International Journal of Phytomedicine 2 (2010) 240-254

http://www.arjournals.org/ijop.html

Research Article

ISSN: 0975-0185

Dermal irritation and sensitization study of *Euphorbia neriifolia* latex and its anti-inflammatory efficacy

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Abstract

The presence of polycyclic diterpene esters in *Euphorbia* plants sap makes it highly irritant and notably corrosive causing burning pain of skin. The toxic nature of the *Euphorbia* is discouraging their uses despite the possible manifold therapeutic potentials. *Euphorbia neriifolia* (Euphorbiaceae) commonly known as Thuar is a sacculant shrub commonly and abundantly found as hedge plant in Central India. This study was designed to screen the physiochemical properties of latex and dermal irritation potential of its different solvent fractions targeting to retain the triterpene content high to extract therapeutic utilization. Physico-chemical, qualitative and quantitative phytochemical, dermal irritation and sensitization profile and topical anti-inflammatory activity of *E. neriifolia* latex were studied. Fresh latex contains 10.95 % solid with 18.32 % total resinous matter, and 24.50 % and 16.23 % of total diterpene and triterpene respectively. Petroleum ether fractionation showed 63.80 % yield with rich presence of steroid and triterpenoid. Pet. ether fraction was found to be apparently nonirritating with a PII score of 0.43/0.11 for erythema and edema according to Draize Dermal Classification System. Chloroform, acetone and water fractions are skin irritating due to presence of high diterpene content where as pet. ether fraction is rich in triterpene showing nonirritant activity. Topically latex pet. ether fraction at 750 and 500 mg/ml dose showed 42.40 and 35.25 % inhibition of carrageenan induced paw edema. Anti-inflammatory activity of latex pet. ether fraction is due to presence of triterpenes euphol, nerifoliol and cycloartenol. This study explores safe topical use profile of *E. neriifolia* latex retaining its anti-inflammatory efficacy.

Key words: *Euphorbia neriifolia*, Latex, Dermal irritation, Anti-inflammatory, Triterpenes, Euphol

Introduction

Euphorbia is one of the most diverse genera in the plant kingdom belonging to the family Euphorbiaceae. The *Euphorbias* are named after a Greek surgeon called Euphorbus. He was physician of Juba II who was the Romanised king of a North African kingdom and is supposed to

doi:10.5138/ijpm.2010.0975.0185.02035

have used *Euphorbia* milky latex as an ingredient for his potions. *Euphorbias* are annual or perennial herbs, woody shrubs or trees with a caustic and poisonous milky sap (latex). Members of this family and genus are sometimes referred as *Spurges*. Spurges are large and variable worldwide, genus of more than 2,000 plants that include small trees, shrubs, vines, herbaceous plants and succulents. Usually latex is white, but in rare cases (e.g. *E. abdelkuri*) yellow also. As it is under pressure, it runs out from the slightest wound and congeals within a few minutes on contact with the air. The presence of polycyclic diterpene esters in *Euphorbia* plants sap makes it highly irritant, which is a deterrent to insects and herbivores [1]. The milky sap of many *Euphorbias* are notably corrosive, on contact with skin may cause temporary burning pain or permanent blindness on contact with eyes. Swallowing of the sap is potentially fatal causing severe inflammation of the walls of the stomach and intestine with perforation in some cases. The active component of *Euphorbia* latex is euphorbon, a cytotoxic protein.

Euphorbia plants are used as raw material for rubber, castor oil and tapioca [2]. Traditionally *Euphorbias* are used as purgative, analgesic, anti-inflammatory, antipyretic, antimicrobial, antiparasitic, in the treatment of cough, asthma, rheumatism, cancer and other maladies as folk remedy [2,3,4]. Latex of some *Euphorbia* species is traditionally used in the treatment of skin diseases, gonorrhea, migraines, intestinal parasites and warts [5]. *Euphorbia* latex and its isolated compounds have molluscicidal, pesticidal, cytotoxic, mitogenic [6], anti-inflammatory and anti-arthritic activity [7]. Terpenes including diterpenes and triterpenes, steroids, cerebrosides, glycerols, phenolics and flavonoids have been frequently found in *Euphorbia* species. The compounds most relevant to the toxicity and considerable biological activities in *Euphorbias* are diterpenes, especially those with abietane, tigliane, and ingenane skeletons [8]. Latex contains diterpene esters of the phorbol, ingenol and 12-deoxyphorbol esters known to be highly active cocarcinogenic and tumor promoting agents [9,10].

Euphorbia neriifolia commonly known as Thuar is a large glabrous erect much branched sacculant shrub or small tree 1.8 - 4.5 meter high. Straight saccular branches occur with a pair of strong stipular spine on tubercles or swelling of the branchlet. These tubercles are more or less confluent in 5 vertical or slightly spinal lines on ribs. The branches are more or less obtusely 5-gonous in section. Bunches of succulent thick leaves occur terminal on the branchlets. The trunk is covered with reticulate bark. Leaves are short living, fleshy, alternate, glabrous, deciduous, nearly sessile, obovate, oblong 6 to 12 cm in length, base tapering. Involucres yellowish, 3-7 in a cyme, usually 3-nate, with a very short fleshy peduncle about 3.8 mm long. In the involucre, the lateral ones pedicelled and bisexual, the central flowers usually male and sessile. Flowering and fruiting occurs during December to May [11].

In Ayurveda whole plant, leaf and roots are used in treatment of abdominal troubles, bronchitis, tumors, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers, fever, bleeding piles and in cough and cold. The milky latex is used as a purgative, expectorant, rubefacient, aphrodisiac and in earache. The latex is pungent, laxative, good for abdominal troubles, tumors and leucoderma. It is liable to cause dermatitis. It is used to remove warts and cutaneous eruptions. The juice of the leaves is a popular cure for earache in the Philipine Islands [12].

The tribal population of Chattishgarh region uses the milky latex as an ingredient of aphrodisiac mixture. The juice of the plant is used in Gujarat for smearing cuts made by tapers on *Borassus flabellifer* (Linn) in order to prevent the palm from the attack of red weevil. Stem or leaf juice is used in case of cough and cold mixed with honey. A succus consisting of equal parts of the juice of this plant and simple syrup was prepared and administered in doses of 10 to 20 minims three times a day in case of asthma and was

found to give relief from the fits of the diseases [13].

E. neriifolia latex is one of the constituents of "Kshaarasootra", which is used in Indian medicine to heal anal-fistula. "Kshaarasootra" is prepared by smearing a surgical linen thread with fresh latex of snuhi or *E. neriifolia* and a specially prepared alkaline powder (kshaara) from plant *Achyranthes aspera* and turmeric powder from the dried rhizomes of *Curcuma longa*. A multicentric randomized controlled trial carried out by Indian Council of Medical Research revealed that the long term out come with "Kshaarasootra" was better than with the surgery offering an effective, ambulatory and safe treatment for patients with fistula-in-ano [14].

The latex of *E. neriifolia* is used for arthritis in Tibbe-e-Unani (Unani Medicine) as Sheer-e-Zaqqoom. Amin et al. [15] reported its oral efficacy and safety on adjuvant arthritis. In a 14-day repeated dose subacute toxicity study, the drug showed to possess striking anti-arthtitic activity. It's ED_{50} and LD_{50} was found to be 8.37 and 630 mg/100gm respectively. At a dose close to its ED_{50}, it produced mild gastric irritation and slight depression of blood glucose and elevation of biochemical markers of liver and kidney function without crossing the normal range and minimal histological changes in the two organs in the subacute toxicity study. Water soluble fraction of *E. neriifolia* latex was evaluated for wound healing activity in guinea pig. *E. neriifolia* latex showed increase in collagen and DNA content improving the tensile strength. It also showed increased epithelization and angiogenesis indicating potential wound healing property [16]. Ilyas et al. [7] reported anti-inflammatory and antiarthritic activity of a novel triterpene (Nerifolione) isolated from the latex of *E. neriifolia* along with total extract of latex in acetone.

Ponsinet and Ourisson [17] examined latex of 75 *Euphorbia* species for triterpene constituents. The groups constituted by chemical criteria can be correlated with sections and sub-sections and particularly with morphological, morphogenetical and geographical characteristics. Fifteen tetra and penta cyclic triterpenes have been identified by TLC and GLC. Euphol was reported in petroleum ether (pet. ether) extract of lyophilized *E. neriifolia* latex along with other plants of *Euphorbia* species like *E. antiquorum* L., *E. canariensis* L., *E. Hernandez-pachecoi* Cab, *E. handiensis* Burch., *E. ingens* E Mey., *E. resinifera* Bioss., *E. tirucalli* L. and *E. triangularis* Desf. Euphol and nerifoliol along with cycloartenol and nerifolione were reported in dried latex of *E. neriifolia* [18]. A new tetracyclic triterpene 9, 19-cyclolanost- 22(22'), 24-diene-3beta-ol, named as nerifoliene along with euphol were isolated from the fresh latex of *E. neriifolia* [19].

The literature review reveals the traditional and scientifically proved multipurpose use of *E. neriifolia* latex. Topical use potential as rubefacient, counterirritant, skin wart derooting and analgesic is higher as orally it causes purgation and mild stomach mucosal irritation with elevation of kidney and liver biochemical markers [7]. Literature survey and folkfore use of latex shows that it is mostly applied topically. Latex is also reported to have good antiarthritic potential with woundhealing activity. These findings have directed us to explore the topical use potentialities of *E. neriifolia* latex. The latex contains di and triterpenes, out of which triterpenes are of more medicinal value and diterpenes causes skin irritation [1]. As the latex is liable to cause dermatitis and skin irritation it is important to study the detailed aspect of latex effect on the skin along with its possible therapeutic implications. Since it causes irritation to skin, the first challenge for topical formulation development is to reduce its irritant effect without compromising the medicinal value of latex. In spite of being the use of latex from ancient times, studies still now has not been conducted to explore the effect of topical and intradermal application of latex. This study was designed to screen the physiochemical properties of latex and dermal irritation potential of its different solvent fractions on rabbit's skin targeting to retain the triterpene content high to extract therapeutic utilization.

Materials And Methods

Collection of plant material

E. neriifolia latex was collected from the Bhopal district of state Madhya Pradesh, India in the month of Sept – Oct 2009.

Physico-chemical Parameters

Refractive Index: Refractive index was determined with the help of Abbe's Refractometer.

Table 1. Physico-chemical parameters of latex

Parameter	Value
Refractive index of latex	1.41 ± 0.12
Weight per ml	1.14 ± 0.08 gm
Percent solid content	10.95 %
pH	5.2 ± 0.17
Resinous matter	18.32 %

Determination of weight per milliliter (density): Weight per ml of latex was determined by pycnometer as described in Indian Pharmacopoeia [20].

Percent solid content: Fresh latex (5 ml) was taken in preweighed petridish and dried in oven at 110^0C upto constant weight. After complete drying the dry weight percent was calculated.

Determination of pH: pH meter was calibrated with known buffer solution. The glass electrode of pH meter was dipped in the test solution. The reading displayed by pH meter is noted down.

Determination of total resinous matter: Fresh latex (10 ml) was fractioned thrice with 10 % ethanol (20 ml each). Ethanol layer was separated and concentrated (25 ml). In the concentrated ethanol fraction equal volume of distilled water was added, it forms flocculated precipitate. The precipitate was collected, dried and weighed. Percentage of total resinous matter was calculated.

Fractionation of latex: Weighed amount (25 gm) of dried latex were treated twice with 50 ml each of water, acetone, chloroform and pet. ether separately for 24 hrs with occasional stirring. The soluble fraction was decanted, dried, weighed and percentage yield (w/w) calculated.

Solubility study: Solubility nature of dried and fractionated latex was determined in water, ethanol, methanol, acetone, diethyl ether, chloroform, ethyl acetate, benzene, pet. ether, propylene glycol, glycerin and ground nut oil.

Table 2. Solubility range study of dried latex and fractions in different solvents

Solvents	Dried latex	Dried latex fraction			
		Pet. Ether	Chlo-roform	Aceto ne	Water
Water	+	–	–	–	++++
Ethanol	+	+++	++	+++	+
Methanol	++	+	+	++	+
Acetone	++	++	++	++++	+
Diethyl ether	++	+++	++++	++++	+++
Chloroform	++	+++	++++	+++	++
Ethyl acetate	++	++	++	+++	+
Benzene	+++	++	++++	++++	+
Pet. Ether	++++	++++	++	++	+
Propylene glycol	+++	+	+	+	+
Glycerine	–	–	–	–	–

Qualitative phytochemical test

Qualitative test like Molescolt, Libermann, Salkowski and Libermann Burchard was performed on the fresh and dried latex for the presence of steroid and triterpenoids as per the procedure given by Paech and Tracey [21].

Table 3. Qualitative test of latex for the presence of steroids and triterpenoids

Test	Fresh		Dried	
	Observation	Inference	Observation	Inference
Molescolts	Yellowish brown ppt. (++)	Steroid	Yellowish brown ppt (+++)	Steroid
Libermann's	Yellowish brown ppt. (++++)	Steroid	Yellowish brown ppt (++++)	Steroid
Salkowski	Lower layer golden yellow (++++)	Triterpene	Lower layer red (+++)	Steroid
Liberman Burchard	Brown ring in junction (++++)	Steroid	Upper layer red (+++)	Triterpene

++++ = Abundantly present
+++ = Moderately present
++ = Mildly present

Salkowski and Libermann Burchard tests were also performed in different latex fractions before and after hydrolysis with dilute hydrochloric acid.

Determination of total diterpene and triterpenoid in latex

E. neriifolia latex was collected over methanol and dried below 40^0C. The dried latex (4 gm) was extracted thrice with acetone (10 ml each). Acetone soluble residue was mixed with 25 ml of methanol: water (50:50). The aqueous phase was fractioned with n-hexane. The n-hexane layer containing total triterpenes was separated and dried. Acetone insoluble residue was extracted with ether and partioned against 1 % sodium carbonate solution to remove phenolic impurities. Ether layer containing diterpenes was seperated and dried. Percentage yield calculated for both diterpene and triterpene [22].

Animals

Healthy laboratory bred New Zealand rabbits (2-3 kg) and Wistar albino rats (150-200 gm) were used for the study. The animals were housed in an air-conditioned room in stainless steel cages at room temperature (22 ± 2ºC) and 60 % relative humidity with 12-hr light/dark cycles. The animals had free access to a nutritionally adequate standard pellet diet and tap water for one week prior to the experiment. Institutional Animal Ethical Committee approval was obtained before carrying out the experiments.

Dermal irritation and sensitization test

Non-occluded dermal irritation test on rabbits: On the day before dosing eighteen animals (three per group) were weighed and hair removed from the right and left side of the animals with a small animal clipper on the back (approximately 1 × 1 inch square of intact skin). Care was taken to avoid abrading the skin during clipping procedure. Ground nut oil was used as vehicle.

On the day of dosing 1 gm/ml (100 %) of drug in ground nut oil was applied on the designated area on the back. Adjacent areas of untreated skin of

each animal serve as control for the test. After 4 hr, the test site was delineated with an indelible marker and rinsed with distilled water. At the appropriate grading interval (1, 4, 24, 48 and 72 hrs) the animals were examined and scored for signs of erythema and edema according to the Draize Dermal System. Animals were kept under observation upto 14 days of test drug application [23].

Table 4. Qualitative test of dried latex fractions for the presence of steroids and triterpenoids

Dried latex fraction	Before hydrolysis		After hydrolysis	
	Liberman Burchard	Salkowski	Liberman Burchard	Salkowski
Pet. Ether	–	Dark brown color (Steroid)	Brown ring in the junction (Steroid)	Lower layer golden yellow (Triterpene)
Chloroform	–	–	Brown ring in the junction (Steroid)	Dark brown color (Steroid)
Acetone	–	Dark brown color (Steroid)	Dark brown color (Steroid)	Dark brown color (Steroid)
Water	–	Lower layer red (Steroid)	Green color (Steroid)	Lower layer golden yellow (Triterpene)

The standard Buehler sensitization test on rats (topical range finding study): On the day of dosing, four closed patches of different concentrations (750 mg/ml, 500 mg/ml, 250 mg/ml and 100 mg/ml) of test substance were applied to the clipped area on the back of each animal (four per group). A dose of 0.4 ml was placed on a 25 mm Webril patch moistened with ground nut oil was applied to the clipped surface as quickly as possible. The trunk of the animal was wrapped with elastic wrap secured with adhesive tape to prevent removal of the patch and the animal returned to its cage. Six hrs after patch application the test substance was removed with distilled water. The test sites of the animals were graded for irritation at 24^{th} and 48^{th} hrs after patch application using the Buehler Dermal Grading System.

A challenge phase was repeated on day 32. The animal's haircoat was again clipped on the day before dosing. On the next day patches containing the test substance were applied to a naive site within the clipped area of the test and control animals. The same procedure described were repeated again. Approximately 20 hrs after patch removal, the test sites was depilated by applying hair remover cream on the test sites and surrounding areas and left on for no more than 15 min. The depilatory was then removed thoroughly with a stream of warm water. The animals was then dried with a towel and returned to their cages. Test sites were graded for dermal irritation at 24^{th} and 48^{th} hr after patch removal using the Buehler dermal grading system. The rechallenge phase was repeated at day 39 with same exposure period, dosing, wrapping and depilation procedures as used in the challenge phase [24].

Buehler dermal sensitization test on rabbit: On the day before dose administration, twelve rabbits (four per group) were weighed and the hair removed from the right and left back side of the animals with a small animal clipper. A positive control group was also provided in order to evaluate the responsivity of the test system. On the next day, four intradermal injections of four different fractions (pet. ether, chlorofrom, acetone and water) of latex 0.1 ml (0.1 gm) were injected using tuberculin syringe with 25 gauge, 5/8 inch hypodermic needle into clipped area of each animal. The test sites of the animals were graded for irritation at 1, 4, 24 and 48 hrs intervals after intradermal injections using Buehler dermal grading system. Examination of

general condition, skin reactions and all abnormal findings occurs as a result of sensitization was also recorded [25].

Topical anti-inflammatory activity against carrageenan induced rat paw edema method

Antiedematiogenic activity was evaluated by the rat paw edema test following method of Lira et

Table 5. Non-occluded dermal irritation test of latex and its different fractions on rabbit

Time interval in hrs	Draize scoring (erythema/edema)					
	Fresh latex	Dried latex	Dried latex fraction			
			Pet. Ether	Chloro-form	Acetone	Water
1	-	-	-	-	-	-
4	-	-	-	-	-	-
24	2.33/1.16	2.5/1.33	1.30/0.33	3.85/3.33	4.0/4.16	3.50/2.33
48	3.66/2.33	3.86/1.16	0	2.25/2.16	3.66/3.17	2.33/1.17
72	> 4/ Very severe burning, in depth injury and tissue necrosis	> 4/ Severe burning, in depth injury and tissue necrosis	0	0.80/0	1.58/1.25	1.04/0
14 days	Escher formation & wound healing		0	0	0	0
PII*	Very severe irritant	Very severe irritant	0.43/0.11	2.30/1.83	3.08/2.86	2.29/1.16
			Non irritant	Mildly irritating	Moderately irritating	Mildly irritating

Table 6. Standard Buehler topical range finding study of latex petroleum ether fraction on rat

Concentration	Scoring					
	Initial		Challenge (32nd day)		Rechallenge (39th day)	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
750 mg/ml (75 %)	0.94	0.24	0.50	0	0	0
500 mg/ml (50 %)	0.56	0	0	0	0	0
250 mg/ml (25 %)	0	0	0	0	0	0
100 mg/ml (10 %)	0	0	0	0	0	0

Patch Scoring

Skin reaction	Value
Very faint erythema usually confluent	± (0.5)
Faint erythema usually confluent	1
Moderate erythema	2
Strong erythema with or without edema	3

al. [26]. Albino rats of either sex were divided into four experimental groups containing six animals in each. Vehicle control group received ground nut oil 0.4 ml, standard drug control group received diclofenac sodium 100 mg/ml and drug treated groups received 700 mg/ml and 500 mg/ml of latex pet. ether fraction. The standard drug and the latex pet. ether fraction were applied in the planter surface of right hind paw after mixing with ground nut oil in a final volume of 0.4 ml per application site. The site of application was gently massaged so as to enable good distribution and penetration of drug, and then housed individually securing the application with a gauge bandage patch. Thirty minutes after the application of drug, 0.1 ml of 1 % w/v carrageenan (Sigma, USA) solution in normal saline was injected beneath the sub-plantar surface of right hind paw of all the animals. For assessment of anti-inflammatory activity, the volume of the paw before and three hours after carrageenan treatment was measured by mercury displacement technique using plethysmometer. Percentage inhibition in paw edema volume calculated.

Statistical evaluation

The data was expressed as mean ± S.E.M. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-test using GraphPad Prism version 4.00. The results were considered statistically significant if the *p*-values were 0.05 or less.

Results And Discussion

Latex is a milky sap-like fluid occurring in 10 % of all flowering plants and angiosperms. It is a complex emulsion consisting of proteins, alkaloids, starches, sugars, oils, tannins, resins, and gums usually exuded after tissue injury that coagulates on exposure to air. This sap averse native herbivores providing defence against grazing and helps to reduce water loss by lowering the potential loss of water through evaporation.

Latex contains 50–1000 % higher concentrations of defense substances than other plant tissues. *Euphorbia* milky sap contains highly irritant toxin polycyclic diterpene esters, triterpenes, alkaloids, glycosides, and ricin-type protein toxins. Steroids, cerebrosides, glycerols, phenolics and flavonoids were also isolated from plants of the genus, but the compounds most relevant to the toxicity and considerable

Table 7. Buehler dermal sensitization test of different latex fractions on rabbit

Time interval in hrs	Patch scoring of dried latex fraction				Any visible change
	Pet. Ether	Chloroform	Acetone	Water	
1	-	-	-	-	-
4	-	-	-	-	-
24	3	3	3	3	Severe necrotic cell damage, red erythematic linings following vascular system, burning and narcosis of skin
48	< 3	< 3	< 3	< 3	

Sensitization (Maximization) Classification System: Grade V (Extreme sensitizer)

latex with pet. ether, chloroform, acetone and water gives 63.80, 25.05, 59.35 and 0.70 % yield respectively.

Solubility profile of dried latex, and the pet. ether, chloroform, acetone and water fractions were explored in a range of solvents. Dried latex showed good solubility in benzene, pet. ether and propylene glycol. Pet. ether fraction was moderately soluble in ethanol, diethyl ether and chloroform on the other hand chloroform fraction was highly soluble in diethyl ether and benzene.

Acetone fraction showed good solubility in ethanol, diethyl ether, chloroform, ethyl acetate and benzene but water extract showed moderate solubility only in diethyl ether. Except acetone all fractions were soluble in ground nut oil.

Table 8. Topical anti-inflammatory activity of petroleum ether fraction of latex on Rat

Treatment	Volume of edema in ml after 3rd hr	% Inhibition
Vehicle control (ground nut oil; 0.4 ml)	1.25 ± 0.18	---
Diclofenac sodium (100 mg/ml)	0.36 ± 0.01***	71.22
Latex petroleum ether fraction (750 mg/ml)	0.81 ± 0.04*	42.40
Latex petroleum ether fraction (500 mg/ml)	0.72 ± 0.03**	35.25

***P<0.001, **P<0.01 and *P<0.05 as compared to vehicle control values (n = 6).

Qualitative phytochemical test

Both dried and fresh latex showed rich presence of steroid and triterpenoid. Chloroform fraction was positive for steroid only after hydrolysis. Acetone fraction was positive for steroid only. Tritepenoid was present in pet. ether and water fraction, detectable after hydrolysis. Pet. ether, acetone and water showed presence of steroid before hydrolysis.

Determination of total diterpene and triterpenoid in latex

Total diterpene and triterpene content in fresh latex was found to be 24.50 % and 16.23 % respectively. Presence of triterpene was confirmed by positive Salkowski test (lower layer golden yellow) and negative Libermann Burchard test. Presence of diterpenes was confirmed by standard procedure of concentrated sulfuric acid and antimony chloride test giving magenta pink colour [30]. Partition of the polar fraction of the latex against hexane enriched the diterpene content by removing steroidal and triterpenoidal compounds. Ether soluble residue of *Euphorbia* latex is highly potent irritants with Irritant Dose (ID 50 %) value in µg range [22]. Skin irritant diterpenes of *Euphorbia* latex are mostly extracted with ethanol, methanol, acetone, ether, chloroform or methanol:water [22,31,32,33,34]. Diterpene isolation from *E. neriifolia* latex has not been attempted so far. Root and bark of *E. neriifolia* is reported to have diterpenes like; 12-deoxy-4β-OH-phorbol-13-dodecanoate -20-acetate, ingenol triacetate, 12-deoxyphorbol-13,20-diacetate, antiquorin and neriifolene [33,35,36].

E. neriifolia dried latex is highly soluble in pet. ether with highest yield. Non polar solvent like pet. ether extract out non polar constituents like fixed oil, fats and waxes [37]. Pet. ether extract are mostly rich in steroidal, saponin and triterpenoidal contents. Triterpenes can also be extracted with semipolar solvents like ethanol, methanol, acetone and chloroform etc but final fractionation with pet. ether or n-hexane enrich triterpenes. Triterpenes occur as true triterpenes, steroids, saponins or glycosides. Acid hydrolysis was carried out to liberate aglycones or sapogenin if any [30,38]. Tritepenoid was detected in pet. ether and water fraction only after hydrolysis. All the fractions give positive test for steroid after hydrolysis. Ponsinet and Ourisson [17] showed presence of euphol, a triterpene alcohol in petroleum ether extract of lyophilized *E. neriifolia* latex. Triterpenes like neriifolione,

euphol and nerifoliol along with cycloartenol and nerifolione was reported in acetone extract of latex [18], A new tetracyclic triterpene 9,19-cyclolanost-22(22'), 24-diene-3beta-ol, named as nerifoliene along with euphol were isolated from the n-hexane extract of fresh latex of *E. neriifolia* [19].

Dermal irritation and sensitization test

Non-occluded dermal irritation test was perform on fresh and dried latex along with four fractions at 1 gm/ml (100 %) topical dose dissolved in ground nut oil in a final volume of 0.4 ml. Draize Dermal Irritation Scoring System was followed to judge the extent of erythema and edema formation. Fresh and dried latex was found to be severely irritating causing very severe burning, in depth injury and necrosis of skin dermal layer with primary irritation index (PII) of more than 4. All the fractions causes mild to well defined erythema and edema 24 hrs after application which decreased after 48 hrs and abolishes on 72 hrs. Pet. ether fraction was found to be apparently nonirritating with a PII score of 0.43/0.11 only for erythema and edema according to Draize Dermal Classification System. Chloroform, acetone and water fractions were found to be mild to moderate irritating, but most irritant activity was shown by acetone fraction. Chloroform, acetone and water fractions are skin irritating due to presence of high diterpene content where as pet. ether fraction is rich in triterpene showing nonirritant activity [22,31]. Diterpenes are also reported to be highly toxic with tumor promoting and proinflammatory activity [9].

Standard Buehler topical range finding study was performed on latex pet. ether fraction only as it was found to be least irritating with rich triterpenoidal content. The doses tested were 750 mg/ml (75 %), 500 mg/ml (50 %), 250 mg/ml (25 %) and 100 mg/ml (10 %) applied topically with occlusions (patches). Initial exposure at 750 mg/ml and 500 mg/ml doses causes faint erythema usually confluent at 24 hrs, which got reduced on 48 hrs and all other doses were free of any skin reaction. Challenge on 32nd day showed very faint confluent erythema only on 750 mg/ml dose, absent on other doses. Rechallenge on 39th day with different doses of latex pet. ether fraction was free of any dermal irritating effect.

Fresh and dried latex was severe dermal irritant when applied topically on rabbit skin so intradermal Buehler sensitization test was carried out only on different latex fractions on rabbit skin. Pet. Ether, chloroform, acetone and water fractions were found to be non irritant topically in 100 % dose. Intradermal injections of four different fractions of latex at 0.1 mg/0.1 ml (100 %) dose caused marked erythema and edema 24 hrs after administration with patch score 3 of Buehler Dermal Grading System. After 48 hrs severe necrotic cell damage occurs with red erythematic linings following vascular system causing burning and narcosis of skin. According to Sensitization (Maximization) Classification System, all the fractions were found to be Grade V, Extreme sensitizer. Intradermal maximization study for range finding was not attempted further due to severe necrotic damage potential of the latex fractions.

Topical anti-inflammatory activity against carrageenan induced rat paw edema method

Topical anti-inflammatory activity of latex pet. ether fraction was accessed on rat at 750 and 500 mg/ml. Topically latex pet. ether fraction at 750 and 500 mg/ml dose showed 42.40 and 35.25 % inhibition of carrageenan induced paw edema in comparison to 71.22 % inhibition of topical diclofenac sodium (100 mg/ml).

It is established that anti-inflammatory substances which exerts their effect by virtue of their irritant property can be distinguished from the true anti-inflammatory agents by administering them locally in the carrageenan test. If a mixture of the plant extract and carrageenan produces reduction in paw edema, the effect of the plant extract are not due to counter irritant activity. It has long been thought that pain can be relieved and healing promoted by irritating an area of the skin. Body initiates an inflammation in response to trauma, this is red swollen and sore event that occours on injury or infection. Although

unpleasant the inflammatory response is necessary to protect the body from spread of infection and to clear up damaged tissue. Body has number of mechanisms to start healing and tissue repair process until inflammation switches off. In some autoimmuno diseases, inflammation becomes semipermanant needing the use of anti-inflammatory agents. Application of a bit of injury to or near the effected area with a needle for example (acupuncture) is enough to trigger a little more inflammation and in turn presses the inflammation switch off button and allow healing to take place as a sort of weak up call for the bodys anti-inflammatory and healing process. Irritants chemical like menthol, turpentine and camphor are mild irritant substances which cause low level inflammation. Counter irritant action of medicinally useful plants are responsible for anti-inflammatory activity. The irritation of tissue could release a substance that act as anti-inflammatory agent, even at a remote site of the body. Administration of inflammatory exudate results in suppression of experimental inflammation [39]. Topical resiniferatoxin isolated from dried latex of *E. resinifera* (Euphorbium) was clinically trialled in human volunteers having diabetic neuropahthic pain, which showed promising analgesic effect. Resiniferatoxin binds with specific membrane recognition sites (referred as venilloid receptors) expressed by sensory neurone mediating pain perception and neurogenic inflammation. Binding with this receptors resiniferatoxin causes desensitization of neurones thus inhibition of pain perception. It is a more potent counter irritant than capsaisin, working in the same pathway but interacting with different modulators [40].

Euphorbia latex triterpenes euphol, euphorbol, tirucallol, nerifolione etc have anti-inflammatory, analgesic and antiarthritic effect [41,7]. Triterpene alcohols and plant sterols have recently been demonstrated to possess inhibitory effects on 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induced inflammation in mice [42,43]. Triterpene alcohol euphol isolated from *Camellia japonica* and *Camellia sasanqua* seed oil inhibited the TPA induced inflammation with 0.2 mg/ear of the 50 % inhibitory dose at a grade almost corresponding to that of indomethacin (ID_{50} 0.3mg/ear), and was far more potent than quercetin (ID_{50} 1.6mg/ear). As far as the euphane/tirucallane type compounds are concerned, the euphanes (euphol; ID_{50} 0.2mg/ear) are more potent than the tirucallanes (tirucallol; ID_{50} 0.4mg/ear). Inhibition of TPA induced inflammation has been demonstrated to have an almost parallel inhibitory effect aganist tumor promotion and cellular proliferation [44]. Presence of triterpenes like, euphol, nerifoliol, cycloartenol, nerifolione and nerifoliene in the fresh and dried latex of *E. neriifolia* are reported by Ponsinet and Ourisson [17], Ilyas et al. [18] and Mallavadhani et al. [19].

The hydrosoluble fraction of *E. royleana* latex, administered by gavage at doses of 50 - 200 mg/kg showed dose-dependent anti-inflammatory and anti-arthritic effects in different acute and chronic test models in rats and mice. It reduced the exudate volume and the migration of leukocytes and showed a poor inhibitory effect on the granuloma formation induced by cotton pellets, while it had a low ulcerogenic score. The oral LD_{50} was more than 1500 mg/kg in both rats and mice [45]. The latex of *E. neriifolia* is used for arthritis in Unani medicine [15]. Ilyas et al. [7] reported anti-inflammatory and antiarthritic activity of a novel triterpene (Nerifolione) isolated from the latex of *E. neriifolia.* Both these activities were explored orally where as post-mortem examination of people killed by *Euphorbia* latex has revealed severe inflammation of the walls of the stomach and intestine and in some cases, the wall of the stomach has been perforated. In folklore practices also the *Euphorbia* latex are mostly used topically as rubefacient, counterirritant, skin wart derooting and analgesic. The active component of *Euphorbia* latex that gives its toxicity is Euphorbon, a protein called cytotoxin (glycoside and resin). Severe pain and inflammation can result from contact with the eyes, nose, mouth and even skin, which may be due to the activation of protein kinase C enzyme [46]. Diterpene esters of the phorbol, ingenol and 12-deoxyphorbol

esters types are known to be highly active tumor promoting agents that typically occur in members of the Euphorbiaceae [9,47].

conclusion

In view of manifold uses of *Euphorbias* it appears surprising that the toxic nature of the phytochemical principles of the plants is discouraging their uses. The biological nature of their toxic chemicals and their possible therapeutic value remained relatively undefined until recently. For safe and effective therapeutic exploitation of *E. neriifolia* latex triterpenoidal content as anti-inflammatory substance irritant diterpenes should be removed to reduce the dermal irritancy properties. This study was designed for effective separation of triterpenoidal content *E. neriifolia* latex and to explore dermal and intradermal skin irritancy property before proceeding for anti inflammatory efficacy study. Pet. ether fractionation showed rich presence of tritrpenes, noirritancy and also a good topical anti-inflammatory activity. This finding may prove as a step forward for large scale therapeutic exploitation of this less known hedge plant *E. neriifolia*. Systematic study on phytoconstituents of *Euphorbia* plants latex, as potential sources of new compounds will lead to promising natural product based new drug development. There arises a need, therefore to screen *E. neriifolia* bioactive compounds for detailed and intensive pharmacological properties.

Authors' Contributions

Dr. P. Bigoniya has made substantial contribution in conception and design of the study. Acquisition, analysis and interpretation of data were also carried out by her. P. Bigoniya participated in drafting and revising of manuscript and performed the statistical analysis. Singh C.S was involved in physic-chemical, quantitative and qualitative estimation and participated in editing of manuscript. Shukla A carried out the animal studies, also participated in study design and helped to draft the manuscript. Each author has participated sufficiently in the work to take public responsibility for appropriate portion of the content.

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International Journal of pharmaceutical Sciences and Drug Research, International Journal of Pharmacology, International Journal of Ayurveda Research and Journal of Young Pharmacists.
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Acknowledgements

The authors are thankful to, P.G. students of Department of Pharmacology, Radharaman College of Pharmacy, M.P., India, for helping in procuring the latex sample and study coordination.

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