Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

Isolation of Vitexin from *Vitex Negudo* of Assam and Development of Its Topical Formulation and Determine Acute Dermal Toxicity Study in Wistar Rats

Chinmoy Bhuyan[*1], Dipankar Saha[2], Bipul Rabha[3]

[1] Assam Science and Technology University, Jalukbari, Guwahati 781013, Assam, India.

[2] School of Pharmaceutical Science, Girijananda Chowdhury University, Guwahati 781 017, Assam, India.

[3]Department of Entomology, Defence Research Laboratory (DRDO), Tezpur, 784001, Assam, India

***(Received:** 16 September 2024 **Revised:** 11 October 2024 **Accepted:** 04 November 2024)*

KEYWORDS

Acute toxicity, Vitexin, Isolation, topical formulation, OECD

ABSTRACT:

Objective: To isolate vitexin from Vitex Negundo and development of its topical formulation and determine acute dermal toxicity study in wistar rats.

Methods: The flavonoid content of the leaves of Vitex negundo was determined by systematically extracting them using hexane, chloroform, ethyl acetate, methanol, and water using a soxhlet apparatus. After loading the methanol extract into the column chromatography, vitexin was separated, purified and identified by FTIR, NMR,DSC, MS and HPTLC. The purity of the vitexin was evaluated using HPLC. The isolated vitexin was incorporated to topical drug delivery vehicle cream and gel. The optimized formulation was evaluated physiochemically and acute dermal toxicity of optimized cream was determined as per OECD guideline (402) in three groups of wistar rats.

Results: Based on the findings, methanolic extract of Vitex negundo depicted the existence of a significant amount of flavonoids with 50-60% yield of vitexin. From the HPLC study's findings, vitexin derived from Vitex negundo leaves was demonstrated to be more pure than vitexin found in other sources. Vitexin was incorporated in topical drug delivery vehicle as cream. Vitexin cream exhibited acceptable stability, viscosity and physical properties. Topical application of vitexin cream (10%) was applied to rat skin to determine acute dermal toxicity.

Conclusions: 10% cream formulation of vitexin did not cause acute dermal toxicity to wistar rats. Results of the analysis indicate that isolated vitexin may be regarded as non- toxic and generally safe.

1. INTRODUCTION

A major factor driving the use of medicinal plants for a range of conditions, from acute to chronic, is the rising cost of western medications and the negative effects associated with long-term use of these medications [1]. An estimated 80% of the world's population uses plant-derived medications to treat a variety of illnesses [2]. Recently, the search for innovative therapeutic agents accounting for lead chemicals or medications to cure a wide range of human illnesses has also relied heavily on natural products. [3-5]. Although traditional herbal treatments are extensively employed, A few have undergone safety and efficacy testing [6-8]. Because

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

of this, during the last decades, there has been a significant increase in studies evaluating the pharmacological and toxicological profiles of medicinal plants, their extracts, and their formulations [9]. Many people utilize medicinal plants as supplementary and alternative remedies across the world. But a little is known about their toxicity. According to research, not all herbal medications are safe [10]. Findings have indicated that some traditional herbs might have adverse effects including diarrhea and skin necrosis [11]. The majority of natural products and herbal medications are now used for prolonged duration of time in the treatment of most diseases without regulatory enforcement, adequate dose monitoring, or consideration of any potential harmful consequences. Therefore, in order to continue using any herbal products regulatory toxicology is necessary [12].

Vitex negundo Linn (Verbenaceae) (VN) is a aromatic, woody shrub. It often has quadrangular branches with trifoliate or pentafoliate leaves, which produce purple-blue flowers. [13]. It has been shown to exist in wastelands, mixed open forests, eastern Africa, Madagascar, Pakistan, India, Sri Lanka, Thailand, and Malaysia. It grows best in moist environments or beside water channels [14]. In addition to being frequently used externally to alleviate rheumatism and joint inflammations, it is also said that VN's leaves and seeds have insecticidal properties. [15,16]. The hydroxy 3,6,7,3′,4′ pentamethoxyflavone, 6′-p-hydroxybenzoyl mussaenosidic acid, and 2′-p-hydroxybenzoyl mussaenosidic acid are all found in the leaves of V. negundo [17,15,18]. Due to the significance of using VN extract and oil topically in various formulations, we examined the acute dermal toxicity of VN extract (topical formulation) in wisrar rats in accordance with recommendations from the OECD (Organization for economic cooperation and development) guidelines. In this study, in comparison to previous reported vitexin yields, a higher grade vitexin was extracted, separated and purified with a better yield (Figure 1).

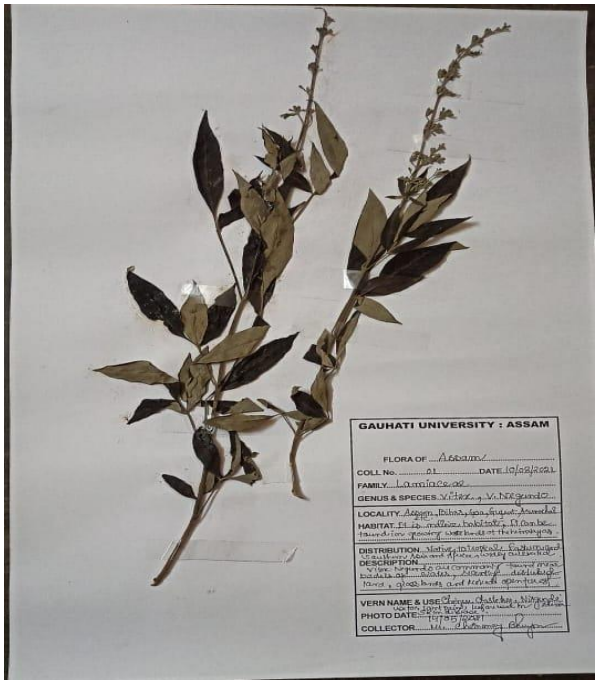


Figure 1. Leaves of *Vitex negundo* prepared in herbarium

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

2. MATERIALS AND METHODS

2.1. Drugs and Chemicals

Pure vitexin (95% purity) was purchased from sigma Aldrich, India. Different solvents including n-hexane, chloroform, ethyl acetate, and methanol were collected from yarrow chem. Mumbai. Stearic acid, cetyl alcohol, glycerin, carbopol were purchased from RANKEM India. Triethanolamine was bought from sigma Aldrich. Demineralized water was used, and all chemials were of the analytical grade.

2.2 Processing and extraction procedure for plant materials

Fresh *Vitex negundo* leaves (VN) were collected from different region of Assam, India (Location: 26°8᾽ North, 91°43᾽ East). Herbariums were prepared and the voucher specimens were identified and authenticated by Botanical survey of India, Shillong, Meghalaya (Ref. no.BSI/ERC/Tech/20-21/241). The plants were properly documented and the specimens were preserved in the dept. of pharmacognosy, GIPS, Azara, Guwahati for future reference. After collection leaves (*Vitex negundo*) were thoroughly cleaned, shed dried and ground into coarse powder. Using a Soxhlet apparatus, the uniformly ground powder material (1 kg) was successively extracted over a 72-hour period using solvents (2.5 L) of increasing polarity (hexane, chloroform, ethyl acetate, and methanol). The resulting extracts were filtered, concentrated under decreased pressure, and designated as methanol extract (VNME), ethyl acetate extract (VNEA), chloroform extract (VNCH), and hexane extract (VNHE).

2.3 Residual solvents in extract by gas chromatography

Gas chromatography (GC) using a Headspace system (clarus 690 perkin Elmer) was performed to the procured extracts to determine the residual solvent content. The sampling rate for the dosages volume was kept at 1250000 points/ second using the oven starting temperature at 40´ C for 2 minutes. Next, a GC run was performed with an offset of 100 µV (Ramp 1 at 5.0 deg/ minute to 100 deg/ min and hold for 1 minute, and Ramp 2 at 10.0 deg/ min to 150 deg/ min). After the extraction samples were placed into vials, the GC run was performed in compliance with the recommended guidelines.

2.4 *Phytochemical studies and physical evaluations of VN successive extract*

Physical parameters of the VN successive extracts

The extracts were assessed for a number of physical characteristics, such as nature of extract, percentage yield, and organoleptic characteristics. The results of pH and solubility tests were also obtained [19].

2.5 *Vitexin from VN: Phytochemical assessments and analytical procedure*

Preliminary phytochemical screening, thin layer chromatography and quantification of total flavonoid content

To find different classes of phytochemicals, a phytochemical screening of the VN consecutive extract was completed [20]. Using thin- layer chromatography, significant phytochemical groups that revealed positive test findings were again verified. TLC analysis was performed for extract high in vitexin using known procedure[21]. The process included developing the chromatogram, choosing the stationary phase, optimizing the mobile phase, preparing sample extracts for analysis, and spotting detection in compliance with pre determined standards (Fig.2). Pre-coated silica gel $60F_{254}$ aluminium sheets (Merck ltd) were used as the stationary phase in TLC, and a 8:1:1 combination of ethyl acetate, methanol and acetic acid made up the mobile phase. After that the TLC plates were analysed in visible lights, short UV light (254nm) and long UV light (366nm) both before and after the detecting reagent was applied. Furthermore, HPLC CAMAG equipment, which included the Linomat V sample applicator, CAMAG TLC visualizer, and WINCATS 4 software, was used to optimise High-Performance liquid Chromatography (HPLC), fingerprinting and analyse the vitexin-rich crude fraction following isolation via column chromatography.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

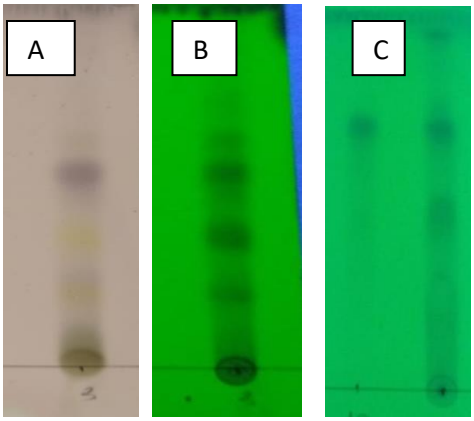


Figure 2. Phytochemical evaluation of VN extract and characterization of vitexin A. TLC chromatogram of VN Extract B. HPTLC fingerprint of isolated vitexin C. Pure isolated chromatogram of vitexin

Additionally, further study was carried out with a UV-Visible spectrophotometer (Shimadzu-AA630) to estimate the total amount of flavonoid based on the findings of the preliminary phytochemical screening and TLC study. The total flavonoid content was determined using colorimetric assay method with slight modification [22]. To prepare each consecutive extract at a concentration of 1 mg/mL, sampling was carried out. Quercetin (1 mg/mL stock solution) was used as the reference standard, and a series of dilutions in the concentration range of 0.02–0.10 mg/mL was performed. The total flavonoid content was estimated using the reported methods. Applying the linear regression equation, each estimation was done in triplicate and statistically represented as Mean±S.E.M

2.5.1 Isolation and characterization of vitexin from VN

Vitexin was found to be exist in significant amounts in the methanol extract (VNME) after analysis using TLC and HPTLC. As a result, a column chromatography technique was used in an attempt to separate vitexin from this extract. VNME (50gm) was absorbed in pure methanol and injected into the column using the wet packing procedure, which packed the column combining silica gel (#60-120 mesh, column grade, merck ltd.) A gradient mixture of hexane (C_6H_{14}) and ethyl acetate ($C_4H_8O_2$) was used for the elution procedure, with an increasing proportion of hexane in ethyl acetate. The gradient was divided into following levels 1%,2.5%,5%,7.5%,10%, 15%,20%,40%,60%,80% and 100%. In, 25 ml fractions, the eluates were collected. Corresponding eluates were pooled based on similar Rf values, and then concentrated using a rotary evaporator. 202 fractions were obtained from the process. Collect the 168 to 185 fractions of elution based on time were collected and tested by TLC and spot were detected by uv 254nm and Anisaldehyde-sulphoric acid. All the fraction mix and evaporate the solvent use rotovap evaporator. After evaporation of the solvent cool the flask add small amount of hexane to get the solid.

Advanced spectrometric methods such as FTIR (Bruker Alpha), ^{1}HNMR and ^{13}CNMR (Bruker avance Neo 500 MHz FT-NMR spectrometer), and mass spectrometer (Waters corporation MALDI – TOF SYNAPT XS HD mass spectrometer) were used to analyze and interpret the structure of the isolated vitexin [23].

2.5.2 Differential scanning calorimetry (DSC)

Using the DSC 3 instrument (Mettler Toledo; Model no. ME5140313), the melting point behaviour of the standard vitexin as well as the isolated and purified vitexin was studied. In an inert nitrogen gas environment, samples were placed in aluminium pans and scanned at a rate of 10 °C per minute between 20 °C and 300 °C. The endothermic peak of the isolated

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

vitexin and that of the standard vitexin were compared in order to verify the identification and purity of the isolated vitexin [24].

2.5.3 HPLC study to assess the extracted vitexin's purity

HPLC (Arc HPLC, Waters) was used to assess the purity of the separated vitexin from *Vitex negundo* extracts. The components of the HPLC system were a UV detector, an injector, and chromatographic pump. Using a C18 column in reverse phase was employed for HPLC separation. At a flow rate of 1 m/min, the mobile phase was made up of 80:20 v/v acetonitrile-acidic water (pH 3 adjusted by acetic acid). 425nm was used as the detecting wavelength and the run time was 10 minutes. (Figure 3)

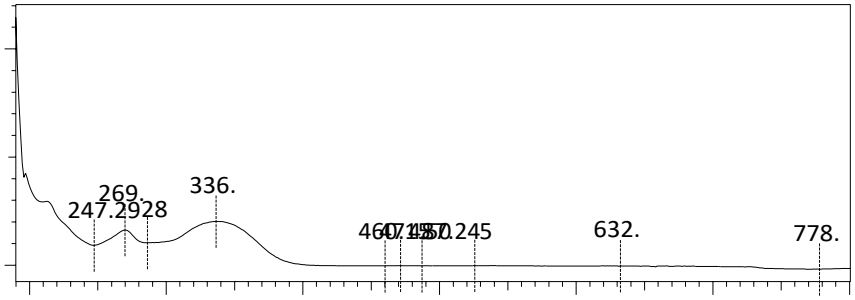


Figure 3. HPLC chromatogram of vitexin isolated from VN

2.5.4 Nuclear magnetic resonance (NMR)

Using a High Resolution Multinuclear FT-NMR Spectrometer (ECX400-Jeol 400 MHz, Japan), the chemical identification of the VNME extracted vitexin was again identified by proton NMR spectrum analysis. The samples were dissolved using dimethyl sulfoxide as a solvent (DMSO d6). As an internal reference, 20 µl of tetra methylsilane (TMS) was added to the sample solution. s (singlet) and d (doublet) were the spin multiplicities that were found. and the chemical shifts were reported in parts per million (ppm) in relation to the expression of tetramethylsilane (TMS) in δ units [25]. (Figure 4)

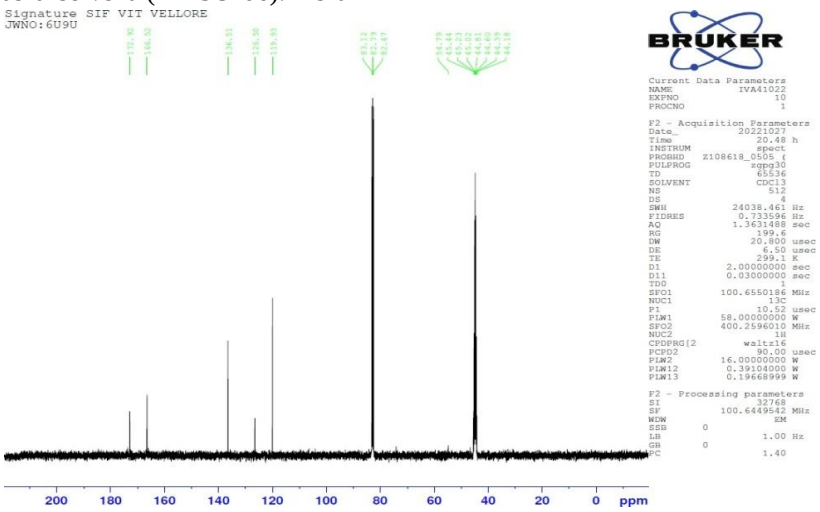


Figure 4. ^{13}C NMR of purified vitexin

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

2.5.5 Fourier-transform infrared spectroscopy (FTIR)

For structural identification of isolated VNME vitexin was identified by comparing its IR spectrum with that of the standard vitexin using an FT-IR spectrophotometer (Perkin Elmer Model: L1600400 Spectrum Two Ft-IR/DTGS). The samples were immediately scanned at 400–4000 cm−1 in a pan coated with KBr[24]. (Figure 5)

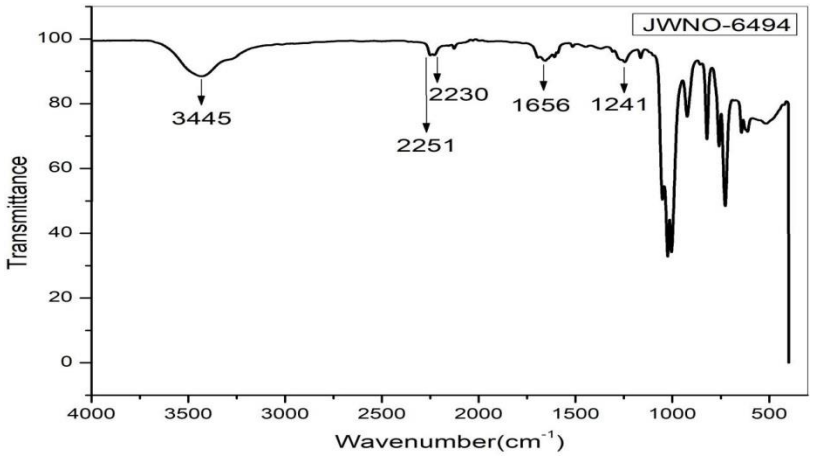


Figure 5. FTIR fingerprinting of purified vitexin

2.5.6 phase inversion temperature technique for cream preparation

The phase inversion temperature method was used to make an essential oil based *vitex negundo* cream. To ensure that there would be sufficient for both qualitative and quantitative assays, a 50 g cream sample was made. In a hot magnetic plate stirrer (Magnetic Stirrer IKA RCT basic), the oil phase was prepared by dissolving the oil soluble excipients and gently heating them to 65°C at 200 rpm. Various water soluble substances were combined with mild heating and stirring to produce the aqueous phase. At a stirring speed of 200 rpm and 55±2 °C, aqueous phase was gradually introduced to the oil phase. The prepared vitexin cream was there after stored for normal temperature for cooling [26].

2.6 Optimization and evaluation of cream

After that, the creams were made and their physicochemical characteristics were optimized by adjusting the polymer's content (0.2–0.5%) and the alkali (triethanolamine). Eg. P^H, Viscosity, Spreadability, Homogeneity, Accelerated stability studies etc.

P^H: Utilising a digital p^H metre (p^H system 361, Systonics), the p^H of the cream was determined. The p^H 4, 7, and 9 standard buffers were used to calibrate the pH meter initially. After weighing and dissolving around 0.5 g of the cream in 50.0 ml of distilled water, the p^H of the mixture was determined.

Viscosity: Using a Brookfield viscometer (DV-E Viscometer; Model No.-LVDVE, USA) with spindle number 7, the viscosity of the produced creams was measured at 30 rpm/min. prior to any readings, a standard glycerin solution was used to calibrate the viscometer.

Spreadability: Two sets of identically sized glass slides were employed to evaluate the creams' spreadability. The cream formulation was spread on one of the slides. When the other slide was placed over the cream, the cream was positioned between the two slides in an area that was 7.5 cm wide. A 100-g weight was placed between the upper slides to evenly press the cream between them into a thin

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

layer. After removing the weight, the extra cream that was adhering to the slides was scraped off. To ensure that only the higher slides could fall away from the force of the weight linked to them, the two slides that were in place were firmly fixed to a platform. The top slide has a 20 g weight fastened to it. The amount of time that the top slides needed to travel 7.5cm before separating from the lower slide due to the weight's force was recorded [27,28]. Equation for calculating spreadability was as follows:

$$S = m \times l/t$$

Where, S= Spreadability, m= Weight tied to upper slide , l= Length of the glass slide and t= time Taken in sec

Stability studies: The ICH recommendations were followed in the performance of the accelerated stability studies. The prepared creams stability was monitored for 30 days while it was stored at 4 °C, 25 °C, and 40 °C. The stability was noted in terms of, pH, viscosity, colour, and appearance [29].

2.7 Experimental Animals

Albino, Unisex Wistar rats weighing between 220±20 g were employed to determine the skin irritation and dermal toxicity. Animal experiment was approved by the Institutional animal ethical committee (IAEC), GCU, Guwahati. (Approval number:GCU/IAEC/Ph.D/PRO/04/2023). The animals employed in the experiment were kept in a regular laboratory setting with a 12-hour day and night cycle, normal room temperature of 25±1°C, and 45–55% relative humidity. They were given commercial pellets of standard quality and had unlimited access to water [30].

2.7.1 Skin irritation test

The skin irritation test was performed on ten male wistar albino rats, weighing between 250 and 300 g, in accordance with OECD standards 404 [31]. There were two groups of five animals each created out of the creatures. Five rats were utilised as control rats (Cr) and five more as test rats (Tr). After shaving the rats' back hair with a sterile razor until it reached the lower midpoint and measured about 20 cm in diameter, every rat was housed in an own cage. To 0.5 g of VNME, 0.2 ml of water was added, and the mixture was then equally applied to the test animals' shaved area. After applying gauze and a adhesive tape without irritation to the skin for one hour, the plant extracts were removed, the skin's surface was cleaned with purified water, and any skin irritation was examined. Additionally, the sites were observed 24 hours after application, 48 hours, and 72 hours later (Table 7). The control rats were received a topical application of sterile water (sterile cotton soaked in sterile water), which was attached with non irritating tape and gauze. The Draize grading method was used to rate the oedema and erythema that were observed (Table 6) [32]

2.7.2 Acute dermal toxicity study

This experiment was carried out in accordance with the OECD guidelines [33]. For this test, ten male wistar albino rats weighing between 250 and 300 g were employed. On the dorsal portions of the rats, a razor blade was used to shave around 10% of their total body surface. The animals were left alone in their clean cages for a full day. Each set of rats consisted of five; the testing group and the control group. According to OECD guideline 404, a limit test at one dosage level should be performed at 2000mg/kg body weight. Consequently, the test group rats' shaved portions were treated with VNME at a dosage of 2000 mg/kg after 24-hour period. Sterile water was applied topically to the control group. Gauze and non-irritating sticky tape were used to fix these. After 24 hours of application, the covers were gently removed, cleaned with purified water, and checked for erythema or oedema. Both the test and control groups received the application every day for 14 days, and daily observations were made of them for any changes in their clinical condition.

3. Results

3.1 phytochemical analyses and physical assessments of the Vitex Negundo consecutive extract

The physical characteristics and yield percentage of the subsequent *Vitex Negundo* extracts are listed in Table1. The quantification of total flavonoid content in the current study is shown in Table1 indicated that

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

the VNME extract (2.56±0.52 mg QE/g of dry weight) followed by VNEA, VNHE. Table2 indicates the findings of the phytochemical screening.

Table1. Physical properties, %yield and total flavonoid content of the successive VN Extract

Solvent	Nature & P^H	Colour & odour	Solubility	% of yield (%w/w)	Total flavonoid content(mg QE/g extract)
VNHE	Semisolid, P^H: 6.8	Green	Highly soluble in hexane,ethyl acetate	3.6%	0.87±0.62
VNCH	Semisolid, P^H: 7.2	Yellow	Highly chloroform-soluble only slightly soluble in methanol	3.1%	0.96±0.25
VNEA	Semisolid, P^H: 7.4	Green	High solubility in ethyl acetate and hexane	2.4%	1.54±0.68
VNME	Semisolid, P^H: 6.9	Brown	Soluble in methanol	8.2%	2.56±0.52

Table 2. Preliminary phytochemical screening of various successive extract of VN

Phytoconstituents	VNHE	VNCH	VNEA	VNME
Alkaloids	-	-	-	-
Glycosides	+	+	+	+
Flavonoids	+	+	+	+
Triterpenoids	+	+	+	+
Phenols/Tanins	-	-	-	-
Saponins	-	-	-	-
Sugar	-	-	-	-
Mucilage	-	-	-	-
Proteins	-	-	-	-
Amino acid	-	-	-	-
Fixed oil	+	+	+	+

3.2 Isolation and structural elucidation of vitexin from *Vitex Negundo*

Total 185 fractions are obtained from the VNME, by column chromatography. Fraction numbers 174-185 from TLC chromatogram revealed the existence of vitexin components. Considering the TLC profile and the existence of a significant dense region of vitexin with trace quantities of isovitexin, fraction 174-180 showed the greatest purity. Additionally this fraction (174-180) was purified by re-crystallization in ethanol as reported in the literature [34] producing yellowish crystals of vitexin. NMR, FT-IR, mass, and UV-visible spectrophotometers were used to characterize the lyophilized pure vitexin (Figure 6).

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

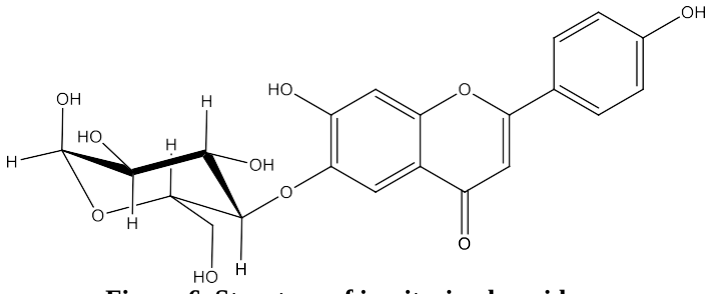


Figure 6. Structure of isovitexin glycoside

The compound's melting point was found to be 204°C and its uv-visible spectrum with λmax was measured at 335nm. IR bands (υ cm^{-1}) revealed that vitexin has strong functional groups at 3445 (O-H Stretching), 2251 (C=C band), 2230 (C=N Stretching), 1656 (Conjugated ketone or N-H bending), 1241 (C=O Carboxylic acid derivatives stretching). Distinct carbon peak (δppm) are represented in the ^{13}C NMR spectrum (125MHz, DMSO) at 54-44 (Glycoside ring carbon), 136-119.9 (Aromatic ring carbon),166.52 (=C-O).172.9 (Ring C=O). Chemical shift of ^{13}C NMR with δ value at 44-54 indicate that carbon atom corresponding to glycoside ring, moreover, δ value at 172.9 indicate the presence of C=O group. Additionally, the mass spectra (TOF–MS-ES+) showed the detection of a molecular ion peak [M+ H]+ at 757.49 and the base peak at m/z 192.13. These outcomes, which are showed in Fig. 5, are accordance with the information documented in the literature and so indicate the identification of vitexin extracted from *Vitex Negundo* [24,34]. (Figure 7)

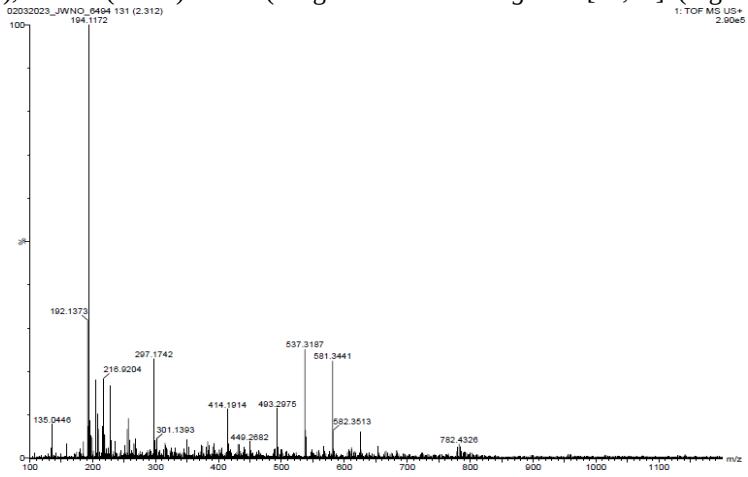


Figure 7. Mass spectra of purified vitexin

3.3 Formulation of vitexin loaded cream

Using the phase inversion temperature method vitexin loaded cream were prepared. Phase inversion temperature method is simple, quick, and easy way to prepare cream formulation shown in Table 3

3.4 Optimization and evaluation of cream

Physicochemical characteristics of vitexin cream such as P^H, viscosity, spreadability were studied in relation to the concentration of vitexin, stearic acid and alkali (triethanolamine). The optimized cream was homogeneous, smooth, transparent, clear and had high spreadability and consistency. The cream

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

exhibited a desirable consistency for topical administration that was acceptable with patients, with a viscosity of 18,480±241 cp. Findings indicated that the spreadability was 20.08±1.2 g/cm^2. The cream exhibited a satisfactory consistency and a slightly acidic P^H of 6.60±0.2, indicating that they are usable topically without risk as shown in Table 4

Table 3. Formula for 50g VN cream

Cream Bases	Ingredients	Quantity (w/w)	Use
	Phase A		
	VN Oil	2-4gm	Protection against microbial infection
Oil Phase	Phase B		
	Stearic Acid	3-6gm	Emulsifying agent
	Cetyl Alcohol	2-5gm	Thickening agent
	Liquid Parafin	2-4gm	Stabilizer
	Triethanolamine	1-3gm	P^H Adjusting agent
	Glycerine	5-8gm	Emolliant
	Sodium Benzoate	0.02-0.06gm	Preservative
	Methyl Paraben	0.1-0.4gm	Preservative
	Vitexin	0.3-0.5gm	Antiinflammatory,antioxidant
Aqueous Phase	Phase C		
	Polyethylene glycol	3-5gm	Humectant
	Tween 20	0.5-1.5gm	Surfactant
	Demineralised water	Q.S 50gm	Solvent

Table 4. Evaluation of optimized VN loaded cream

Formulation	Physical Appearance	Viscosity cp±SD	P^H±SD	Spreadability g/cm^2±SD
F3	Homogeneous and smooth	18,480±241cp	6.60±0.2	20.08±1.2

3.5 Accelerated stability study

According to ICH guideline, stability study of optimized formulation were carried out for 30 days. The stability study showed that optimized formulation had acceptable stability behavior in terms of P^H, Physical appearance, viscosity,

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

spreadability as shown in Table 5. At room temperature 4°C and 40°C no specific alteration in the cream characteristics are noted.

3.6 Toxicity studies

After three days of testing, neither the test nor the control animals revealed any indication of erythema or oedema in the skin irritation test. No significant ($P > 0.05$) clinical change was seen in any of the treated rat groups when testing the dermal toxic effect of vitexin loaded cream in the acute dermal toxicity bioassay, with the exception of the rats' initial response within the first 30 minutes of patch attachment when they attempted to rip the patch off. After one hour and twelve hours following the administration of test drugs, we observed the rats' general look in both the test and control groups. . When the skin was shaved and test compounds were applied, no change indicated that the animals' post-acclimatization behavior remained unchanged. For the duration of 14 day study period, neither the control nor the vitexin treated animals shown any indication of erythema or oedema.

Over the course of the study, the rats' observations revealed no toxic effect, which helped to determine the dermal toxicity study of vitexin loaded cream in the repeated dosage dermal toxicity bioassay. Both the control and vitexin treated rats did not exhibit any erythema or oedema over the 21-day trial period, despite the rats' attempts to remove the patch during the first 30 minutes after it was attached. For a repeated dose toxicity test, the maximum limit dosage

Table 5. Accelerated stability testing of optimized VN cream

Days	Temp.	Formulation	Parameters						
			p^H	A1	A2	A3	A4	A5	A6
0	Room Temp.	F3	6.48	**	NCC	**	E	NG	ES
0	(40±1)°C	F3	6.52	**	NCC	**	E	NG	ES
10	Room Temp.	F3	6.55	**	NCC	**	E	NG	ES
10	(40±1)°C	F3	6.58	**	NCC	**	E	NG	ES
15	Room Temp.	F3	6.55	**	NCC	**	E	NG	ES
15	(40±1)°C	F3	6.60	**	NCC	**	E	NG	ES
30	Room Temp.	F3	6.54	**	NCC	**	E	NG	ES
30	(40±1)°C	F3	6.55	**	NCC	**	E	NG	ES
45	Room Temp.	F3	6.89	**	NCC	**	E	NG	ES
45	(40±1)°C	F3	6.76	**	NCC	**	E	NG	ES
60	Room Temp.	F3	6.45	**	NCC	**	E	NG	ES
60	40±1)°C	F3	6.54	**	NCC	**	E	NG	ES
75	Room	F3	6.47	**	NCC	**	E	NG	ES

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

	Temp.								
75	40±1)°C	F3	6.35	**	NCC	**	E	NG	ES
90	Room Temp.	F3	6.32	**	NCC	**	E	NG	ES
90	40±1)°C	F3	6.27	**	NCC	**	E	NG	ES

A1: Homogeneity, A2: Appearance, A3: Spreadability, A4: After feel, A5: Type of smear, A6: Removal, **: Good, *: Satisfactory, E: Emollient, NG: Non greasy, ES: Easy, NCC: Not change in colour

should not be more than 2000 mg/kg. An additional two dosage levels, 500 mg/kg and 1000 mg/kg, were proportionately determined. No rats died over the course of the investigation.

Table 6. Draize dermal grading method

Score	Translation
0	No erythema or oedema
1	Very inappreciable oedema or erythema
2	Small oedema with raised skin at the edges of the area
3	Moderate to severe erythema or oedema
4	Severe erythema or oedema

Table 7. Erythema and oedema score after application of VN cream

Reaction	1hr		24hr		48hr		72hr		7th day		14th day	
	Cl	trt	Cl	trt	Cl	trt	Cl	trt	Cl	trt	Cl	trt
Erythema	0	0	0	0	0	0	0	0	0	0	0	0
Oedema	0	0	0	0	0	0	0	0	0	0	0	0

Cl – Control groups of animals, trt- treatment groups of animals

4. DISCUSSION

Vitex negundo contains a large amount of vitexin, which have several biological functions. Particularly clinical and pre clinical study have demonstrated the broad range of therapeutic benefit of vitexin. The biological properties of vitexin analogues have recently been demonstrated, isovitexin has anti-inflammatory and anti-oxidant properties on lipopolysaccharides induced acute lung injury by inhibiting MAPK and NF-κB[35]. Thus, in order to investigate the biological properties of vitexin, it is necessary to isolate them with a high degree of purity. The current study investigated the procedure of isolation and purification of vitexin from *Vitex negundo* of Assam and formulation of topical cream and determination of acute dermal toxicity study in experimental animals. Our data showed that VNME extract had the highest percentage yield among the Vitex negundo consecutive extract. During the phytochemical screening of the VNME, the density of colour change suggested the existence of a considerable quantity of flavonoid, phenolic, tannins and steroids. The results we obtained are in line with other studies [34,35] in which the samples of *Vitex negundo* collected worldwide demonstrated similar and comparable phytochemical profiles. According to the findings of Gas Chromatography investigations, showed that the extract has no hazardous solvent residue. The total residual solvent contained in all subsequent extracts was determined to be very

Journal of Chemical Health Risks

www.jchr.org

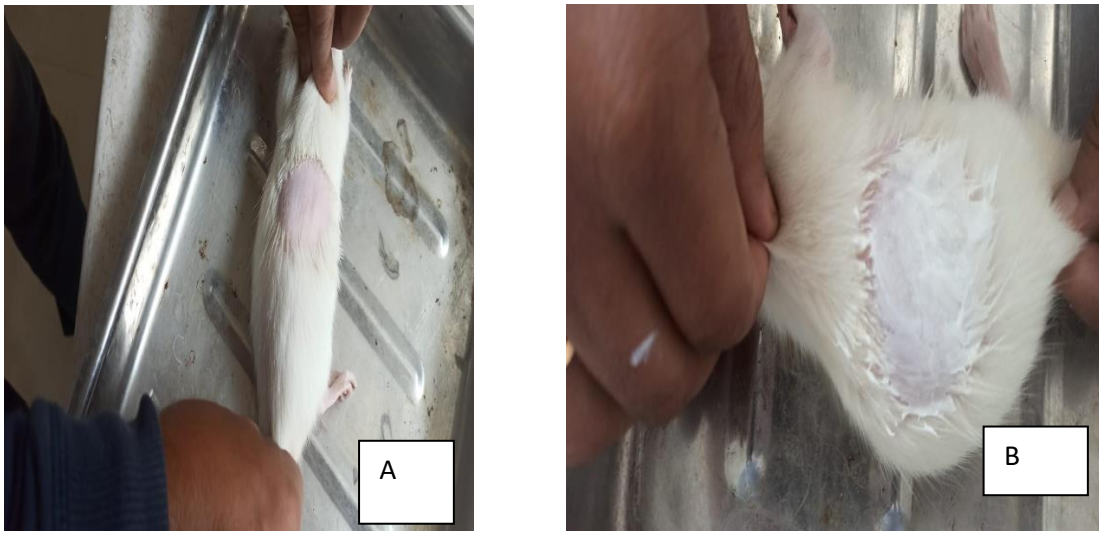
JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

minimal. Research has shown that the presence of residual solvent in finished herbal medical products over safety limits may have harmful effects such as neurotoxicity, teratogenicity, and carcinogenicity [36]. According to the quantitative estimation, the extract from VNME had the highest total flavonoid content (2.56±0.52 mg QE/g of dry weight extract) considering other subsequent extract. It is speculated that vitexin and other flavonoids components contribute to *Vitex negundo* high flavonoid content.

The isolated vitexin *Vitex Negundo* based cream was prepared using the phase inversion temperature technique. The vitexin cream was optimized by examining the effects of alkali (Triethanolamine) and concentration of polymer. Moreover P^H, viscosity, spreadability, appearance also plays a major role. It was found that optimized vitexin cream was homogeneous, smooth, transparent, clear and had good spreadability and consistency. The produced cream's qualities were determined to be safe and appropriate for topical administration, specifically in the context of dermal toxicity.

In skin irritation, localised inflammation results from direct skin damage and manifests as erythema and oedema [37]. This type of direct skin damage can result from a single, recurring, or extended chemical substance interaction with the skin [38]. The results of the skin irritation test suggested that vitexin was not irritating because the test group did not exhibit erythema or oedema for the whole duration of the trial. All of the erythema and oedema scores were zero (0).

The OECD guidelines were followed in the performance of the acute dermal toxicity study. There was no significant clinical change in the animals. The animals under test and those under control behaved similarly. Throughout the whole study period, no erythema or oedema was seen. (Figure 8)



Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

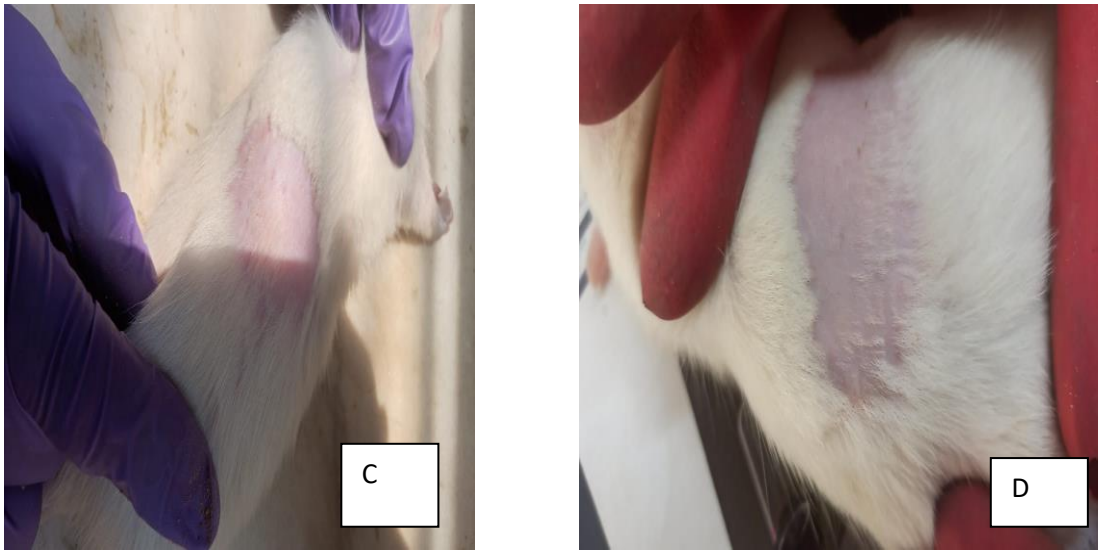


Figure 8. Dermal toxicity study of wistar albino rats

A: Removal of skin surface at 0 days, **B:** Application of VN cream in skin surface of rats **C: Day-7:** No erythema and oedema were observed, **D: Day-14**: No skin irritation erythema, oedema were seen at the last day of animal experiment

5. CONCLUSION

The result of the study concluded that the *Vitex negundo* has high vitexin content and that its methanolic extract yields a greater amount of pure vitexin with significant anti-inflammatory activity. Isolated vitexin was incorporated as topical drug delivery vehicle. By using phase inversion temperature method vitexin cream was prepared with suitable polymers. This vitexin cream showed that there will be no significant dermal toxicity in experimental animals. Due to *Vitex negundo* has a high and pure vitexin content, it may be further investigated. Results of the analysis indicate that isolated vitexin may be regarded as non- toxic and generally safe. Additionally appropriate nano formulation might be developed for improved therapeutic use of vitexin.

Acknowledgement

Ministry of science and technology, Department of science and technology (DST), Government of India, New Delhi, is gratefully acknowledged. (INSPIRE Scheme No. DST/INSPIRE Fellowship/2019/IF190482). Authors are thankful to school of pharmaceutical science, Girijananda Chowdhury university and Assam science and technology university for providing necessary research facility.

References

1. Leonti M, Casu L.,2013. Traditional medicines and globalization: current and future perspectives in ethnopharmacology. Front. Pharmacol.(4) 92.

2. World Health Organization WHO, WHO Launches the First Global Strategy on Traditional and Alternative Medicine. [Cited 2016 20 July]. Available from: 2002.

3. Engel N, Falodun A, Kuhn J, Kragl U, Langer P, Nebe B.,2014. Pro-apoptotic and anti adhesive effects of four African plant extracts on the breast cancer cell line MCF-7 BMC Complement. Altern. Med, (14) 334.

4. Abu N, Akhtar MN, Yeap SK, Lim KL, Ho WY, Zulfadli AJ.,2014. Alitheen, Flavokawain A induces

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 740-755 | ISSN:2251-6727

apoptosis in MCF-7 and MDA-MB231 and inhibits the metastatic process in vitro. PLoS One, 4.

5. Hossen MN, Ali MY, Jahurul MH, Daim MM, Gan SH, Khalil MI.,2017. Beneficial roles of honey polyphenols against some human degenerative diseases: a review. Pharmacol. Rep, 69: 1194–1205.

6. Bae JW, Kim DH, Lee WW, Kim HY, Son CG.,2015. Characterizing the human equivalent dose of herbal medicines in animal toxicity studies. J. Ethnopharmacol,162: 1–6.

7. Costa C, Tsatsakis A, Mamoulakis C, Teodoro M, Briguglio G, Caruso E.,2017. Current evidence on the effect of dietary polyphenols intake on chronic diseases. Food Chem. Toxicol.,110: 286–299.

8. Seremet OC, Olaru OT, Gutu CM, Nitulescu GM, Ilie M, Negres S.,2018.Toxicity of plant extracts containing pyrrolizidine alkaloids using alternative invertebrate models. Mol. Med. Rep,17: 7757–7763.

9. Dicson SM, Samuthirapandi M, Govindaraju A, Kasi PD.,2015. Evaluation of in vitro and in vivo safety profile of the Indian traditional medicinal plant *Grewia tiliaefolia*. Regul. Toxicol. Pharmacol;73: 241–247.

10. Wannissorn B, Jarikasem S, Siriwangchai T, Thubthimthed S.,2005.Antibacterial properties of essential oils from Thai medicinal plants, Fitoterapia;76:233–236.

11. Limenih Y, Umer S, Wolde-Mariam M.,2015. Ethnobotanical study on traditional medicinal plants in Dega Damot woreda, Amhara Region, North Ethiopia. Int. J. Res. Pharm. Chem.;5: 258–273

12. Viyda ADB, Devasagayam TPA.,2007. Current status of herbal drugs in India: an overview. J Clin Biochem Nutr 41:1–11.

13. Chattopadhyay P, Hazarika S, Dhiman S.,2012. Vitex negundo inhibits cyclooxygenase-2 inflammatory cytokine-mediated inflammation on carrageenan-induced rat hind paw edema. Pharmaconosy Res, 4: 134–137.

14. Jabeen A, Khan M, Ahmad M, Zafar M, Ahmad F.,1999. Indigenous uses of economically important flora of Margallah Hills National Park Islamabad Pakistan. Afr J Biotech, 8: 763-84.

15. Sehgal CK, Taneja SC, Dhar KL, Atal CK.,1982. 20 -p-hydroxybenzoyl mussaenosidic acid a new iridoid glucoside from Vitex negundo. Phytochem, 21: 363–366.

16. Vishwanathan AS, Basavaraju R.,2010. A review on Vitex negundo L. – a medicinally important plant. Eur J Behav Sci, 3:30–42.

17. Banerji A, Chadha MS, Malshet VG.,1969. Isolation of 5-hydroxy-36- 73'4'-pentamethoxyflavone from Vitex negundo. Phytochem, 8:511-2.

18. Sehgal CK, Taneja SC, Dhar KL, Atal CK.,1983. '6'-p-hydroxybenzoyl mussaenosidic acid an iridoid glucoside from Vitex negundo. Phytochemi.;22:1036-8.

19. Laloo D, TahbildarR, SmithK, Ahmed A, Nath J, Shil D.,2020. Impact of quality control standardization parameters and antioxidant potential of the aerial parts of potentilla fulgens wall: a comprehensive monographic study.J Biol Act Prod Nat.10 (4) 338-356.

20. Trease GE, Evans WC. Pharmacognosy.,2002.Saunders Company London.

21. Wagne H, Bladt R, Zgainsk EM.,1948. Plant Drug Analysis: A Thin Layer Chromatography Atlas 2 Springer-Verlag Berlin Heidelberg. New York.

22. Shi JY, Zou XB, Zhao JW.,2012. Determination of total flavonoids content in fresh Ginkgo biloba leaf with different colors using near infrared spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc. 94: 271–276.

23. Cordenonsi LM, Sponchiado RM, Campanharo SC,Garcia CV, Rafn RP.,2017.Study of favonoids present in pomelo (*Citrus maxima*) by DSC, UV-vis, IR, ^{1}H and ^{13}C NMR and MS, Drug Anal Res. (1) 31-37.

24. Yoksan R, Noivoil L.,2023 Fabrication, characterization and in vitro release of vitexin-loaded chitosan nanoparticles. J Polym Environ;4:1557-1572.

25. Bharali A, Sarma H, Biswas N, Kalita JM, Das B, Sahu BP.,2023. Green synthesis of silver nanoparticles using hydroalcoholic root extract of Potentilla fulgens and evaluation of its cutaneous wound healing potential. Mater Today Commun. 35:106.

26. Hazarika H, Krishnatreyya H, Tyagi V, Islam J, Gogoi N.,2022 The fabrication and assessment of mosquito repellent cream for outdoor protection. Sci. Rep. 12:2180.

27. Aiyalu R, Govindarjan A, Ramasamy A.,2017.Formulation and evaluation of topical herbal gel for the treatment of arthritis in animal model. Braz J Pharm Sci.52:493-507.

28. Harish NM, Prabhu P, Charyulu RN, Gulzar MA, Subrahmanyam EV.,2009. Formulation and evaluation of in situ gels containing clotrimazole for oral candidiasis. Indian J Pharm Sci. 4: 421-427.

29. Ubaid M, Ilyas S, Mir S, Khan AK, Rashid R, Khan MZ.,2016. Formulation and in vitro evaluation of carbopol 934-based modifed clotrimazole gel for topical application. An Acad Bras Cienc; 88(4)2304 - 2317.

30. Savenije B, Strubbe J, Ritshes-Hoitinga M.,2005. Nutrition, feeding and animal welfare. The Care and Management of Laboratory and Other Research Animals. 183–193.

31. OECD, Acute dermal irritation/corrosion, OECD Guidelines For Testing of Chemicals,No.404, 1981,Organization for Economic Cooperation and Development, Paris, France.

32. Draize JH.,1959. The Appraisal of Chemicals in Foods, Drugs and Cosmetics. The Association of Food and Drug Officials of the United States, Austin, TE, USA, 46-48.

33.Hefernan C, Ukrainczyk M, Gamidi RK, Hodnett BK, Rasmuson AC.,2017. Extraction and purifcation of curcuminoids from crude curcumin by a combination of crystallization and chromatography.Org Process Res Dev. 21 (6) 821-826.

34. Thenmozi S, Subasini U.,2016. Isolation, characterization and in vitro cytoxicity study of vitexin from *vitex pinnata* linn. Leaves. Int J Res Pharmacol pharmacotherapy.1:84-89.

35. Hongming LV, Zhenxiang YU, Yuwei Z, Lidong W, Xiaofeng Q.,2016. Isovitexin Exerts Anti-Inflammatory and Anti-Oxidant Activities on Lipopolysaccharide-Induced Acute Lung Injury by Inhibiting MAPK and NF-κB and Activating HO-1/Nrf2 Pathway. Int.J.Biol.Sci.12(1):72-86.

36. Opuni S, Nkansah P, Fosu A, Akonnor SO, Bekoe AN.,2021. Monitoring and risk assessment of pesticide residues in selected herbal medicinal products in Ghana. Environ Monit Assess.193(8)470.

37. Alavi A, Sibbald RG, Ladizinski BS.,2016.Wound-related allergic/irritant contact dermatitis. Adv Wound Care. 29(6) 278–286.

38. Nyigo VA, Mdegela R, Mabiki F, Malebo HM.,2015 Assessment of dermal irritation and acute toxicity potential of extracts from Synadenium glaucescens on healthy rabbits, wistar albino rats and albino mice. European J Med Plants. 10(4)1–11.