Original article:

Standardadization and skin irritation potential of herbal analgesic cream containing *Nigella sativa seed oil*

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Abstract:

Background & Objective*:** The aim of present study was to evaluate herbal analgesic cream containing *Nigella sativa* oil as an active ingredient, standardization of *Nigella sativa* oil and evaluation of irritation/sensitization potential of formulation. ***Material & Method: For this, HPLC analysis of oil, skin irritation test on rabbits and patch testing on human skin was conducted. HPLC analysis using C-18 column, using an isocratic mobile phase of water: methanol (10:90) at flow rate of 1 ml/inactive ingredient: thymoquinone purified from the oil was found to be 1.42g (28.4 %). Safety assessment of analgesic cream was based on Primary Dermal Irritation Index (PDII) by Draize method. ***Result:*** According to Draize standard scoring system of reactions PDII was found 0.04 which comes under the category of non irritant. Patch testing on human volunteers revealed that none of volunteers showed any sign of skin reactions.

Keywords: *Nigella sativa* HPLC; standardization; skin irritancy; rabbits; human volunteers.

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Introduction

Nigella sativa, belonging to botanical family; Ranunculaceae, used as medicinal plant. It is native to North Africa, Southern Europe and Asia but widely cultivated in Pakistan and India. The plant reproduces dicotyledonous capsulated fruits containing numerous white trigonal seeds, which turn black on exposure to air; thus commonly known as Black Seeds[1,2]. It is well known as *Habbat Albarakah*, *Alhabahat Alsawda*, and *Alkamoun Alaswad* among Muslim communities.[3]As established historical and religious basis, it is recommended for a broad range of health problems, and is one of the herbal medicines that are being actively investigated and has worldwide recognition[4].Its oil comprised of 30% by weight of p-cymene, which is the most original composition, and 61.48% of the weight is composed of the volatile oil. *Nigella sativa* seeds are rich source of essential fatty acids, unsaturated acids, vitamins, minerals, proteins, and carbohydrates[5].There are also other compounds in seeds, such as phospholipids, carotene, calcium, iron, and potassium. The biological effects of these compounds on the human body are largely positive. They have shown prominent effects in the treatment of many diseases such as hypertension, headache, diabetes, inflammation, eczema, fever, dizziness, influenza, asthma and bronchitis.[6]

The pharmacological properties of *N.sativa* were also reported by *in vitro* and *in vivo* studies conducted on human and laboratory animals. These studies showed that *N. sativa* and its ingredients have a broad range of medicinal effects such as antimicrobial, antidiabetic, antinephrotoxic,antiinflammatory,antio xidant,antihypertensive,antiasthmatic, antiparasitic and anticancer.[7,8,9.] Acute and chronic toxicity studies on laboratory animals reported that *Nigella sativa* seeds, its oil and thymoquinone(the most plentiful and broadly studied active compound) are safe,

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particularly when given orally. [10,11,12]

According to a mobility disability caused by osteoarthritis among the elderly people , a lot of side effects of synthetic drugs and multiple medications exist with no certain cure for it; thus the prevalence of musculoskeletal disorders has been reported in older people in Sabzevar City, especially in the knee joint to evaluate the topical impact of *Nigella sativa* oil and oral acetaminophen on knee osteoarthritis in the elderly residing in a parents 'nursing home in Sabzevar City of Iran.[13,14]

In present work *Nigella sativa* oil has been used in cream formulation that is locally used for the treatment of muscular pain, inflammatory conditions, to relieve headache, neuralgic, rheumatic and other allied pains (lumbago, sciatica, stiff muscles and joint pains). This herbal analgesic cream is also recommended in conditions like allergies, eruptions, swellings, warts and insect bite etc. It is prepared from indigenous raw material and can be applied safely. It has an advantage over oral administered preparations as it does not cause gastrointestinal disturbances.

The aim of present study is to standardize the *Nigella sativa* oil as it is an active ingredient and to evaluate skin irritation potential of analgesic cream when long term use is expected. Safety assessment of topical formulations is a primary interest; this must be done by means of *in vitro* and *in vivo* tests to determine the risk of irritation by the contact with human skin.

Material and Method

Chemicals and Equipment.All chemicals used in this study were laboratory grade which included liquid paraffin, white petroleum, white wax, sodium borate, N.hexane(analytical grade) purchased from E.Merck Chemical company.

HPLC used was Prominence LC-20A Series, Shimadzu, Japan with PDA. Alumina sheets (Kieselgel 60 F254 0.2mm; Merck, Munich Germany) were used for thin layer chromatography (TLC) and silica gel (230-400 mesh) was used for column chromatography. Visualization of the TLC was carried out by iodine vapors and under UV light at 254 and by spraying with Ceric Sulphate reagent solution (with heating). Thymoquinone used as standard purchased from Aldrich. All solutions used were of HPLC grade.

Oil Extraction. *Nigella sativa* seeds were purchased from local market and submitted to botanist with whole plant for identification. The seeds were dried in shade and grinded in an electrical grinder and wrapped in a filter paper and closed with a second filter paper in a manner so as to prevent the escape of the material. The second filter paper is left open at the top placed in the top of thimble to distribute the solvent as it drops on a sample. The wrapped seeds were placed in the Butt extraction tube and assemble the apparatus according to AOAC, 2004.[15] N-Hexane (1000ml) was taken in round bottom flask before attaching to the tube. The solvent was removed through distillation and the residue was dried over anhydrous sodium sulphate then filtered off. The rest of the solvent was removed under the gentle stream of Nitrogen then weighed the oil content, repeated the drying procedure until a constant weight was obtained.

Standardization of oil

Preparation of standard.30 mg of standard thymoquinone was taken in 10 ml volumetric flask and dissolved in methanol. The solution was made up to the mark with methanol to give a concentration of 3 mg/ml of thymoquinone.

Preparation of sample.10 ml of methanol was added to 5 g of oil extracted from *Nigella sativa* seeds in a 25 ml flask. It was sonicated for 20 minutes upper methanolic layers was separated and concentrated over vacuum on rotary evaporator. Concentrated viscous liquid was loaded on silica column and eluted with petroleum ether. The fastest moving yellow band was collected in vials and compared on TLC with standard thymoquinone using 10 % chloroform in petroleum ether. It was found to be comparable with standard thymoquinone with an Rf value of 0.35. The vials were combined and evaporated to give 1.836 g of impure thymoquinone. Out of this 30 mg of sample was taken in 10 ml volumetric flask and dissolved in methanol. The solution was made up to the mark with methanol.

HPLC analysis .The sample and standard were subjected to HPLC analysis using C-18 column, using an isocratic mobile phase of water: methanol (10:90) at flow rate of 1 ml/min. [16] The detection was made at 254 nm. The peak for extracted thymoquinone matched with that of standard at RT of 1.93 min. The amount was calculated by comparing the area of peak of the standard with that of sample. Fig 1 & 2.

Preparation of Formulation

O/W formulation was prepared by adding oily phase to the liquid phase with continuous stirring. In oil phase, the emulsifier (stearic acid) and oil soluble components (cetyl alcohol, *Nigella sativa* oil) were dissolved and heated up to 70+5C(Part A).At the same time the aqueous phase containing water soluble components like methyl paraban, propyl paraban,propylene glycol (Part B) was heated up

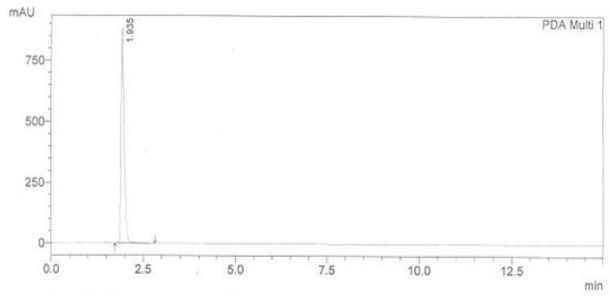

Fig 1: Chromatogram of Standard Thymoquinone

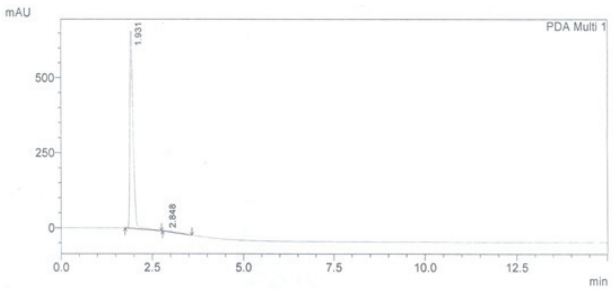

Fig 2: Chromatogram of Thymoquinone extracted from oil

to 70+5.After heating, the oily phase was added to aqueous phase with continuous stirring at 2000 rpm by the mechanical mixer for about 20 minutes. Then speed of mixer was reduced to 1000 rpm for homogenization until the emulsion cooled to room temperature. The formulation of cream is given in table 1.

Table 1. Formulation of ointment

Ingredients	Formula % w/w
Borax	1
Wax	15
Propylene glycol	4
Liquid paraffin	20
Stearic acid	0.5
Cetyl Alcohol	5
Propyl paraban	0.5
Methyl paraban	5
Nigella sativa Oil	49
Water	

Evaluation of Analgesic Cream (Physical Parameters of cream)

pH of the Cream. pH meter was calibrated with standard buffer solutions then about 1g of the cream was dissolved in 50.0 ml of distilled water and its pH was measured.

Homogeneity. Homogeneity of herbal analgesic cream was checked by visual appearance and by touching.

Appearance. The appearance of the cream was evaluated by its color, and roughness.

After feel. After applying the fix amount of cream on skin its emollient, slipperiness and amount of residue left was checked.

Type of smear. After application of cream, the type of smear formed either greasy or non greasy on the skin was checked.

Removal. The ease of removal of the analgesic cream applied was checked by washing with tap water.

Acid value. 10 gram of herbal analgesic cream dissolved in 50 ml mixture of equal volume of alcohol and ether, the flask was connected to reflux condenser and slowly heated, until sample dissolved completely. 1 ml phenolphthalein added to this mixture and titrated with 0.1N NaOH, until faintly pink color appears after shaking for 30 seconds.

Acid value = n*5.61/w

n = the number of ml of NaOH required.

w = the weight of substance.

Saponification value. Herbal analgesic cream(2g) refluxed with 25 ml of 0.5 N alcoholic KOH for 30 minutes, to this 1 ml of phenolphthalein added and titrated immediately, with 0.5 N HCL.

Saponification value = (b-a)*28.05/w

The volume in ml of titrant = a

The volume in ml of titrant =b

The weight of substance in gm = w

Centrifugation Test. Centrifugation test was performed immediately after preparation of formulation and repeated after 24 hours, 7 days, 14 days and 30 days of preparation. This test was performed at 25°C and at 5000 rpm for 10 minutes by placing the 5 g sample in stopper centrifugal tubes. [17]

Acute Dermal Irritant Test

The acute dermal irritant test was carried out in accordance with the OECD Guideline 404.[18]

Animal Husbandry and Maintenance. Healthy, adult white male rabbits (05) weighing about 1.5-2.5kg kept carefully following an acclimitation period of 7 days to ensure their suitability for the study. The temperature and humidity of experimental animal room were controlled at 22±3°C and not exceed 70% respectively. Light and dark cycle was also maintained by artificial lightening. Conventional laboratory feed was used with an unlimited supply of drinking water.

Animal preparation for Testing. The experiment was carried out using 06 adult white male rabbits. The fur on the back of animals on both side of the

spinal column was clipped carefully, exposing approximately 6cm^2 of skin. Avoid mechanical irritation or trauma remove loose hair by means of vacuum then swab the skin lightly with 70% alcohol and dry the skin prior to application of cream.

Test procedure.0.5g cream was applied to the test area of approximately 6cm^2 of skin and 0.5 g of the base containing all ingredients except *Nigella sativa* oil was applied to control sites held in contact with the skin by a non occlusive bandage. After 4 hours, the test and control material was removed and 1hour later the sites were examined for signs of erythema and edema at 1, 24, 48 and 72 hours according to scoring criteria for skin reactions (table 2).

Table 2. Standard scoring system for skin reactions

Reaction	Score
Erythema	
No erythema	0
Very slight erythema	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation	4
Edema	
No edema	0
Very slight edema	1
Well defined edema (edges of the area well defined by defined raising)	2
Moderate edema (raising approximately 1 mm)	3
Severe edema (raising more than 1 mm and extended beyond the area of exposure	4
Total possible score for primary irritation	8

The Primary Irritation Indexes of test substance were calculated using the formula given below.

Primary Dermal Irritation Index (PDII)

$$= \frac{\text{sum of erythema / oedema}}{\text{No. of test sites x grading interval}}$$

By using the PDII the test sample was then classified according to Draize method[19] of classification as shown in table 3.

Table 3. Primary Dermal Irritation Index system

Primary Irritation Index	Classification of irritancy
0 to 0.4	non- irritant
0.5 to 1.9	slightly irritation
2 to 4.9	moderately irritation
5 to 8	Severe irritation

Ethical clearance: This study was approved by ethics committee of Pharmaceutical Research Centre, PCSIR LABS COMPLEX, Karachi.

Results and Discussion

Herbal medicines are being utilized from time immemorial as the major source of treating various ailments and diseases however the major objection on herbal medicines is the lack of scientific data for their quality control. Therefore as pointed out by WHO, guidelines for the production and quality assurance of herbal products needs to be standardize to ensure quality and optimum levels of active principles for their bio-potency. One of the basic requirements is to standardize the raw material for active ingredient. Therefore the oil extracted from *Nigella sativa* seeds used as active ingredient in this formulation was standardized for its thymoquinone content by the method of Ghosheh,1999 i.e. first by extraction from oil and then by using column chromatography followed by HPLC. The quantity of thymoquinone purified from the oil used in the formulation by column chromatography followed by HPLC was found to be 1.42 g (28.4 %).as showed in fig 1&2. This percentage is close to reported percentage of thymoquinone (30- 48%) as various studies showed that thymoquinone also known as 2-isopropy l-5-methyl-1,4-benzoquinone is mainly responsible for the biological activity of *Nigella sativa* seeds which is pre-dominantly. [20,21,22]All physical parameters of cream were evaluated and the result was presented in table 4 .

Table:4 Physical Parameters of cream

Days	pH	Homogeneity	Appearance	After feel	Smear type	Acid value	Centrifugation Test	Saponification value
0	6.5	Good	No change	Emollient	NG	5.5	NSL	75%
5	6.5	Good	No change	Emollient	NG	5.5	NSL	75%
10	6.5	Good	No change	Emollient	NG	5.5	NSL	75%
15	6.5	Good	No change	Emollient	NG	5.5	NSL	75%
30	6.5	Good	No change	Emollient	NG	5.5	NSL	75%

NSL=No separation of layer, NG=Non greasy

No change was observed in pH, homogeneity appearance, acid value and saponification value of cream at 7th ,14th,21 and 28 days **.** Formulation was found to be stable when stored for long time, having constant pH, emollient non greasy and homogeneous. As it is an oil water base emulsion, hence can be removed easily with plan water that gives better customer compliance and its external application produced good feeling upon use.

Assessment of irritation and sensitization potential of pharmaceutical and herbal skin care products with natural compounds is significant step in the evaluation of their biocompatibility. Researchers and regulatory agencies recognize the importance of *in vitro* and *in vivo* testing of pharmaceutical and herbal skin products for their biological evaluation. Based on the results of this *in vivo* investigation, the irritant and sensitization properties of *Nigella sativa* oil based cream after direct application to rabbit and human skin were evaluated respectively. The result of acute dermal irritant test is presented in table 5.

Table 5. Dermal reactions (Time after patch removal)

Rabbit NO.	Herbal analgesic Cream								Cream Base							
	1hour		24 hours		48hours		72hours		1hour		24 hours		48hours		72hours	
	E	O	E	O	E	O	E	O	E	O	E	O	E	O	E	O
1.	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

E=Erythema, O=Oedema. PDII=0.04

The result reflects negligible erythema at I hour observation after removing the patch in two animals of both test and control groups. After 24 hours erythema was disappeared and sites were cleared. After 72 hours of observation period, primary irritation index score of test and control group calculated i.e. 0.04 which comes under the category of non irritant(0-0.4) according to Draize method of classification as shown in table 3.This index of irritation indicates the safety of formulation.

Conclusion

Based on physical, chemical and biological studies, it is concluded that herbal analgesic cream containing *Nigella sativa* oil is stable formulation and safe for long term topical use for various muscular problems.

Conflict of Interest

The Authors declared there is no conflict of interests.

Individual Contribution of the Authors:

Conceptual work: Sohail T

Data collection: Sohail T, Yasmeen S

Manuscript writing: Sohail T

Editing of final manuscript: Sohail T, Yasmeen S, Imran H, Ferheen S, Rehman A, Khan RA

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