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EVALUATION OF ACUTE DERMAL IRRITATION, SENSITIZATION, AND IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF CANNABIS-BASED PAIN-RELIEVING TOPICAL OIL – CANNAQUIC™

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ABSTRACT

Cannabis is known and considered a sacred plant because of its analgesic, anti-inflammatory, and stimulating properties. Other herbs like Shallaki, Bala, Nirgundi, Kantakari, and Kupilu are also known as medicinal herbs and have tremendous pain-relieving properties. **Objectives:** A formulation with an infusion of cannabis leaves extract and other herbs in sesame oil can be a good therapy for relieving different types of pain when applied topically. An evaluation study was conducted in animals for the evaluation of dermal irritation, sensitization, and anti-inflammatory properties. **Methods:** This study was designed to evaluate acute dermal irritation/corrosion, sensitization on skin and anti-inflammatory activity of Cannaquic™ according to OECD guidelines. No erythema, edema, or any skin lesion was observed after application. **Results & Conclusion:** The results revealed no irrita-

tion/corrosion from Cannaquic™. In-vitro Anti-Inflammatory activity against LPS-induced toxicity in Mouse Macrophage (RAW 264.7) cell line exhibits a significant percentage reduction in IL-6, Nitric Oxide (NO), and TNF-alpha in Mouse Macrophage (RAW 264.7) cell-line against LPS-induced inflammation.

Keywords: Pain relieving oil, Cannabis sativa, Preclinical trial, Anti-inflammatory

INTRODUCTION

Pain is often described as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Pain can be caused by a variety of factors, including injury, disease, inflammation, and psychological factors. Pain can have a significant impact on a person's life. It can interfere with sleep, work, relationships, and activities that they enjoy. It can also lead to depression, anxiety, and other mental health problems.

In Ayurvedic texts, various terms such as Ruk, Ruja, Vedana & Shoola are commonly used for pain, however, 'Shoola' is more appropriate term amongst all of which means – a condition with the state of discomfort to the body & mind [1] In Ayurveda, pain is seen as a sign of imbalance in the body's doshas - vata, pitta, and kapha [2]. For example, if Vata is imbalanced, it can lead to pain in the joints, muscles, and nerves. If Pitta is imbalanced, it can lead to pain in the muscles and joints. If Kapha is imbalanced, it can lead to inflammation in the joints, muscles, and lungs.

Modern science defined the mechanism of pain as a complex process that involves the nervous system, the brain, and the spinal cord. It can be divided into five stages. Transduction is the first stage of pain, where the nociceptive nerve endings in the body detect a noxious stimulus. Once the nociceptive nerve endings have been activated, they send electrical signals to the spinal cord through nerve fibers. The spinal cord transmits the signals to the brain and the brain receives the signals from the spinal cord and interprets them as pain. The brain can increase or decrease the perception of pain. Certain factors such as the release of neurotransmitters can influence pain modulation.

Several steps have been adopted in Ayurveda to manage pain. Snehana (oleation) is one of the processes as Vata dosha gets pacified by oleation. Sesame oil is commonly used as the base material for snehan process in Ayurveda. The use of herbal oil allows for deeper penetration of muscle and greater relaxation. Some of the good, medicated oils like Mahanarayan oil. Karpooradi oil, and Kottamchukadi oil, are used as ayurvedic pain relieving oil.

In this study, the Ayurvedic polyherbal oil, CANNAQUIC™ treatment for all types of pain has been studied for its safety and efficacy and looked for symptomatic relief without harmful side effects in animals which provides a fundamental characterization of the potential hazards of this developed formulation.

MATERIAL & METHODS

Plant material and formulation preparation

Cannaquic™ is a medicated oil infused with several medicinal herbs including cannabis. Kwatha of Rajika (*Brassica juncea*) seed, Sigru (*Moringa oleifera*) bark, Shallaki *(Boswellia serrata*) exudates, Bala (*Sida cordifolia*) Wh Pl., Gokshura (*Tribulaster-restris*) fruit, Kantakari (*Solanum virginianum*) leaves, Nirgundi (*Vitex negundo*) leaves, Ashwagandha (*Withania somnifera*) root, Rasna (*Alpinia calcarata*) rhizome, Hingu (*Ferulaassa-foetida*) exudates, Kupilu (*Nux vomica*) seed and Cannabis (*Cannabis stativa*) leaves extract along with rock salt (Saindhava Lavana) infused in sesame oil at low flame. Then the oil was filtered out and packed in the bottle.

Animals

Irritation/ Corrosion test: The current study was conducted in accordance with OECD guidelines using three female rabbits. The animal experiment was conducted following the guidelines of the committee for control and supervision of experiments on animals (CPCSEA Registration Number-1803/PO/RcBi/S/2015/CPCSEA). Animals were

housed under a temperature of 22 ± 3ºC, relative humidity 30 - 70%, 12-hour light, and 12-hour dark cycle in a standard polypropylene cage with Sterile Corncob as bedding material. A normal chow diet and fresh, uncontaminated water was provided to all the animals throughout the experiment. The study was commenced after the protocol was reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Radiant Research Services Pvt. Ltd.

Skin sensitization test: This study was conducted as per the guideline of OECD –406- *In-vivo* Skin Sensitization Guinea Pig Maximization Test, adopted on 30th June 2022. Out of 15 female guinea pigs used in the study, 5 guinea pigs were assigned to Vehicle control group (Gr.1) and 10 guinea pigs were assigned to Treatment group (Gr.2). Animals were provided a contaminant-free normal chow diet and drinking water during the experimental period.

All the animals were observed daily for general health. Body weights were recorded before treatment and once a week after treatment.

Irritation/ Corrosion study

Selection of animal and preparation:

Healthy young adult female rabbits with body weight in the range of 2000 - 2500gm, were used for this study. The dorsal area of the trunk of the animals was shaved free of fur 24 hours before the application of pain-relieving oil.

Application of Cannaquic™:

A dose of 0.5 ml pain relieving oil (Cannaquic™) was applied topically to each rabbit to an area of 6cm^2 of skin and covered with a gauze patch. After 4 hours of exposure, the residual oil was removed.

The initial test was performed using one animal. Up to three test patches were applied sequentially to the animal. The first patch was removed after three minutes. A second patch was applied at a different site and removed after one hour. A third patch was applied and removed after four hours, and the response was graded. Two additional animals were used to confirm irritant or negative response. Cannaquic™ was applied to each animal with one patch, for an exposure period of four hours. The animals were observed for 14 days after the removal of the patches.

All animals were examined for signs of erythema and oedema and the responses scored at 1, 24, 48, and 72 hours after patch removal. For the initial test in one animal, the test site was also examined immediately after the patch removal.

Dermal reactions were graded and recorded as in Table 1.

Erythema and Eschar Formation	
Signs of formation	**Grade**
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight edema (edges of the area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

Table 1: Dermal reactions and the grades

Sensitization study

Table 02: 15 female guinea pigs were used in 2 groups for the study.

Sl. No.	Group	Sex	Numbers	Treatment
1	Gr. 1 - Vehicle control group	Female	5	Control (Saline)
2	Gr. 2 - Treatment group	Female	10	Cannaquic™

Table 03: *Intradermal Induction phase:* A pair of 0.1mL intradermal injections was made for each treatment, into each animal, at the injection sites (A, B, and C) in the clipped intrascapular region.

Sites	Control group	Treatment group
Site A	Saline solvent	1:1 (V/V) stable emulation of Freund's complete adjuvant
Site B	Saline solvent	Cannaquic™, the pain-relieving oil in a varied concentration
Site C	An emulsion of the blank liquid (Saline) with adjuvant.	Cannaquic™ was emulsified in a 1:1 (V/V) stable emulation of Freund's complete adjuvant with the saline solvent (50%) in the same concentration as in site B.

Topical induction phase: After completion of intradermal induction phase, at 6±1 days, 0.5 mL Cannaquic™ was applied topically to the intrascapular region of each animal, using a patch of area approximately 2cm x 4cm (absorbent gauge), to cover the intradermal injection sites.

Challenge phase: At 20 ± 1 day after the completion of the topical induction phase, all the test and control animals with the test sample were challenged. Absorbent gauges (2cm x 4cm) were soaked respectively with 0.5 mL test substance and 0.5 mL control blank (Saline) and were applied topically to two sites that were not treated during the induction stage. The patches were removed after (24 ±2) h.

Table 04: Observation for all animals was recorded after 6 hrs of application. Scoring was recorded following the patch test as mentioned below.

Patch test reaction	Grading scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

Anti-inflammatory effect study

The in vitro cytotoxicity was performed for the test substance on Mouse Macrophage (RAW 264.7) cell line to find a toxic concentration of the test substance and to evaluate its modulatory effect of anti-inflammatory activity against LPS-induced toxicity.

Preparation of test solution:

For Cytotoxicity studies, 10mg of Cannaquic™ were separately dissolved and the volume was made up of DMEM-HG (Dulbecco's Modified Eagle Medium - HG, Gibco.) supplemented with 2% inactivated FBS (Fetal Bovine Serum, Gibco.) to obtain a stock solution of 1 mg/mL concentration and two-fold serial dilutions were prepared as working solution.

Cell line and Culture medium:

Mouse Macrophage (RAW 264.7) cell line was cultured in DMEM-HG media supplemented with 10% inactivated FBS, penicillin (100 IU/mL), streptomycin (100 ug/mL), and amphotericin B (5ug/mL) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The stock cultures were grown in 25 cm^2 culture flasks. Cytotoxicity studies and anti-inflammatory studies were carried out in 96 well and 6 well microtitre plates respectively.

In vitro IL-6, Nitric Oxide (NO), TNF-α inhibitory activities study:

Mouse Macrophage (RAW 264.7) cell line was seeded into 6 well culture dishes at a cell population 1.5x105 cells/ml in DMEM with 10% FBS. After 24 h, the cells were treated with a known non-toxic concentration of Cannaquic™ (Pain relieving oil) along with 5ug/mL of lipopolysaccharide (LPS) and incubated at 37°C with 5% CO_2 for 24 hr. After incubation, the cell supernatant is collected, centrifuged, separated, and stored at -20°C till use. IL-6, Nitric Oxide (NO), and TNF-α were estimated as per the manufacturer's protocol.

DISCUSSION

Cannaquic™ is a multi-herbal formulation with sesame as base oil and cannabis leaves as one of ingredients. Cannabinoids are the active compound present in cannabis leaves and the most studied cannabinoids are tetrahydrocannabinol (THC) and cannabidiol (CBD). These compounds mediate their effects through cannabinoid receptors (CB1 and CB2). Mainly the anti-inflammatory activity of cannabinoids exerts through induction of apoptosis, inhibition of cell proliferation, suppression of cytokine production and induction of T-regulatory cells (Tregs)[3]. Extracts derived from the cannabis plant have been applied to wounds for thousands of years. After the discovery of the human endo-cannabinoid system and its dominant function throughout the human system provides a strong scientific foundation for use in medicine for pain in recent times. One animal study has also shown significant peripherally mediated anti-nociception using the synthetic cannabinoid agonist WIN55,212–2 (WIN-2) applied topically [4][5].

Sesame oil has a significant role on neuro-hormonal effects within body in bahyasnehan process, so is used as a base for preparation of various medicated oil. Sesame oil helps in regulating eicosanoids which inhibits inflammation[6]. Research conducted by TOUCH Research Institute, Univ. of Miami, states that sesame oil helps increase the level of neurohormones like dopamine, serotonin, epinephrine, and endorphins and reduces the response to pain sensation [7].

Herbs like Rajika (*Brassica juncea*) seed[8], Sigru (*Moringa oleifera*) bark[9], Shallaki (*Boswellia serrata*) exudates[10], Bala (*Sida cordifolia*) wh pl.[11], Gokshura (*Tribulasterrestris*) fruit, Kantakari (*Solanum virginianum*) leaves[12], Nirgundi (*Vitex negundo*) leaves[13], Ashwagandha (*Withaniasomnifera*) root[14], Rasna (*Alpinia calcarata*) Rhizome[15], Hingu (*Ferulaassa-foetida*) exudates, Kupilu (*Nux vomica*) seed[16] have also pain relieving properties.

Irritation and corrosion study

Assessment of dermal irritation/corrosion of Cannaquic™ is a significant step in the evaluation of dermal toxicity. At a dose of 0.5ml, the initial test could not show any sign of toxicity which was confirmed by testing two more animals (rabbits) fora 14-day observation period. Weight of individual animals was also increased from 0 to 14 days.

No reversibility and local toxic effects occurred before 14 days of the observation. The Score of grading skin reaction was calculated for each rabbit. Scores for erythema and oedema at 60mins, 24 hours, 48 hours, and 72 hours were summed, and the number of observations for the treated sites. Oedema and erythema with a score of 0 were found in all the animals on sites with Cannaquic™ (Table 2). Hence there was no oedema and erythema observed in the animals.

Grading of Skin Reaction								
Animal No.	**60 Mins.**		**24 Hrs.**		**48 Hrs.**		**72 Hrs.**	
	E	**Oe**	**E**	**Oe**	**E**	**Oe**	**E**	**Oe**
RB01	0	0	0	0	0	0	0	0
RB02	0	0	0	0	0	0	0	0
RB03	0	0	0	0	0	0	0	0

Score	00	00	00	00	00	00	00	00
Hours	01		24		48		72	

*__E__ = Erythema, **Oe** = Oedema, **0** = No reaction

Table 2: Individual score on the skin following application of test substance to the rabbits Grading of Skin Reactions Test.

Skin sensitization test

During the study no animals (Guinea pig) died. There is no significant decrease in animal body weight in the treatment group compared to that of body weight of the control group. There were no clinical symptoms observed during the entire treatment period before and after the treatment. In the present study, no evidence of sensitization was observed (Table 3). The result of the study showed that Cannaquic™ does not cause any sensitization in animals.

			Control Group	**Test Group**
	Day	**Hour**	**Gauge Patch**	**Cannaquic™**
INDUCTION PHASE	Day 01	0 Hr	0	0
		6 Hrs	0	0
	Day 02	0 Hr	0	0
		6 Hrs	0	0
	Day 03	0 Hr	0	0
		6 Hrs	0	0
	Day 08	0 Hr	0	0
		6 Hrs	0	0
	Day 09	0 Hr	0	0
		6 Hrs	0	0
	Day 10	0 Hr	0	0
		6 Hrs	0	0
CHALLENGE PHASE	Day 15	0 Hr	0	0
		6 Hrs	0	0
	Day 16	0 Hr	0	0
		6 Hrs	0	0
	Day 17	0 Hr	0	0
		6 Hrs	0	0

Table 3: Summary of Skin observation during the induction phase and challenge phase

Evaluation of Anti-inflammatory activity

Sl No.	**Samples**	**Concentration tested (µg/mL)**	**% Reduction level of TNF-α**	**% Reduction level of Nitric Oxide (NO)**	**% Reduction level of IL-6**
1	LPS + CANNAQUIC™	5+1000	36.51±2.65	83.02±0.85	98.31±3.39
		5+500	15.46±0.82	67.92±0.85	63.56±5.93
2	LPS CONTROL	5	-	-	-

Table 4: Anti-inflammatory effect of Cannaquic™ in Mouse Macrophage (RAW 264.7) cell line.

Cannaquic™ was evaluated for its cytotoxicity with seven different concentrations on Mouse Macrophage (RAW264.7) cell line. The safest concentration of the pain-relieving oil that exhibited less than or equal to 20% cytotoxicity was selected for performing anti-inflammatory studies.

IL-6, Nitric Oxide (NO), and TNF-α are the three major molecules that are upregulated and increased at the site of injury and inflammation and carry the signal to the brain for pain. It has also been found that pain score is directly related to the concentration of these 3 signaling molecules, so considered as biomarkers for measuring pain.

Cannaquic™ exhibited a significant reduction in markers i.e., IL-6, Nitric Oxide (NO), TNF-α. In TNF-α estimation assay, it exhibited a percentage reduction by 36.51±2.65%, 15.46±0.82% at 1000µg/mL, 500µg/mL respectively as compared with LPS. In Nitric oxide estimation assay, it exhibited a percentage reduction by 83.02±0.85%, 67.92±0.85% at 1000µg/mL, 500µg/mL as compared to the LPS control. In IL-6 estimation assay, exhibited a significant percentage reduction by 98.31±3.39%, 63.56±5.93% at 1000µg/mL, 500µg/mL as compared to the LPS control. (Table 4)

CONCLUSION

From the above studies, it could be concluded that Cannaquic™ -a pain relieving oil is completely safe and exhibits no dermal toxic effects and does not cause any adverse effects on the skin. This formulation also showed a significant anti-inflammatory effect on animals. Further studies are required in humans on efficacy in relieving different types of pain in humans.

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