of *M. lucida* at doses of 100 and 200 mg/kg and Ranferon®, respectively.

3.3.3 Effect of *Morinda lucida* extract on hematocrit

Table 2 shows the effect of aqueous root bark extract of *Morinda lucida* and Ranferon® on hematocrit in anemic rats. Phenylhydrazine-induced anemia was evidenced by a significant (P < .0001) decrease in hematocrit on day 2 (D2) in rats of groups 2, 3, 4 and 5 compared with that in normal control rats (47.67 ± 0.88%). The hematocrit in these rats, estimated from 46.93 ± 1.06 to 47.47 ± 1.01% before induction of anemia (D0), decreased to values ranging from 23.10 ± 0.23 to 25.43 ± 0.59% at D2.

Treatment of anemic rats with aqueous extract of *M. lucida* and Ranferon® for 7 days (D8) resulted in a significant increase in hematocrit in all treated groups compared to anemic control rats (27.83 ± 0.44%). The hematocrit values recorded in treated rat groups ranged from 30.58 ± 0.36% to 31.10 ± 0.49% and were lower than those of normal control rats (46.67±0.67%). On the 14th day, the hematocrit of rats treated with *M. lucida* extract at 100 mg/kg bw (46.60±1.14%) and Ranferon® (45.87±1.62%) was statistically identical to that of normal controls (46.07 ± 0.58%). After 21 days of treatment (D22), this hematological parameter was also normalized in rats treated with the plant extract at 200 mg/kg b.w. Values obtained were 47 ± 1.1, 47.37 ± 0.63% and 46.20 ± 0.92% in animals treated with Ranferon® and *M. lucida* extract at 100 and 200 mg/kg b.w. respectively. The value for normal control rats was 46.27 ± 0.41%.

3.3.4 Effect of *Morinda lucida* extract on mean corpuscular volume

The effect of aqueous root bark extract of *Morinda lucida* and Ranferon® on mean corpuscular volume (MCV) in anemic rats is shown in Fig. 1. A significant (P < .05) increase in mean corpuscular volume was observed in all phenylhydrazine-intoxicated rats compared with normal control rats (63.77 ± 0.39 fL) (D2). The MCV in intoxicated rats, ranging from 60.63 ± 0.95 to 61.83 ± 1.68 fL before phenylhydrazine administration (D0), increased to values ranging from 79.27 ± 0.59 to 83.6 ± 1 fL at D2.

Treatment of rats for 7 days (D8) with the aqueous extract of *M. lucida* and Ranferon® resulted in a significant reduction in the MCV of the treated rat groups compared with that of the anemic control rats (73.67 ± 0.33 fL). However, the value of the normal control rats (62.57 ± 0.3 fL) was not reached. Two weeks after treatment (D15), MCV in rats receiving *M. lucida* extract at 100 mg/kg b.w. (60.70 ± 0.35 fL) and 200 mg/kg b.w. (61.87 ± 0.94) reached normal control levels (62 ± 0.51 fL). After 21 days of treatment (D22), MCV was restored in all treated rats. A better effect was observed for *M. lucida* extract at 100 mg/kg b.w. (58.53 ± 0.53 fL), followed by the extract at 200 mg/kg b.w. (60 ± 0.58 fL) and Ranferon® (62 ± 1 fL). The MCV of normal control rats did not change significantly during the experiment.

3.3.5 Effect of *Morinda lucida* extract on mean corpuscular hemoglobin

The changes in the mean corpuscular hemoglobin (MCH) of anemic rats treated with aqueous root bark extract of *Morida lucida* and Ranferon® are shown in Fig. 2. Administration of phenylhydrazine for two consecutive days (D0 and D1) resulted in a significant increase (P < .0001) in MCH in intoxicated animals compared to normal controls (18.50 ± 0.26 pg). Rats given phenylhydrazine showed MCH levels from 28.33 ± 1.76 to 32.13 ± 0.61 pg at D2. Treatment with the aqueous extract of *M. lucida* and Ranferon® for seven days did not result in any significant change (P > .05) in the MCH of these rats. The effect of plant extract and Ranferon® was only noticeable in anemic rats after 2 weeks of treatment (D15), where MCH was significantly reduced in all groups treated compared to anemic control rats (21.33 ± 0.33 pg). Values of 17.3 ± 0.4 pg, 18.23 ± 0.22 and 19.40 ± 0.31 obtained in animals treated with Ranferon® and *M. lucida* extract at 100 and 200 mg/kg b.w. respectively, reached those of normal control rats (18.63 ± 0.38 pg). At the end of treatment (D22), MCH was significantly reduced in rats treated with Ranferon® and *M. lucida* extract at 200 mg/kg b.w. compared to normal control rats

(18.43±0.9 pg). MCH levels were 16.47 ± 0.27 pg, 17.87 ± 0.19 pg and 17.23 ± 0.15 pg in rats treated with Ranferon® and M. lucida extract at 100 and 200 mg/kg b.w.

Table 2. Effect of aqueous root bark extract of *Morinda lucida* and Ranferon® on hematocrit in anemic rats

Parameters	Groups	D0	D2	D8	D15	D22
Hematocrit (%)	Normal	47.13±0.91	47.67±0.88	46.67±0.67****	46.07±0.58***	46.27±0.41**
	Anemic	47.53±1.68^{ns}	25.43±0.59$^{\#\#\#\#}$	27.83±0.44	36.07±0.52	40.0±0.58
	Ranferon®	47.03±1.65^{ns}	23.33±0.63$^{\#\#\#\#}$	31.10±0.49***	45.87±1.62***	47.0±1.10***
	AEMl (100 mg/kg b.w.)	46.93±1.06^{ns}	23.10±0.23$^{\#\#\#\#}$	30.58±0.36**	46.60±1.14***	47.37±0.63**
	AEMl (200 mg/kg b.w.)	47.47±1.01^{ns}	23.60±0.23$^{\#\#\#\#}$	30.70±0.46**	45.27±0.64***	46.20±0.72**

*AEMl: aqueous extract of Morinda lucida. Values are expressed as mean ± SEM, with n=5. Symbols (# and *) indicate statistical significance. For each parameter, comparisons were made by column. Values of normal control group were compared with those of other groups from D0 to D3. Values of anemic control group were compared with those of other groups from D8 to D22. $^{\#\#\#\#}P < .0001$: significant difference between normal control group and other groups. **P < .01; ***P < .001; ****P < .0001: significant difference between anemic control group and other groups.*

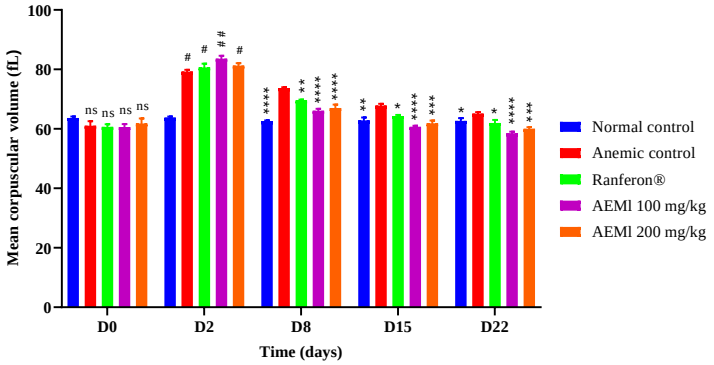

Fig. 1. Mean corpuscular volume variation in anemic rats treated with aqueous root bark extract of *Morinda lucida* and Ranferon®

*AEMl: aqueous extract of Morinda lucida. Values are expressed as mean ±SEM, with n=5. Symbols (# and *) indicate statistical significance. $^{\#}P < .05$; $^{\#\#}P < .01$: significant difference between normal control group and other groups. *P < .05; **P < .01; ***P < .001; ****P < .0001: significant difference between anemic control group and other groups.*

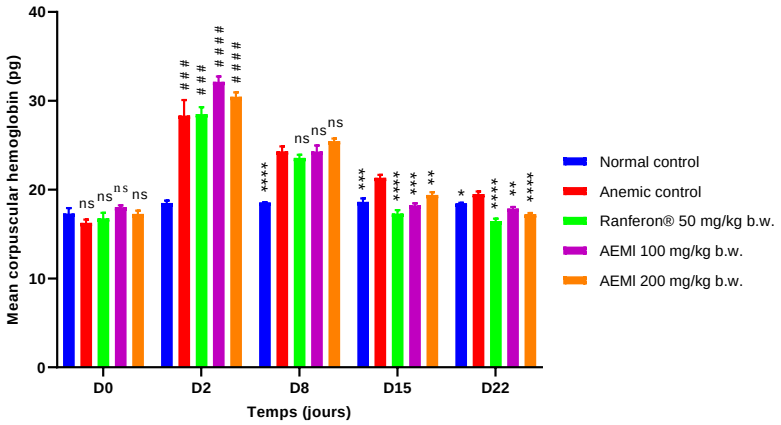

Fig. 2. Mean corpuscular hemoglobin variation in anemic rats treated with aqueous root bark extract of *Morinda lucida* and Ranferon®

*AEMl: aqueous extract of Morinda lucida. Values are expressed as mean ±SEM, with n=5. Symbols (# and *) indicate statistical significance. $^{\#\#}P < .05$; $^{\#\#\#}P < .01$; $^{\#\#\#\#}P < .01$: significant difference between normal control group and other groups. *P < .05; **P < .01; ***P < .001; ****P < .0001: significant difference between anemic control group and other groups.*

3.3.6 Effect of *Morinda lucida* extract on mean corpuscular hemoglobin concentration

Fig. 3 shows the changes in the mean corpuscular hemoglobin concentration (MCHC) during the treatment of anemic rats with aqueous root bark extract of *Morinda lucida* and Ranferon®. A significant ($P < .05$) reduction in MCHC was observed on day 2 (D2) after phenylhydrazine administration for two days (D0 and D1) to rats in groups 2, 3, 4 and 5 compared to normal control rats (45,1 ± 1,43 g/dL). With initial values ranging from 41.07 ± 0.24 to 43.33 ± 1.22, the MCHC decreased in these rats to values ranging from 35.40±0.31 to 37.5 ± 0.46 g/dL at D2. One week after treatment (D8), aqueous extract of *M. lucida* at a dose of 100 mg/kg b.w. and Ranferon® induced a significant increase ($P < .05$) in MCHC of treated rats compared to that of anemic control rats (36.53 ± 0.29 g/dL). On the 14th day of treatment (D15), all treated rats show a significantly higher MCHC than the anemic control rats (38 ± 0.23 g/dL). At the end of treatment (D22), MCHC levels were 41.47 ± 0.32, 42.53 ± 0.74 and 43.1 ± 0.49 g/dL in rats treated with Ranferon® and *M. lucida* extract at 100 and 200 mg/kg b.w. respectively. Treatment of anemic rats did not reach normal control MCHC level (45.77 ± 1.48 g/dL), but the effect of the extract was better than that of Ranferon®.

4. DISCUSSION

In this study, the aqueous root bark extract of *Morinda lucida* was tested for its efficacy to reverse phenylhydrazine-induced anemia in rats. The total phenol content of this extract was first determined. Phenolic compounds in plants have redox properties, and these properties allow them to act as antioxidants (Soobrattee et al., 2005), reducing free radical generation and alleviating diseases caused by oxidative stress (Ballard and Junior, 2019). The results showed the presence of these compounds at an estimated level of 0.169 ± 0.015 mg GAE/g extract. The phenolic content of *Morinda lucida* leaf, stem and root bark extracts were reported to be 4.84, 4.84 and 1.05 mg/100 g extract respectively (Adeleye et al., 2018). This shows that the phytochemical content of the same plant can vary according to its parts and terroir.

In the acute oral toxicity study of the aqueous root bark extract of *M. lucida*, behavioral observations of the experimental animals did not show any sign of toxicity and any mortality. The lethal dose 50 (LD_{50}) was greater than 2000 mg/kg b.w. This extract is therefore assigned to category 5 of the Globally Harmonised System of Classification of Chemicals, the category of substances with low toxicity (OECD, 2001). This is in agreement with the findings of Joppa et al. (2008) who showed that the hydroethanolic extract of *M. lucida* leaves with a LD_{50} above 5000 mg/kg b.w. is almost non-toxic.

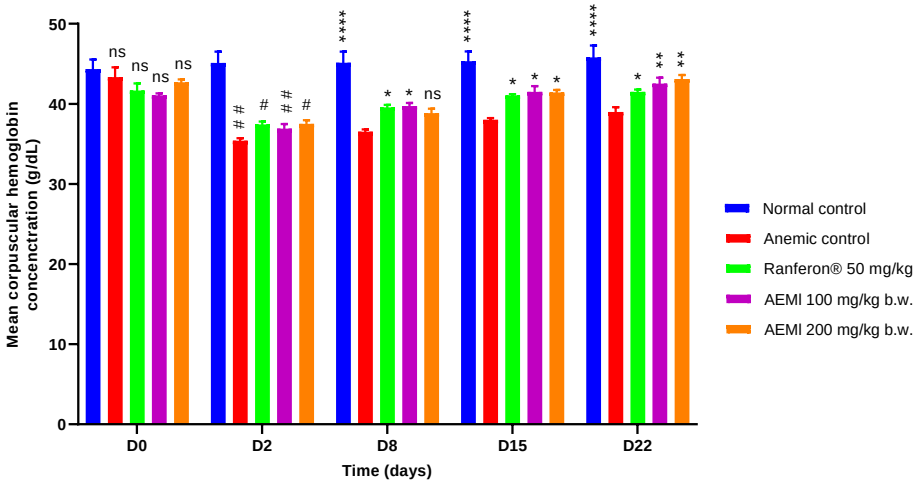

Fig. 3. Mean corpuscular hemoglobin concentration variation in anemic rats treated with aqueous root bark extract of *Morinda lucida* and Ranferon®

*AEMl: aqueous extract of Morinda lucida. Values are expressed as mean ±SEM, with n=5. Symbols (# and *) indicate statistical significance. $^{\#}P < .05$; $^{\#\#}P < .01$: significant difference between normal control group and other groups. *P < .05; **P < .01; ***P < .001; ****P < .0001: significant difference between anemic control group and other groups.*

For the anti-anemic activity test, hematological parameters were measured before and after inducing anemia and while treating rats with the aqueous extract of *Morinda lucida*. Intraperitoneal administration of phenylhydrazine (40 mg/kg b.w. for two days) to rats resulted in significant changes in hematological parameters. A very marked decrease in red blood cell (RBC) count, hemoglobin level and hematocrit (HCT) was observed, as well as a significant decrease in mean corpuscular hemoglobin concentration (MCHC). On the other hand, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) increased significantly. This very significant decrease in RBC count, hemoglobin level and hematocrit indicates anemia, probably caused by increased destruction of erythrocytes or impairment in their production.

Phenylhydrazine was mainly used for experimental induction of anemia in animals. It is known to cause hemolytic anemia by damaging the membranes of red blood cells, leading to their premature destruction (Berger, 2007). Its interaction with red blood cells leads to the formation of free radicals. These initiate the peroxidation of membrane lipids, leading to the lysis of red blood cells (Cohen and Hochstein, 1964; Jain and Hochstein, 1979). This results in a marked decrease in the number of red blood cells, hemoglobin levels and hematocrit, as observed in all rats given phenyhydrazine. The significant decrease in MCHC, which represents the concentration of hemoglobin in red blood cells, may indicate a relative dilatation of these blood cells or a reduction in hemoglobin production in anemic rats (Hoffbrand et al., 2006). In contrast, the increases in MCV and MCH observed in rats given phenylhydrazine may reflect the body's compensation for the reduced number of red blood cells. Higher MCV may indicate macrocytosis, where red blood cells are larger in response to anemia (Dacie and Lewis, 1991). The increase in MCH may suggest that the remaining red blood cells contain more hemoglobin, which may be an attempt by the body to maintain oxygen transport despite the reduction in the total number of red blood cells. Phenylhydrazine-induced anemia was therefore manifested by a significant decrease in red blood cell (RBC) count, hemoglobin level, hematocrit and mean corpuscular hemoglobin concentration (MCHC), and an increase in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH).

Treatment with aqueous root bark extract of *Morinda lucida* at doses of 100 and 200 mg/kg b.w. and Ranferon®, the standard anti-anemic drug at 50 mg/kg b.w., improved RBC count, hemoglobin level, hematocrit, MCV, MCH and MCHC in anemic rats. Hemoglobin levels, a key parameter in anemia, increased progressively during treatment of anemic rats. After two weeks of treatment (D15), this parameter was corrected by Ranferon® and *M. lucida* extract at 100 mg/kg b.w. At the end of treatment (D22), the hemoglobin level of all treated rats were restored to the level of normal control rats. There was also a progressive increase in RBC count during the treatment of anemic rats with Ranferon® and *M. lucida* extract. The results show that RBC count of all groups of treated rats were reduced to those of normal control rats after three weeks of treatment (D22). Red blood cells contain hemoglobin, so their production is associated with an increase in hemoglobin levels. The hematocrit reached that of normal control rats in rats treated with *M. lucida* extract at 100 mg/kg bw and Ranferon® on the 14th day of treatment (D15). After 21 days of treatment (D22), the plant extract at both doses (100 and 200 mg/kg b.w.) and Ranferon® normalized this parameter, i.e. the values observed in treated rats reached those of normal control rats. MCHC levels increased during the treatment of anemic rats, but did not reach those of normal control rats in rats treated with *M. lucida* extract at the end of treatment, with a better effect than Ranferon®. Finally, MCV and MCH decreased during treatment until they reached the values of normal control rats at the end of treatment. This was the case for rats treated with 100 mg/kg b.w. extract as well as for those treated with Ranferon®. The partial or complete recovery of hematological parameters in anemic rats during 3 weeks of treatment suggests that *M. lucida* extract restores erythrocyte size and hemoglobin content, synonymous with normal cell proliferation and hemoglobin synthesis. These observations show that the aqueous root bark extract of *M. lucida* is effective in repairing phenylhydrazine-induced damage to red blood cells and therefore in the treatment of anemia. The results are in agreement with those of Oladiji et al (2007) who showed that *M. lucida* leaf extracts were able to restore hemoglobin levels in anemic rats. Since phenylhydrazine-induced hemolysis is due to free radical peroxidation of erythrocyte membrane lipids, the effect of *M. lucida* extract may be due to the presence of phenolic compounds, notably flavonoids within it. Flavonoids are powerful antioxidants that can

prevent and repair free radical/reactive oxygen species-induced cellular oxidative damage (Shen et al., 2022). Sheth et al. (2021) reported that most anti-anemic compounds are known to scavenge free radicals and can improve anemia. Furthermore, the hematological parameters of the anemic control rats showed a progressive improvement during the treatment period of the other rats. This may be due to the body's ability to regenerate after phenylhydrazine-induced damage.

5. CONCLUSION

The aqueous root bark extract of *Morinda lucida* was evaluated for its anti-anemic potential in this study. The results showed that this extract is effective in repairing phenylhydrazine-induced erythrocyte damage and therefore in treating anemia by improving and correcting hematological parameters after two to three weeks of treatment. These findings suggest that this plant could provide a therapeutic alternative to conventional treatments for anemia.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Exploring the Therapeutic Potential of Ashwagandha (*Withania somnifera*) in Alleviating Stress and Anxiety

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Authors' contributions

This work was carried out in collaboration among all author. All authors read and approved the final manuscript.

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ABSTRACT

Background: Stress is a known causative factor in modulating cognitive health, on which the overall well-being and quality of life are dependent. Long-term stress has shown to disrupt the balance of the hypothalamic–pituitary–adrenal (HPA) axis. Adaptogens, such as *Withania somnifera* (Ashwagandha), are commonly used in ayurvedic medicine for stress relief and ameliorating HPA-axis dysfunction.

Aims & Objectives: To collect available literature in *Ayurveda* and modern science along with research had been studied published in International Journals till date.

Methods: A comprehensive search was conducted in MEDLINE, EMBASE, PubMed, PsychINFO and the Cochrane Library. Randomized controlled trials that examined the effects of Ashwagandha on stress and anxiety were included. Both subjective and objective measures of stress and anxiety were assessed as outcome variables.

Conclusion: The findings from the included studies indicated that *Ashwagandha* formulations had beneficial effects on stress and anxiety. The adverse effects associated with *Ashwagandha* are limited; however, further information is required to determine its safety with long-term administration.

Keywords: Anxiety; ayurveda; Perceived Stress Scale (PSS); psychosomatic; stress; Withania somnifera: ashwagandha.

1. INTRODUCTION

Ayurveda, the traditional system of medicine from India, offers a holistic approach to managing anxiety, focusing on balancing the body's energies (doshas), mind, and spirit. The Ayurvedic perspective on anxiety is that it results from an imbalance in the mind-body connection, often due to factors like stress, poor diet, lack of sleep, or unresolved emotional issues. Anxiety is commonly associated with the Vata dosha, which governs movement, energy, and the nervous system. An imbalance in Vata can lead to restlessness, worry, and fear, which are common symptoms of anxiety (Naidu et al., 2024). Pitta dosha, which controls digestion and metabolism, can also contribute to anxiety when it becomes excessive, leading to irritability and impatience (Mills et al., 2019). Kapha dosha imbalances, though less common, can contribute to anxiety in the form of lethargy and depression (Pratte et al., 2014).

"Anxiety is often accompanied by stress, which is the body's physiologic response to mental or physical threats. While brief exposure to the stress response is meant to be a beneficial coping mechanism, long-term stress is likely to result in the decline of overall health and the complication of existing diseases" (Archana & Namasivayam, 1999). According to the World Health Organization (WHO), approximately 4.4% of the global population is affected by anxiety disorders. This equates to more than 300 million people worldwide (Chodavadia et al., 2023). Anxiety and Depression are one of the symptoms in various diseases but are not a disease itself. It may be due to various reasons such as the aspects involving both personal and professional life of an individual.

2. DRUG REVIEW: ASHWAGANDHA

"Ashwagandha (*Withania somnifera*) is an adaptogenic herb, has a long history of use for its health benefits, particularly for reducing stress and anxiety. The species name somnifera comes from the latin word for sleep-inducing, signifying another purported property of this botanical" (Abdel-Magied et al., 2001). "Ashwagandha (*Withania somnifera*, fam. Solanaceae) is commonly known as "Indian Winter cherry" or "Indian Ginseng". It is one of the most important herbs of Ayurveda (the traditional system of medicine in India) used for millennia as a Rasayana for its wide ranging health benefits. Ashwagandha is commonly promoted for its well-known assistance in stress and anxiety reduction, in addition to stimulating a sound sleep. The Nagori Ashwagandha is the supreme among all the known Ashwagandha varieties. It is known to provide maximum benefit when fresh Ashwagandha powder is used" (Singh, 1983).

Ashwagandha offers numerous benefits. Some common usages of the drug are summarized here. *Stress and Anxiety Reduction*: "Ashwagandha is known for its ability to reduce stress by lowering cortisol levels (the stress hormone). It helps the body to adapt stress and promotes a sense of calm. A study on Ashwagandha has shown similar anti-stress activity in rats" (Archana & Namasivayam, 1999)

Table 1. Pharmacological properties of *Withania somnifera*

S.No.	Nighantu	Ras	Virya	Vipaka	Gun	Doshaghnata
1.	R.N. (Abdel-Magied et al., 2001)	*Katu,Tikta*	*Ushna*			Vata
2.	P.N. (Sharma, 2004)	*Tikta, Katu, Madhur*	*Ushna*	*Madhura*	*Laghu, Snigdha*	*Vata*
3.	S.N. (Panday, 2009)	-	-	-	-	*Vata*
4.	D.N. (Sharma, 2004)	*Kashaya,Tikta*	*Ushna*	-	-	*Vata, Kapha*
5.	B.P.N. (Chunakar, 2004)	*Tikta, Kashaya*	*Ushna*	-	-	*Vata, Kapha*
6.	K.N. (Sharma, 2006)	*Tikta, Kashaya*	*Ushna*	-	-	*Kapha*
7.	M.P.N. (Panday, 2012)	*Kashaya, Tikta*	*Ushna*	-	-	*Vata,Kapha*
8.	M.V.N. (Sri Khenraj, 2004)	*Tikta, Kashaya*	*Ushna*	-	-	*Vata,Kapha*

(R.N.- Raj Nighantu, P.N.-Priya Nighantu,S.N.- Sodal Nighantu, D.N.- Dhanwantri Nighantu, B.P.N- Bhav Prakash Nighantu, K.N.- Kaiyadev Nighantu, M.P.N.- Madan Pal Nighantu, M.V.N.- Madan Vinod Nighantu)

"It also exhibited an antidepressant effect, comparable with that induced by imipramine, in two standard tests, the forced swim-induced 'behavioral despair' and 'learned helplessness' tests. Another investigation supports the use of Ashwagandha as a mood stabilizer in clinical conditions of anxiety and depression" (Abdel-Magied et al., 2001). *Improved Sleep*: Due to its calming effects, Ashwagandha may help improve sleep quality, making it a popular remedy for insomnia. *Boosts Energy and Stamina*: It is often used to enhance physical endurance, strength, and energy. Some studies suggest that Ashwagandha may help in improving physical performance and recovery, especially in athletes. *Cognitive Function*: Ashwagandha has been linked to improved brain function, memory, and focus. It may also support cognitive health by reducing oxidative stress and inflammation in the brain. *Mood Enhancement*: It can help stabilize mood and may have antidepressant-like effects, particularly in people with mild to moderate depression. *Anti-Inflammatory Properties*: Ashwagandha contains compounds that may reduce inflammation, supporting overall health and recovery from physical ailments. *Hormonal Balance*: In men, Ashwagandha may support healthy testosterone levels and fertility. It is also believed to help balance thyroid hormones.

3. Ayurvedic view on Ashwagandha (*Withania somnifera*)

Pharmacodynamic properties:

Properties: *Vajikara** (Increases sexual craving) is a one of eight major specialty of the Ashtanga Ayurveda. It is an important treatment modality and has benefits of increased sexual capacity and improving health of future progeny as well as in treatment of many common sexual disorders like infertility, erectile dysfunction and premature ejaculation. *Rasayani** (Revitalizes the body). Rasayana chikitsa or therapy, is an ayurvedic rejuvenation therapy that focuses on strengthening the body and mind. It replenishes the vital fluids of the body; boosts the Ojas (vital force of life) and the immune system, thus keeping away from diseases and prevents against ill effects of advanced age. *Balya** (develops strength) involves treatment and substances that enhance strength and vitality, promoting overall well-being and recovery from debility. *Ati Shukrala** (enhances quality and amount of semen) drugs enhance the quality and quantity of Shukra. It enhances the Shukra (semen and sperm) quantitatively and qualitatively and facilitates its ejaculation. *Shwitrapaha** (Useful in treating of white staining of the skin) Shwitra is mentioned in *Kushta Roga Chikitsa* in classics. It is useful in treating of white staining of the skin. *Shothahara** (Useful in treating of edematous conditions and assists with clearing pollutants (Ama) from the different regions of the body) It is useful in treating of edematous conditions and assists with clearing pollutants (*Ama*) from the different regions of the body. *Kshayapaha** (Useful in treating thinness and under nutritive conditions) is useful in treatment for malnutrition, thinness and under nutritive conditions". (Khare, 2004; Khory & Katrak, 1999).

4. MODE OF ACTION

"Ashwagandha is considered an adaptogen, which means it helps the body adapt to stress and maintain balance (homeostasis). It contains bioactive compounds called withanolides, which are believed to contribute to its therapeutic effects. The biologically active chemical constituents of *Withania somnifera* include alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine, etc.), steroidal lactones (withanolides, withaferins) and saponins" (Sri Khenraj, 2004; Pratte et al., 2014). "Sitoindosides and acylsterylglucosides in Ashwagandha are anti-stress agents. Active principles of Ashwagandha, for instance the sitoindosides VII-X and Withaferin-A, have been shown to have significant anti-stress activity against acute models of experimental stress" (Bhattacharya et al., 1987). Many of its constituents support immunomodulatory actions (Ghosal et al., 1989). "The aerial parts of *Withania somnifera* yields 5-dehydroxy withanolide-R and withasomniferin-A" (Atta-ur-Rahman et al., 1991).

A. Anti-Stress Effects

- Results from several clinical trials suggest that Ashwagandha extracts may help reduce stress and anxiety. A 2021 systematic review identified seven studies that investigated the use of Ashwagandha to treat stress and anxiety" (Lopresti & Smith, 2021). "A total of 491 adults, all from India, with either self-reported high stress and anxiety or a diagnosed anxiety disorder, were randomized to take Ashwagandha or placebo for 6 to 8 weeks. Six of the studies used extracts made from Ashwagandha root alone (three studies, KSM-66), root and leaf

(two studies, Sensoril or Shoden), or unspecified parts (one study), while the seventh study used dried root powder made into granules. The Ashwagandha dose varied from 240 to 1,250 mg/day of extract or 12,000 mg/day of whole root granules, which is equivalent to 6,000 mg of root powder. Overall, the studies found that Ashwagandha significantly reduced stress and anxiety levels (subjectively measured by validated rating scales), reduced sleeplessness and fatigue, and reduced serum cortisol levels (a stress hormone) when compared with placebo. In several studies, the benefits appeared to be greater with doses of 500 to 600 mg/day than with lower doses. In India, at two health centers 130 healthy men and women age 20 to 55 years with self-reported stress were randomized to take a sustained-released Ashwagandha root extract (Prolanza) or placebo for 90 days" (Gopukumar et al., 2021). The extract was standardized to contain 15 mg withanolides per 300-mg capsule, and participants took one capsule daily. Compared with those who received placebo, participants who took Ashwagandha extract reported improvements in stress levels and sleep quality as measured by validated rating scales. They also had lower serum cortisol levels. In addition, participants reported improvements in psychological well-being, memory, and focus.

- Another study in India randomized 54 participants with mild to moderate stress and anxiety to receive either Ashwagandha root extract (Shagandha) or placebo (Majeed et al., 2023). The participants in the Ashwagandha group were given tablets that were standardized to contain 2.5% withanolides; each tablet included 500 mg of the root extract and 5 mg of piperine. At day 60, participants in the Ashwagandha group had significantly lower scores for stress and anxiety on two validated rating scales than those in the placebo group. In addition, the quality of life scores increased significantly for people in the Ashwagandha group between baseline and day 60. Moreover, the researchers noted improvements in multitasking and concentration among the participants in this group.

- At the University of Colorado, in Colorado Springs, 60 students (age 18–50 years) were randomized to take an Ashwagandha root extract (Gaia Herbs) or placebo for 30 days in a double-blind trial" (Baker et al., 2022; ClinicalTrials.gov, 2022). The extract contained 2.5 mg withanolides per 350-mg capsule, and participants took two capsules daily. The investigators gathered qualitative, subjective information from participants during daily check-ins and focus groups. Participants who took Ashwagandha root extract reported increased well-being, including a sense of calm; improved energy levels; heightened mental clarity; and enhanced sleep quality. While the descriptions of stress were comparable in both groups, participants who took Ashwagandha were more likely to describe their stress as manageable compared with those taking placebo.

- A randomized clinical trials (RCTs) that investigate the effect of Ashwagandha extract on anxiety and stress is included here. The overall effect size was pooled by random-effects model and the standardized mean difference (SMD) and 95% confidence interval (CIs) for outcomes were applied. Overall, 12 eligible papers with a total sample size of 1,002 participants and age range between 25 and 48 years were included in the current systematic review and meta-analysis. It was found that Ashwagandha supplementation significantly reduced anxiety (SMD: -1.55, 95% CI: -2.37, -0.74; $p = .005$; $I^2 = 93.8\%$) and stress level (SMD: -1.75; 95% CI: -2.29, -1.22; $p = .005$; $I^2 = 83.1\%$) compared to the placebo. Additionally, the non-linear dose-response analysis indicated a favorable effect of Ashwagandha supplementation on anxiety until 12,000 mg/d and stress at dose of 300-600 mg/d" (Akhgarjand et al., 2022).

B. Anti-Anxiety Effects

- WS has been used to stabilize mood in patients with behavioural disturbances. A study investigated the anxiolytic and antidepressant actions of the bioactive glycowithanolides (WSG), isolated from WS roots, in rats" (Bhattacharya et al., 2000). WSG (20 and 50 mg/kg) was administered orally once daily for 5 days and the results were compared by those elicited by the benzodiazepine lorazepam (0.5 mg/kg, i.p.) for anxiolytic studies, and by the tricyclic anti-depressant, imipramine (10 mg/kg, i.p.), for the antidepressant investigations. Both these standard drugs were administered once, 30

min prior to the tests. WSG induced an anxiolytic effect, comparable to that produced by lorazepam, in the elevated plus-maze, social interaction and feeding latency in an unfamiliar environment, tests. Further, both WSG and lorazepam, reduced rat brain levels of tribulin, an endocoid marker of clinical anxiety, when the levels were increased following administration of the anxiogenic agent, pentylenetetrazole. WSG also exhibited an antidepressant effect, comparable with that induced by imipramine, in the forced swim-induced 'behavioural despair' and 'learned helplessness' tests. The investigations support the use of WS as a mood stabilizer in clinical conditions of anxiety and depression in Ayurveda.

- Results from studies published after 2021 review also suggest that Ashwagandha has a beneficial impact on perceived stress (Remenapp et al., 2022). For example, one clinical trial was conducted in Florida over 60 men and women (mean age 34 years) who reported experiencing stress. Participants took capsules that contained 225 mg/day or 400 mg/day of a proprietary Ashwagandha root and leaf extract (NooGandha) or placebo for 30 days. Compared with participants in the placebo group, those in both Ashwagandha groups reported positive effects on stress, anxiety, depression, and food cravings as measured by validated rating scales. In addition, participants who took the 225-mg dose had lower saliva cortisol levels than those in the placebo group.

- Another randomized clinical trial included 120 healthy men and women (mean age 54–55 years) who were overweight or mildly obese and experiencing low energy and fatigue (Smith et al., 2023). Participants took an Ashwagandha root extract (Witholytin, which contains 200 mg hydroalcoholic extract of Ashwagandha root standardized to 1.5% withanolides) or placebo twice daily for 12 weeks. Results showed that Ashwagandha helped in reducing fatigue.

C. Sleep-Promoting Effects

- At one study center in India, 150 healthy men and women aged 18 to 65 years with self-reported sleep problems characterized by insomnia and lack of sound sleep were randomized to take Ashwagandha root and leaf extract (Shoden) or placebo for 6 weeks (Deshpande et al., 2020). The extract was standardized to contain 21 mg of with anolide glycosides per 60-mg capsule, and participants took two capsules each day. Both groups reported improvements in sleep quality as measured by a validated rating scale, but the improvements were greater in the Ashwagandha group (72%) than in the placebo group (29%). In addition, participants who took Ashwagandha extract showed improvements in sleep efficiency (time in bed spent in sleep), total sleep time, sleep latency (time taken to fall asleep), and awakening after sleep onset.

- In another trial conducted in India, 80 healthy men and women age 18 to 50 years, half of them with insomnia, were randomized to take Ashwagandha root extract (KSM-66) or placebo for 8 weeks. The extract was standardized with anolide content of more than 5% per 300-mg capsule, and participants took two capsules each day. Participants with insomnia who took Ashwagandha extract showed improvements in sleep quality, sleep onset latency, mental alertness on rising, and perceived anxiety symptoms compared with those taking placebo, as measured by actigraphy and validated rating scales. Participants without insomnia who took Ashwagandha also reported that Ashwagandha improved their sleep but it did not improve their perceived anxiety symptoms or their mental alertness on awakening" (Langade et al., 2021).

- A Bayesian hierarchical models were developed for a pre-specified subgroup meta-analysis on strength/power, cardiorespiratory fitness and fatigue/recovery variables. A total of 13 studies met the requirements of this systematic review, although only 12 were included in the quantitative analysis" (Bonilla et al., 2021). The meta-analytic approaches of the included studies revealed that Ashwagandha supplementation was more efficacious than placebo for improving variables related to physical performance in healthy men and female. In fact, the Bayesian models showed that future interventions might be at least in some way beneficial on the analyzed outcomes considering the 95% credible intervals for the meta-analytic effect size.

- In an eight-week, prospective, randomized, double-blind, placebo-controlled study, the stress-relieving effect of Ashwagandha root extract was investigated in stressed healthy adults. Sixty male and female participants with a baseline perceived stress scale (PSS) score >20 were randomized to receive capsules of Ashwagandha extract 125 mg, Ashwagandha extract 300 mg or identical placebo twice daily for eight weeks in a 1:1:1 ratio. Two participants (one each in 250 mg/day Ashwagandha and placebo) were lost to follow-up and 58 participants completed the study. A significant reduction in PSS scores was observed with Ashwagandha 250 mg/day ($P < 0.05$) and 600 mg/day ($P < 0.001$). Serum cortisol levels reduced with both Ashwagandha 250 mg/day ($P < 0.05$) and Ashwagandha 600 mg/day ($P < 0.0001$). Compared to the placebo group participants, the participants receiving Ashwagandha had significant improvement in sleep quality" (Salve et al., 2019).

- Ashwagandha extract appears to have a beneficial effect in improving sleep in adults (Cheah et al., 2021). A total of five randomized controlled trials containing 400 participants were analyzed. Ashwagandha extract exhibited a small but significant effect on overall sleep (Standardized Mean Difference -0.59; 95% Confidence Interval -0.75 to -0.42; I2 = 62%). The effects on sleep were more prominent in the subgroup of adults diagnosed with insomnia, treatment dosage ≥600 mg/day, and treatment duration ≥8 weeks. Ashwagandha extract was also found to improve mental alertness on rising and anxiety level, but no significant effect on quality of life. No serious side effects were reported.

D. Adaptogenic Effects

Adaptogens are herbs that improve an individual's ability to cope with stress and adapt to change. The most recent definition of an adaptogen is "a class of metabolic regulators that enhances the body's ability to adapt to environmental factors and avoid the damage they could imply." The ideal adaptogen should reduce negative changes caused by stress, be safe and act beneficially even when the dose given is higher than required, and be free of adverse side effects, such as not affecting the functioning of the body more than needed (Kuttan, 1996). Based on the above-mentioned characteristics, Ashwagandha can be considered as an adaptogen.

E. Immunomodulation and Hematopoiesis

A series of animal studies show *Ashwagandha* to have profound effects on the hematopoietic system, acting as an immunoregulator and a chemo protective agent" (Singhal et al., 2014).

"In a mouse study, administration of a powdered root extract from *Ashwagandha* was found to enhance total white blood cell count. In addition, this extract inhibited delayed-type hypersensitivity reactions and enhanced phagocyte activity of macrophages when compared to a control group" (Kurapati et al., 2013).

F. Neuroprotective and Anti-Neurodegenerative Effects

Neurodegenerative diseases cause the destruction of the central nervous system, resulting in irreversible damage. Over the course of Alzheimer's disease, an abnormal deposition of β-amyloid protein in the brain is observed. In its fibrillar form, it has a neurotoxic effect because it induces the formation of free radicals and impairs glucose transport in neurons, which leads to cell damage and death.

In studies conducted on human nerve cells, Ashwagandha has been shown to neutralize the toxic effects of β-amyloid, an implication in neurocognitive impairment during HIV infection (Pandey et al., 2018).

A study was conducted on rats that were orally administered vitanon—an ingredient isolated from the root of Whitania somnifera. Significant improvements in cognitive function were observed as a result of the inhibition of amyloid β-42, and a reduction in pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and MCP-1, nitric oxide, and lipid peroxidation was also observed. There was also a decrease in the activity of β and γ-secretase, enzymes responsible for the formation of insoluble neurotoxic aggregates of β-amyloid.

5. DISCUSSION

This meta-analysis provides the first comprehensive evaluation of *Ashwagandha* effects on stress, anxiety, and cortisol levels.

The findings demonstrate statistically significant improvements in overall stress and anxiety, with a notable reduction in serum cortisol levels. These results suggest that *Ashwagandha* may be a promising natural intervention for the stress and anxiety management.

Ashwagandha, the Indian Ginseng, is an important herb in Ayurveda that has been used for its various health benefits including, management of stress and anxiety, improving sleep pattern, boosting stamina and energy level, improving brain function and stimulating antidepressant-like effects, thereby enhancing mood. 6–8-week treatment of over 491 individuals in India with different extracts of the herb namely, root, root & leaf and other with unspecified part indicated lowering of stress and anxiety level. Quality of life scores increased significantly with much improvement in multitasking and concentration level in the individuals taking Ashwagandha for over 60 days. One double blind study in Colorado showed that the herb induced a sense of calmness; improved energy levels; heightened mental clarity; and enhanced sleep quality.

A study in rats was performed to investigate the anxiolytic and antidepressant actions of Ashwagandha. This study showed that this drug exhibited an antidepressant effect, comparable with that induced by imipramine. This supports the use of WS as a mood stabilizer in clinical conditions of anxiety and depression in Ayurveda. Positive effects on stress, anxiety, depression, and food cravings as measured by standards in clinical trials conducted in Florida over 60 men and women experiencing stress. It has also reduced fatigue and increase energy level in 120 healthy men and women with overweight or mildly obese physique.

In another studies, Ashwagandha has shown improvement in sleep problems characterized by insomnia and lack of sound sleep. Moreover, it has shown far better results than placebo in terms of sleep efficiency, total sleep time, sleep latency, and awakening after sleep onset. It has been shown in studies that Ashwagandha demonstrates improvement in mental alertness on rising, and perceived anxiety symptoms compared with placebo, as measured by actigraphy and validated rating scales. Bayesian hierarchical model developed for meta-analysis on strength/power, cardiorespiratory fitness and fatigue/recovery variables proved the efficacy of Ashwagandha supplementation. A eight-week long, randomized, double-blind, placebo-controlled study on sixty male and female participants receiving Ashwagandha displayed significant improvement in sleep quality. Another controlled trials on 400 participants with Ashwagandha dosage over eight weeks showed improvement in sleep quality, mental alertness on rising and anxiety levels.

Ashwagandha can therefore be considered as an adaptogen as it reduces negative changes caused by stress, is safe and acts beneficially even when the dose given is higher than required, and is free from any adverse side effects. Ashwagandha is found to be immunoregulator, as it enhances total white blood cell count in addition to inhibiting delayed-type hypersensitivity reactions. Considering the human nervous system, Ashwagandha has shown to neutralize the toxic effects of β-amyloid in brain caused during the course of Alzheimer's disease. It shows significant improvement in cognitive functions as a result of inhibition of amyloid β-42, inflammatory cytokines TNF-α, IL-1β, IL-6, and MCP-1, nitric oxide, and lipid peroxidation. Finally, we examined the physiological impact of Ashwagandha supplementation in stressed adults and identified several changes in hormones associated with the adrenal and steroidal system.

6. CONCLUSION

Rasayana, a subfield of Ayurvedic medicine, seeks to prevent aging, boost intelligence and vigor, and strengthen the body's resilience to illness. One of the best examples of the Rasyana medicinal plant, *Withania somnifera*, has biological qualities such as immunomodulation, anti-cancer, anti-depressant, and neuroprotective. The drawbacks of contemporary conventional medications include elevated resistance, inevitable adverse effects, diminished effectiveness from extended usage, and exorbitant expense. Therefore, it becomes necessary to concentrate on herbal and natural medications like Withania that can offer general protection for the health of the brain and neurons. Ashwagandha contains a wide variety of bioactive substances, including phenols, flavonoids, steroids, and alkaloids. The potential of WS extract to treat a number of pathological disorders has been the subject of extensive research. The findings from the included studies indicate that Ashwagandha formulations have beneficial effects on stress and anxiety. The adverse effects associated with Ashwagandha are limited; however, further information is

required to determine its safety with long-term administration.

CONCENT AND ETHICAL APPROVAL

It is not applicable.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

We hereby declared that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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