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Formulation of Water-Soluble Neem Leaf Bitters and Identification of Their Efficacy in Dermocosmetic and Nutraceutical Application

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ABSTRACT

Neem (*Azadirachta indica*) is important in the global context due to its potential to offer solutions to the many major concerns facing mankind from time immemorial. The present study aimed to develop methods for better solubilisation of neem leaf bitter principles in water. More particularly, the present study relates to a method of solubilizing bitter principles that achieve priorly unknown free aqueous solubility of bitters in water by combining polyvinyl pyrrolidone (a solubilizing agent) with polyoxyethylene sorbitan monooleate (a surfactant). We created a formulation with highest concentration of bitter principles (8% -15% w/w) and total flavonoids (5% w/w). This newly developed method has enabled the achievement of a previously unknown free aqueous solubility of bitters in water, thereby increasing the bioavailability and effectiveness of the extract. This study reports better safety and biological activities of neem leaf water-soluble bitters against Anti-acne, anti-cancer, anti-inflammatory, anti-oxidant, and α-Amylase and α-glucosidase inhibitory activity. Considering the therapeutic potential of this formulation, it can used in the pharmacological industry as a dermocosmetic and Nutraceutical.

INTRODUCTION

Neem (*Azadirachta indica*) has become a significant player in the global context due to its potential to offer solutions to the major concerns facing mankind. This versatile medicinal plant contains various types of compounds with diverse chemical structures, making it a unique source for medicinal purposes [1]. Despite its extensive use in traditional medicine, very little work has been done on the biological activity and plausible medicinal applications of these compounds [2]. Therefore, further investigation is necessary to exploit their therapeutic potential to combat diseases. Modern drugs can only be developed after extensive research into the bioactivity, mechanism of action, pharmacotherapeutics, toxicity, and standardization of neem. As the global scenario shifts towards the use of non-toxic plant products with traditional medicinal uses, the development of modern drugs from neem should be emphasized for the control of various diseases [3,4]. It is crucial to make good use of centuries-old knowledge of neem through modern approaches to drug development. Research into the biological activity and medicinal properties of neem is crucial to unlocking its full potential as a therapeutic agent. Its extensive use in traditional medicine highlights its potential in treating various diseases, and modern drug development from neem can provide safe and effective treatments for a range of health issues [5-7]. Therefore, it is imperative to continue to explore the potential of this versatile medicinal plant to address the major concerns facing mankind.

Neem leaf extract is a natural remedy that has been used for centuries in Ayurvedic medicine for its various health benefits [8]. The neem tree is native to India and its surrounding regions, and its leaves have been traditionally used for medicinal purposes [9]. Neem leaf extract contains several bioactive compounds, including nimbin, nimbinin, nimbidin, and nimbolide, which have been shown to possess anti-inflammatory, antioxidant, and antimicrobial properties [10-12]. These compounds are believed to be responsible for the various health benefits associated with neem leaf extract. Neem leaf extract has been used to treat a wide range of health conditions, including skin infections, digestive disorders, and fever, among others [13]. It is also used in the manufacturing of personal care products such as soaps, shampoos, and lotions due to its antibacterial and antifungal properties [14]. In recent years, neem leaf extract has gained attention from the scientific community due to its potential therapeutic properties. Ongoing research is exploring its potential in treating various diseases, including cancer and diabetes, among others.

Neem leaf bitters are a natural health supplement that is made from the leaves of the neem tree. One of the main advantages of neem leaf bitters is their ability to support digestive health. The bitter compounds in neem leaves help to stimulate the production of digestive enzymes and promote healthy bowel movements [13]. Neem leaf bitters are also believed to support liver function, which plays a vital role in detoxifying the body. Neem leaf bitters are also used to support immune function, which is essential for overall health. The antimicrobial properties of neem leaf bitters help to support the body's natural defences against harmful pathogens, viruses, and bacteria. Studies have shown that neem leaf extract has *in vitro* antibacterial activity against both *Staphylococcus aureus* and MRSA [6]. Another advantage of neem leaf bitters is their potential to support skin health. The anti-inflammatory properties of neem leaf bitters can help to soothe skin irritation, and their antimicrobial properties make them an excellent natural remedy for acne and other skin conditions. Neem has also been found to be a potential natural active for use in cosmetics due to its antimicrobial properties [14]. Additionally, nimbolide, a compound found in neem extracts, has been shown to downregulate cell survival proteins and upregulate genes involved in apoptosis in cancer cells [3].

Neem leaf powder is an integral part of Ayurvedic medicine and is used to balance pitta and kapha dosha, purify the blood, battle free radical damage, flush out toxins, treat insect bites, and cure ulcers. Neem, a versatile plant widely used in traditional medicine, has numerous beneficial properties. Both neem seed and neem leaf contain active compounds that have been shown to have medicinal properties, including antimicrobial, anti-inflammatory, and antioxidant effects [13]. However, there are distinct advantages to using neem leaf over neem seed, particularly in regards to its active content, toxicity, and cosmeceutical and nutraceutical applications. One significant advantage of neem leaf over neem seed is its higher concentration of active compounds. Neem leaves contain a higher amount of flavonoids, triterpenoids, and other bioactive compounds than neem seeds, making them more effective in treating various ailments [6, 13]. For example, studies have shown that neem leaf extract has a stronger antibacterial effect against certain strains of bacteria than neem seed extract [15]. Another advantage of using neem leaf over neem seed is its lower toxicity profile. While both neem seed and neem leaf are relatively safe for use in traditional medicine, neem seed can be toxic if consumed in large quantities. In contrast, neem leaf has been shown to have a lower toxicity profile, making it a safer choice for consumption [6,15]. In addition to its medicinal properties, neem leaf is increasingly being used in the

cosmeceutical and nutraceutical industry. Cosmeceuticals are products that combine cosmetic and pharmaceutical properties, while nutraceuticals are products that contain bioactive compounds that provide health benefits beyond basic nutrition. Neem leaf is particularly well-suited for use in these industries due to its numerous health benefits, including its anti-inflammatory and antioxidant effects [16].

The development of new preparations that enhance the solubilization of neem leaf bitter principles in water is a significant advancement in the field of therapeutics [17,18]. These new formulations have the potential to expand the therapeutic potential of neem and improve its clinical usefulness. The use of PVP K-30 as a solubilizing agent, along with selective surfactants that modulate the surface tension of water, enables the solubilization of bitters to a level of 100% w/v in water. This method minimizes the quantity of PVP normally used in solubilizing water-insoluble material in aqueous media, making it safe and stable for use in nutraceuticals, pharmaceutical drugs, and pesticide aqueous formulations.

With the development of new formulations that enhance the solubilization of neem bitter principles in water, its pharmacological efficacy can be further improved. Further research is needed to fully understand the bioactivity of neem and how these new formulations could further improve its clinical usefulness. However, the development of these new preparations is a promising step towards unlocking the full potential of neem in the field of therapeutics.

1. MATERIALS AND METHODS

1.1 Preparation of water-soluble neem bitters:

In the preparation of solubilized bitters, a five-step process was followed. In the initial step, a mixture of polyvinyl pyrrolidone (PVP) and polyoxyethylene sorbitan monooleate was continuously stirred in ethanol at room temperature until a clear solution was obtained. In the second step, neem leaf hydro alcohol extract was added to the solution, and continuous stirring for 30 minutes resulted in a clear solution. The ethanol in the solution from the second step was evaporated using a Rota evaporator in the third step, and distilled water was added to the residue to obtain a clear mixture. The fourth step involved filtration to remove any insoluble material from the clear mixture. Finally, in the fifth step, the resulting mixture from steps 4 and 5 was spray-dried at a temperature range of 120-140 °C using a spray dryer to obtain the final product of solubilized bitters.

The quality of the final product was ensured by maintaining an assay of bitter principles indicating not less than 8% w/w of bitter principles. The content of water-soluble neem bitters was determined by gravimetry, which is a method used to measure the mass or weight of a substance. Total flavonoids are measured using UV spectroscopy, which is a technique that uses light absorption to determine the concentration of a substance in solution. The LOB is an indicator of the purity of the product and is determined by measuring the amount of butanol-insoluble fraction present in the sample.

1.2 Solubilisation of bitter principles:

Polyvinylpyrrolidone K30 (27.5g) and polyoxyethylene sorbitan monooleate (2.5g) taken in ethanol (500 ml) were stirred continuously at room temperature till the solution of the said mixture (mixture solution 1) became clear. Neem leaf hydroalcoholic extract wherein the chemical assay of said extract defines the content of neem leaf bitter principles as not less than 15% w/w and not more than 20% w/w on a dry basis. 70 grams of the aforesaid neem leaf hydroalcoholic extract was added to mixture solution 1 and continuously stirred for 30 min at room temperature to get mixture solution 2. Ethanol was evaporated from mixture solution 2 using a Rota Evaporator to get the mixture solution 3. 500 ml of distilled water was added to mixture solution 3 and filtered to remove insoluble materials to form a mixture solution 4. Using a spray dryer, mixture solution 4 was spray dried at 120-140 °C.

1.3 Anti-oxidant activity:

The antioxidant activity of neem water-soluble bitters was compared with that of neem water-insoluble bitters at various concentrations. Ascorbic acid (1 mg/ 100µl) was employed as the reference standard. The different concentrations tested for neem water soluble bitters and neem water insoluble bitters include 1 µg/µl, 2.5 µg/µl, 5 µg/µl and 10 µg/µl. For ascorbic acid, concentrations of 200 µg/µl, 400 µg/µl, 600 µg/µl, 800 µg/µl, and 1000 µg/µl were examined. After adding DPPH, the reaction was mixed uniformly and allowed to incubate at 25°C for 30 minutes. The semi-auto analyser was used to detect the absorbance at 517 nm. By comparing the absorbance readings of the test and reference samples, the percentage of inhibition was calculated [19, 20].

1.4 Glutathione reduction activity:

The present purpose of this study is to evaluate the ability of water-soluble neem bitters to generate ROS by using a Glutathione Reduction assay. Neem water soluble and Neem water

insoluble bitters were tested for their ability to generate ROS. Ascorbic acid was used as a reference standard (1 mg/ml). 1 µg/µl of Neem water soluble and Neem water insoluble bitters were tested against 1 µg/µl concentration of the reference standard (Ascorbic acid). The contents were mixed according to manufacturer protocol and incubated for 30 minutes. The absorbance was measured at 412 nm using a semi-auto analyzer [21].

1.5 *In vitro* evaluation of the α-Amylase and α-glucosidase inhibitory activity

In brief, a 1 mg/ml concentration of neem water soluble bitter and neem water insoluble bitter was prepared and tested for their ability to block the enzyme α-Amylase/ α-glucosidase using the substrate starch azure. The absorbance was measured with a spectrophotometer at 540 nm. This approach was used to determine each bitter's IC50 value and percentage of amylase inhibitory action [22].

1.6 *In vitro* anti-inflammatory Activity:

The COX-2 Inhibitor Screening Kit offers a rapid, simple, sensitive, and reliable test for screening COX-2 inhibitors. In this experiment, prostaglandin G2, an intermediate produced by the COX enzyme, was detected fluorometrically. The COX Probe (Ex = 535 nm/Em = 587 nm) provides a fluorescent signal proportional to the quantity of prostaglandin G2 [23]. This conversion is mediated by the COX-2 enzyme. Prostaglandin production is inhibited in the presence of COX-2 inhibitors, reducing the symptoms of inflammation.

1.7 Anti-acne Activity:

Anti-bacterial activity is evaluated by the Agar well diffusion assay and by Minimal Inhibitory concentration (MIC) evaluation.

a. Anti-acne Activity of Neem water soluble bitters against *Propionibacterium acnes*

Agar well diffusion assay for *Propionibacterium acnes:*

A good diffusion test was conducted using the BHI medium as the strain *P. acnes* grows in Brain Heart Infusion medium (BHI). A poisoned food technique was applied for this study. The inoculums used were prepared using *P. acnes* from a 48-hour incubation on BHI agar. A suspension was made by transferring 4–5 colonies into 9 ml of sterile deionized water to yield a concentration of approximately $1.0 \cdot 10^{8}$ CFU/ml. For the poisoned food technique, aliquots of molten BHI agar (100 mL) were cooled to 50 °C before being inoculated with 0.5 mL of the *P. acnes* suspension. The inoculated agar was mixed, then poured and left to

solidify. Once the agar solidified, four wells (8 mm in diameter) were cut out of the seeded agar using a sterile cork borer. The wells were filled with about 100 µl of plant extracts (1 mg/ml of plant extracts dissolved in sterile deionized water). All plates were incubated in anaerobic jars for 72 hours at 37 °C. After this period, the antibacterial activity was evaluated by measuring the inhibition zone diameter in mm around the wells. MIC is determined as the minimum concentration of the drug showing maximum inhibition.

b. Anti-acne Activity of Neem water soluble bitters against *Staphylococcus epidermidis*

Agar well diffusion assay for *S. epidermidis*

A good diffusion test was conducted using the nutrient agar medium as the strain *S. epidermidis* grows in the nutrient agar medium. A poisoned food technique was applied for this study. The inoculums used were prepared using nutrient agar from a 48-h incubation on a nutrient agar medium. A suspension was made by transferring 4–5 colonies into 9 ml of sterile deionized water to yield a concentration of approximately $1.0 \cdot 10^{8}$ CFU/ml. For the poisoned food technique, aliquots of molten nutrient agar (100 mL) were cooled to 50 °C before being inoculated with 0.5 mL of the *S. epidermidis* suspension. The inoculated agar was mixed, then poured and left to solidify. Once the agar solidified, four wells (8 mm in diameter) were cut out of the seeded agar using a sterile cork borer. The wells were filled with about 100 µl of plant extracts (1 mg/ml of plant extracts dissolved in sterile deionized water). All plates were incubated at 37 °C for 72 h. After this period, the antibacterial activity was evaluated by measuring the inhibition zone diameter in mm around the wells. Minimal Inhibitor Concentration (MIC) was evaluated by adding various concentrations of plant extracts in sterile 50 mm petriplates. The extracts were diluted with molten agar to make up the volume to 4 ml. After solidification of the media, the plates are inoculated with 20 µl of broth culture of *S. epidermidis.* Plates are incubated at 37 ^{o}C for 72 h.

***1.8 In vitro* anticancer activity:**

A 96-well plate was seeded with 7,500–8,000 Breast cancer cells (MDA-MB-468) and Breast cancer cells (MDA-MB-231) and incubated overnight. Before treating with the neem water soluble and neem water insoluble bitters, the culture medium was removed and replaced with new media. For Neem water soluble and neem water insoluble bitters, four different concentrations were tested: 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml. DMSO was used as a negative control. As a positive control, doxorubicin (50 mM, 25 mM, 10 mM, and 1 mM/l) was utilized. The MTT assay was carried out with the addition of Thiazolyl Blue

Tetrazolium Bromide (MTT; Sigma; St Louis, MO, USA) and incubated for 4 hours after addition to check for the formation of insoluble purple crystals. Cells were cultured for 48 and 72 hours along with the neem water soluble and neem insoluble bitters. To dissolve the generated purple-coloured formazan crystals, 100 µl of DMSO was added to each well. Using a 96-well Tecan Microplate Reader, the multi-well plate's spectrophotometric absorbance was measured at 570 nm (Tecan Instruments, Switzerland). The Megalan software (Tecan Instruments, Inc.) was used to retrieve the data, which was then exported in Microsoft Excel format for additional analysis [24].

1.9 Safety evaluation of Neem water soluble bitters:

A 96-well plate was seeded with 7500–8000 Kidney Epithelial cells (Hek293T) and Skin Fibroblast cells (HFF-1) and incubated overnight. Before treating with the neem water soluble and neem water insoluble bitters, the culture medium was removed and replaced with new media. For Neem water soluble and neem water insoluble bitters, four different concentrations were tested: 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml. DMSO was used as a negative control. As a positive control, doxorubicin (50 mM, 25 mM, 10 mM, and 1 mM/l) was utilized. The MTT assay was carried out with the addition of Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma; St Louis, MO, USA) and incubated for 4 hours after addition to check for the formation of insoluble purple crystals. Cells were cultured for 48 and 72 hours along with the neem water soluble and neem insoluble bitters. In order to dissolve the generated purple-coloured formazan crystals, 100 µl of DMSO was added to each well. Using a 96-well Tecan Microplate Reader, the multi-well plate's spectrophotometric absorbance was measured at 570 nm (Tecan Instruments, Switzerland). The Megalan software (Tecan Instruments, Inc.) was used to retrieve the data, which was then exported in Microsoft Excel format for additional analysis [24].

2 RESULTS AND DISCUSSION

Neem has become important in the global context today because it offers answers to the major concerns facing mankind. Neem, a versatile medicinal plant, is a unique source of various types of compounds having diverse chemical structures. Hence, very little work has been done on the biological activity and plausible medicinal applications of these compounds, and hence extensive investigation is needed to exploit their therapeutic utility to combat diseases [18, 25]. Although crude extracts from various parts of neem have been used for medicinal purposes since time immemorial, modern drugs can only be developed after

extensive research into its bioactivity, mechanism of action, pharmacotherapeutics, toxicity, and standardization [18]. As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal uses, the development of modern drugs from neem should be emphasized for the control of various diseases. Time has come to make good use of centuries-old knowledge of neem through modern approaches to drug development [5].

2.1 Preparation of Water-soluble neem bitters

The present study relates to a method of solubilizing bitter principles using polyvinyl pyrrolidone (PVP) and polyoxyethylene sorbitan monooleate. PVP is a water-soluble polymer that has been used in drug delivery systems. PVP is also used as a food additive and is safe for human consumption. The use of PVP in the solubilization of bitter principles can enhance their aqueous solubility to 100% w/v in water, making them more bioavailable and easier to administer. This method could have potential applications in the pharmaceutical industry for improving the delivery of drugs with bitter taste profiles.

2.2 Quality check of bitter principles

The Solubilised bitter principles (green-brownish fine powder) obtained by the aforesaid process, wherein the aqueous solubility of bitter principles was completely and freely soluble in water 100% w/v was collected and stored in polyethylene covers. The yield of the final product was 90 grams. It is to be noted that the solubility of bitter principles may be understood as a physical state where bitters go into solution in a manner such that upon filtration of said solution, no solids (insoluble neem particles) are retained in the interposing medium used in filtration.

The specification established for water-soluble neem bitters include the content of water-soluble neem bitters by gravimetry: (≥ 8% w/w); total flavonoids by UV (≥ 5% w/w); and LOB (limit of butanol-insoluble fraction): (≤1.5% w/w). These specifications are important to ensure the quality and consistency of the product.

Neem-derived extracts have been shown to work anywhere from insect repellent to supplements to lower inflammation, diabetic control, combat cancer, and even application in cosmetics [6]. Herein, we state the health benefits found with Neem water soluble bitters, highlighting the free radical scavenging activity, anti-diabetic activity, anti-inflammatory activity, anti-cancer, anti-acne, and *in vitro* safety evaluation [26].

2.3 Anti-oxidant activity:

A. indica (neem) shows a therapeutic role in health management due to its rich source of various types of ingredients. The most important active constituents are bitters and polyphenolic flavonoids. Neem protects against chemically induced carcinogens and liver damage by boosting antioxidant levels [3].

It is known that ROS and free radicals are involved in cancer, DNA damage, and even aging, and the addition of antioxidants in dermo-cosmetics can minimize these effects. Thus, in the present invention, neem water soluble bitters along with flavonoids are screened for their antioxidant activity by the DPPH method. The results for the anti-oxidant potential of neem water soluble and neem water insoluble bitters are interpreted in the table 1.

The study examined the antioxidant activity of neem bitters and compared it with the control substance ascorbic acid. The results indicated that the water-soluble neem bitters had a greater antioxidant activity than the water-insoluble neem bitters. The water-soluble neem bitters had an IC50 value of approximately 42.34 µg/ml, indicating that it exhibited potent antioxidant activity. On the other hand, the water-insoluble neem bitters had an IC50 value of approximately 98.43 µg/ml, indicating a lower antioxidant activity in comparison. These findings suggest that the implementation of neem water-soluble bitters could be more significant in promoting antioxidant activity than the use of ascorbic acid as a control.

2.4 Glutathione reduction activity:

Quercetin is one of the major flavonoids identified in Neem leaf extract. Quercetin has been widely employed as a chemoprevention drug in several cancer cells because of its potent pro-oxidant activity. Previous research has shown that cancer cells have lower levels of antioxidant enzymes and are therefore more likely to be affected by quercetin treatment, resulting in ROS-induced cell damage [27, 28]. In this study, we have evaluated the ability of water-soluble neem bitters to generate ROS by using a Glutathione Reduction assay. The results for the Glutathione reduction potential of neem water soluble and neem water insoluble bitters are interpreted in the following graph (Figure 1).

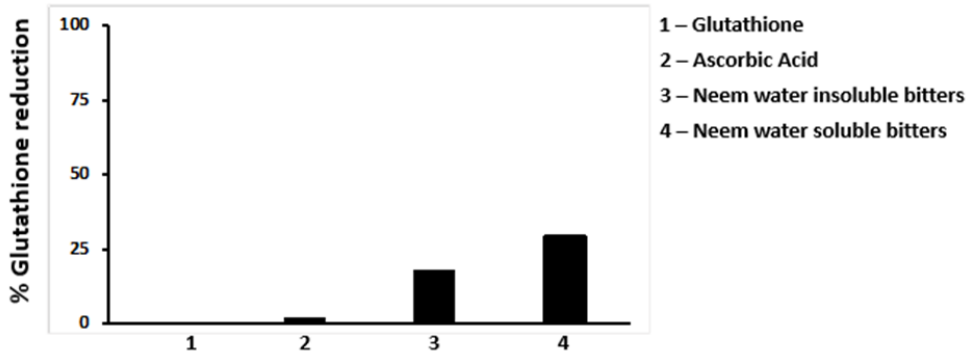

Figure 1: Graph representing the percentage Glutathione Reduction for Neem Water soluble and Neem water insoluble Bitters

In this study, we investigated the effect of ascorbic acid and neem bitters on glutathione levels. Ascorbic acid was used as the control substance, and it was found to cause a modest 7% reduction in glutathione levels at a concentration of 1 µg/µl. The neem bitters, however, showed a more significant impact on glutathione levels. The water-insoluble neem bitters caused a 20% reduction, while the water-soluble neem bitters caused a more substantial 33% reduction at the same concentration. These findings suggest that neem bitters, particularly the water-soluble form, have a more potent effect on glutathione levels compared to ascorbic acid. Therefore, implementing neem water-soluble bitters may have a more significant impact on glutathione levels than using ascorbic acid as a control substance.

2.5 *In vitro* evaluation of the α-Amylase and α-glucosidase inhibitory activity

In recent decades, there has been a drastic increase in the incidence and prevalence of diabetes mellitus. The presence of phytochemicals such as flavonoids, tannins, and saponins in this plant may be responsible for its inhibitory activity on the two enzymes studied. Flavonoids contained in the ethanolic extract of A. indica could contribute to its antihyperglycemic or hypoglycaemic effect. Thus, these flavonoids, in association with their antioxidant property, could be employed as an alternative source of medicine to mitigate or counteract the pathogenesis of diabetes and its complications [29].

The present invention of this study is to evaluate the *in vitro* inhibitory effect of water-soluble neem bitters on the activity of α-Amylase and α-glucosidase as a means of alleviating hyperglycaemia and managing diabetes mellitus.

The results for the anti-diabetic potential of neem water soluble and neem water insoluble bitters are interpreted in the following graph (Figures 2 and 3).

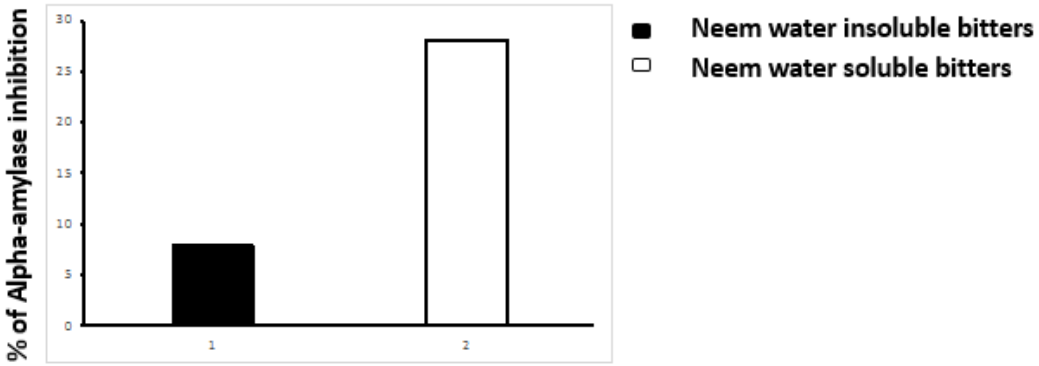

Figure 2: Graph representing the percentage inhibition of α-amylase activity by Neem water soluble and Neem water insoluble Bitters

The study investigated the α-amylase inhibitory activity of neem bitters and compared it with each other. The results showed that the neem water-soluble bitters had appreciable α-amylase inhibitory activity, with an IC50 value of 28 µg/ml. In comparison, the water-insoluble neem bitters had a lower α-amylase inhibitory activity, with an IC50 value of 8 µg/ml. These findings suggest that implementing neem water-soluble bitters may have a more significant impact as an anti-diabetic agent than using neem water-insoluble bitters.

Figure 3: Graph representing the percentage inhibition of α-glucosidase activity by Neem water soluble and Neem water insoluble Bitters

The study examined the α-glucosidase inhibitory activity of neem bitters and compared it with each other. The results showed that the neem water-soluble bitters had appreciable α-glucosidase inhibitory activity, with an IC50 value of 42 µg/ml. However, the water-insoluble neem bitters had a lower α-glucosidase inhibitory activity with an IC50 value of 14 µg/ml. These findings suggest that the implementation of neem water-soluble bitters may be more

significant in inhibiting α-glucosidase activity compared to neem water-insoluble bitters. The study implies that neem water-soluble bitters may have potential in the management of diabetes by inhibiting α-amylase activity.

2.6 *In vitro* anti-inflammatory Activity

An important property found in neem extracts is their ability to work as anti-inflammatory agents. Inflammation is a pathophysiological condition involved in a plethora of diseases like cancer and diabetes. Now, a main bioactive compound found in Neem is limonoid, a bitter principle known for its inhibitory properties in the production of inflammatory mediators [6]. Inflammation leads to the activation of the cyclooxygenase pathway, and in the present invention, inhibition of cyclooxygenases is evaluated by using neem water soluble bitters. The results for the anti-inflammatory activity of neem water soluble and neem water insoluble bitters are interpreted in the following graph (Figure 4).

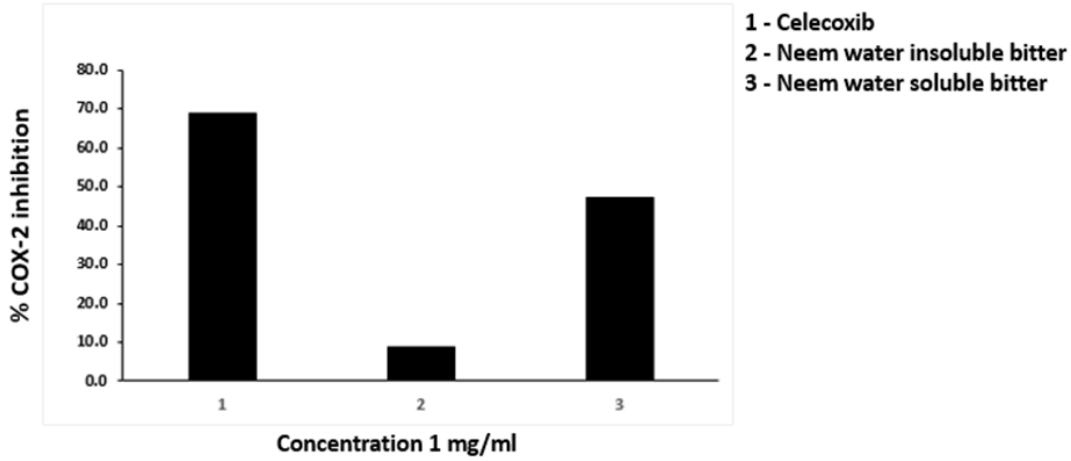

Figure 4: Graph representing the percentage of COX-2 Inhibition by Neem water soluble bitters in comparison with Neem water insoluble bitters.

The study evaluated the COX-2 inhibitory activity of neem bitters and compared it with celecoxib as a control. The results showed that celecoxib was a potential COX-2 inhibitor, with 69% inhibition at ~ 1 mg/ml. The neem water-soluble bitters exhibited COX-2 inhibitory activity with 47% inhibition at ~ 1 mg/ml, which was lower than celecoxib but still significant. On the other hand, the neem water-insoluble bitters had a lower COX-2 inhibitory activity, with 8.9% inhibition at ~ 1 mg/ml. These findings suggest that implementing neem water-soluble bitters could be a more potent COX-2 inhibitor than neem water-insoluble bitters. The study implies that neem water-soluble bitters may have potential in the treatment of inflammation-related disorders by inhibiting COX-2 activity. However, further studies are

required to validate these findings and assess the potential of neem water-soluble bitters as an alternative to celecoxib in COX-2 inhibition.

2.7 Anti-acne Activity:

Acne vulgaris is a chronic inflammatory skin disorder involving the pilosebaceous follicles that is characterized by comedones that are papules, pustules, cysts, nodules, and often scars, chiefly on the face, neck, and upper trunk. It is produced by hyperkeratosis, which retains keratin and sebum, and the main microorganisms involved are *P. acnes* and *S. epidermidis*. For many years, antibiotics have been used to treat acne vulgaris. However, antibiotic resistance has been increasing in prevalence within the dermatologic setting. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatments [30]. The present invention describes an *in vitro* method for determining the effect of neem water soluble bitters on the growth of the skin flora such as *P. acnes* and *S. epidermidis.* Results are recorded in the following table (Table 1).

Table 1: Effect of neem water soluble bitters on the growth of the skin bacterium *Propionibacterium acnes* and *Staphylococcus epidermidis in vitro*

Organism Tested	Extract	Zone of Inhibition	Minimum Inhibitory Concentration
P. acne	Neem water-soluble bitters	13.2 ± 0.2 mm	1 mg
	Chloramphenicol	19.5±0.6 mm	0.064 mg
S. epidermidis	Neem water-soluble bitters	11.9 ± 0.2 mm	2 mg
	Chloramphenicol	19.5±0.6 mm	1.35 mg

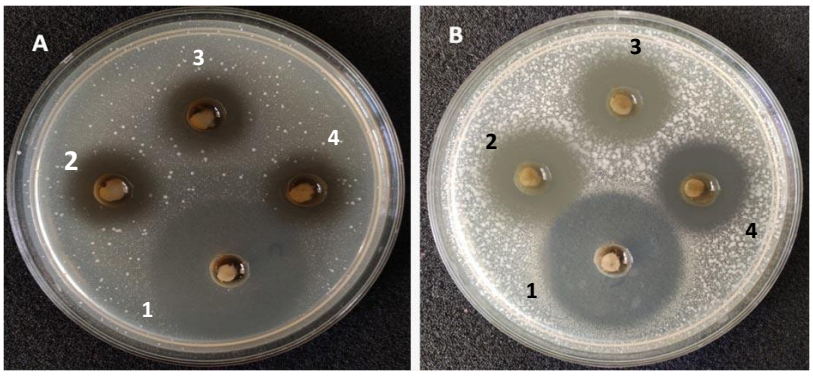

Figure 5: Agar well diffusion assay of Neem water soluble bitters. (A) 1) Chloramphenicol; 2, 3 and 4 represents neem water soluble bitters against *S. epidermidis*. **(B)**

1) chloramphenicol, 2, 3 and 4 represents neem water soluble bitters against represents plants extracts against *P. acnes.*

The study found that neem water soluble bitters have demonstrated antibacterial activity against two types of bacteria, *P. acne* and *S. epidermidis* (Figure 5). The results indicated that P. acne showed a zone of inhibition of 13.2 ± 0.2 mm and a Minimum Inhibitory Concentration (MIC) at 1 mg, whereas *S. epidermidis* showed a zone of inhibition of 11.9 ± 0.2 mm and a Minimum Inhibitory Concentration (MIC) at 2 mg. These findings suggest that neem water soluble bitters could be a promising natural treatment option for acne and other bacterial skin infections caused by *P. acne* and S. epidermidis.

2.8 *In vitro* anticancer activity

The present study provides a water-soluble neem bitter that, along with its flavonoids, is being studied for its anticancer property. Studies state that the extract of neem leaves can destroy cancer cells. Constituents of Neem leaves prevent cancer proliferation and can also aid in cancer treatment by improving the immune response, eliminating free radicals, and inhibiting cell division and inflammation [31].

The Neem water soluble bitters exhibited a lower IC50 value of ~0.09 mg/ml for MBA-MD-468 cells, indicating stronger inhibition compared to Neem water insoluble bitters with an IC50 value of ~0.4 mg/ml, while for MBA-MD-231 cells, the Neem water soluble bitters had a lower IC50 value of ~0.38 mg/ml compared to Neem water insoluble bitters with an IC50 value of ~0.55 mg/ml, suggesting stronger inhibition as well. The results of this study demonstrated that Neem bitters may have a potential anticancer activity against MDA-MB-468 and MDA-MB-231 cells. The present findings also suggest that Neem water-soluble bitters may be more effective in inhibiting the growth of MDA-MB-468 and MBA-MD-231 cells compared to Neem water insoluble bitters (Figure 6, 7). Further studies are warranted to understand the underlying mechanisms of action and to explore the potential therapeutic applications of these compounds.

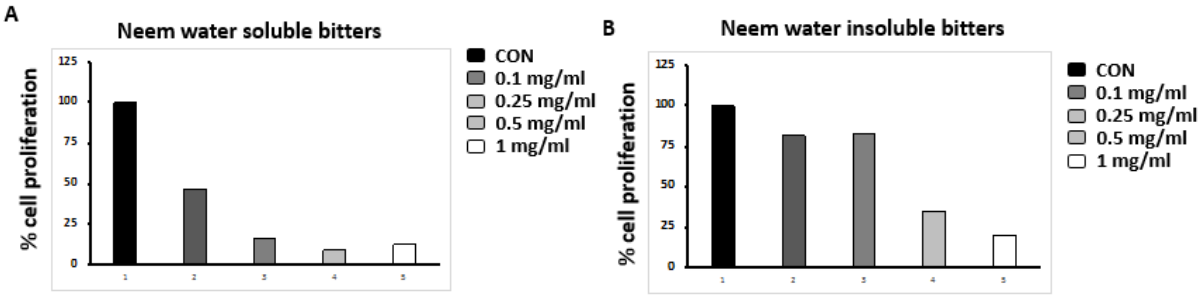

Figure 6: Graph illustrating the percentage inhibition of proliferation of MBA-MB-468 cells on treatment with the (A) Neem water soluble and (B) Neem water insoluble bitters.

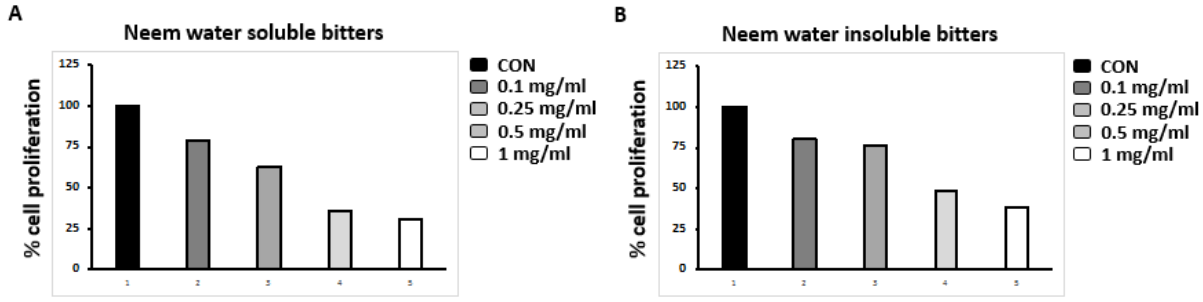

Figure 7: Graph illustrating the percentage inhibition of proliferation of MBA-MB-231 cells on treatment with the (A) Neem water soluble and (B) Neem water insoluble bitters.

2.9 Safety evaluation of Neem water-soluble bitters:

The present study provides a novel neem water-soluble bitter along with flavonoids wherein the said composition functions synergistically to provide a safe dosage for therapeutical usage. The novel neem water soluble bitters are several times safer when compared to the novel neem water insoluble bitters.

Neem water-soluble bitters and novel neem water-insoluble bitters are tested for toxicity against Kidney Epithelial cells (Hek293T) and Skin Fibroblast cells (HFF-1) using the MTT Cytotoxicity assay. An essential method for determining the safety of a drug is to test it for *in vitro* cytotoxicity. Cytotoxicity results from interfering with features or structures required for cell survival, growth, or function [32]. The assay was performed by using four different concentrations of each bitter ranging from 0.1 mg/ml to 1 mg/ml. Data was collected by using

a Tecan Multimode reader and plotted in a non-linear regression curve against the log concentration versus absorbance (Figures 8 and 9).

The results showed that both Neem water soluble and Neem water insoluble bitters exhibited a dose-dependent cytotoxic effect on the tested cells. The higher the concentration of the bitters, the greater the cytotoxicity observed.

The study demonstrated that both Neem water-soluble and water-insoluble bitters were safe and non-toxic to Hek293T and HFF-1 cells. The Neem water-soluble bitters had an IC50 value of ~1.95 mg/ml against Hek293T cells, which was significantly lower than the Neem water-insoluble bitters' IC50 value of ~0.85 mg/ml. Additionally, the Neem water-soluble bitters were found to be safe and non-toxic to HFF-1 cells, with an IC50 value of approximately 7.2 mg/ml, which was higher than the Neem water-insoluble bitters' IC50 value of ~6.8 mg/ml. These results suggest that the Neem water-soluble bitters may have stronger potential for applications in pharmaceutical and cosmetic products than the water-insoluble bitters.

These findings suggest that the bitters may have selective cytotoxicity towards cancer cells while being safe and non-toxic to normal cells. However, further studies are needed to confirm these results and to explore the underlying mechanisms of selectivity.

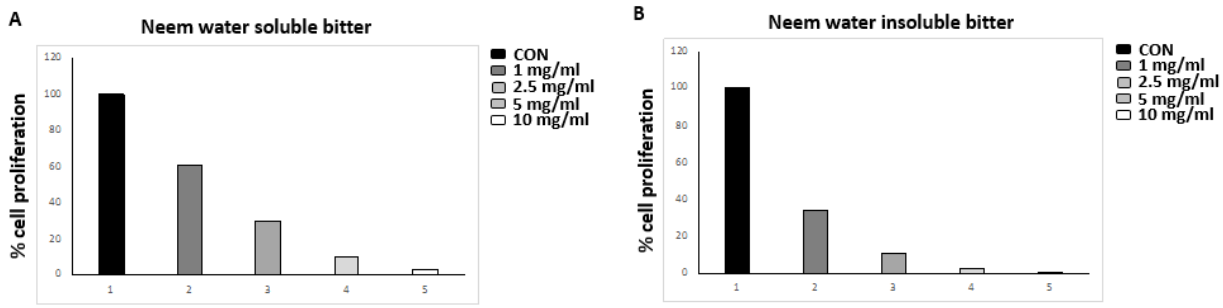

Figure 8: Graph illustrating the percentage inhibition of proliferation of Hek293T cells on treatment with the (A) Neem water soluble and (B) Neem water insoluble bitters.

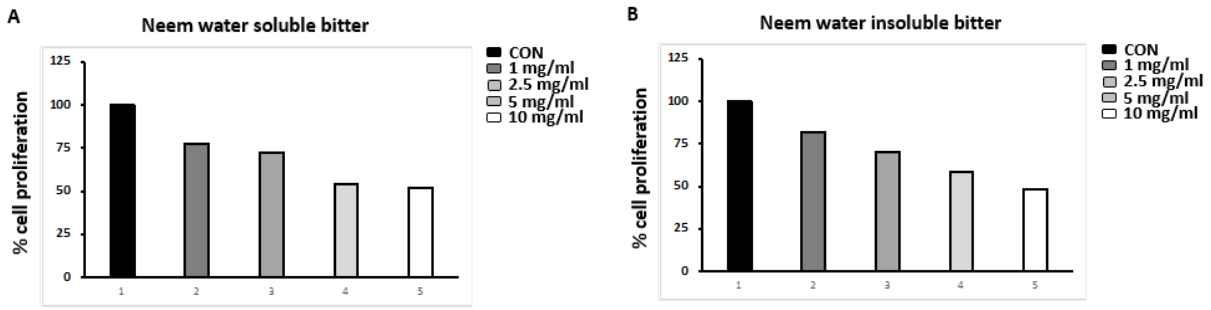

Figure 9: Graph illustrating the percentage inhibition of proliferation of HFF-1 cells on treatment with the (A) Neem water soluble and (B) Neem water insoluble bitters.

3 Conclusion

In conclusion, the present study provides a significant breakthrough in enhancing the solubilization of neem leaf bitter principles in water. The newly developed method using polyvinyl pyrrolidone and polyoxyethylene sorbitan monooleate has enabled the achievement of a previously unknown free aqueous solubility of bitters in water, thereby increasing the bioavailability and effectiveness of the extract. Additionally, the study has also focused on the therapeutic functions of neem and the biological activities of its water-soluble bitters, which have shown great promise in treating various health conditions.

Moving forward, future research can build upon these findings and explore the potential of neem leaf extract in developing new and effective treatments for various diseases. The increased solubility of bitter principles in water may open up new avenues for incorporating neem extract into various pharmaceutical, nutraceutical, and cosmetic products. Further research can also explore the use of neem extract in combination with other natural compounds to enhance its therapeutic potential.

In summary, the current study provides a solid foundation for future research to continue exploring the therapeutic potential of neem extract and its various applications in different fields. With ongoing research and development, the use of neem extract may become even more widespread, providing effective treatments for a wide range of health conditions.

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