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EVALUATION OF ANTI-INFLAMMATORY INFLUENCES OF SAUSSUREA LAPPA ROOTS ETHANOL EXTRACT

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ABSTRACT: Long time ago, phytotherapy constitutes a major health practice all over the world. Many medicinal plants such as Saussurea lappa (S. lappa) has wide beneficial roles. This study was contrived to explore the antiphlogistic effect of S. lappa roots extract treated by 100% ethanol solvent. Carrageenan induced paw edema model was used to carry out the experiment on laboratory Wistar albino rats. Before testing extract on animals, it was necessary to check the safety of the plant according to performed testing conditions albeit S. lappa is generally recognized as safe plant, hence the acute oral toxicity test by the fixed-dose procedure method (FDP) was performed. Roots' ethanol extract tested at two doses (100 and 200 mg/kg), ibuprofen used as a positive control, and negative control was distilled water. SPSS statistical analysis was undertaken to check the variance between tested groups via one-way analysis of variance, then pursued by Tukey and Dunnett two-sided tests. FDP experiment disclosed that extract was safe for further in-vivo testing for the dose up to 2000 mg/kg < LD50 < 5000 mg/kg, without reporting of any toxicity signs. The extract manifested a significant ($P < 0.05$ - 0.01) anti-inflammatory effects at both tested doses. The ethanol extract exposed a clear decrease in edema size by a percentage of 13.73 % at 200 mg/kg dose when compared to that of standard agent which minimized edema by 22.79 %. It is presumed that plant roots contain phytoconstituents that are responsible to share this effect like sesquiterpene lactones (dehydrocostus lactone and cynaropicrin mainly), in which many researches revealed that such compounds which found in many other plants have a potent anti-inflammatory action.

KEY WORDS: Anti-inflammatory effect, Saussurea lappa, Ethanol extract, Soxhlet extraction.

I. INTRODUCTION

Inflammation is a protective reaction served by body tissues to react against harmful stimuli such as chemical irritants, trauma, injury, and infection of pathogens [1]. It constitutes a part of complex biological responses which involve the intervention of particular cells and inflammatory and molecular mediators of body tissues to get rid of the cause of this reaction, to eliminate damaged cells and tissues, and to help in the healing process [2]. Medically, the major symptoms and signs of inflammation involve redness, heat, swelling, pain, and loss of function [3]. In case of acute inflammation, these merits resulted from increased flow of plasma and white blood cells from the blood through blood vessels into the harmed tissues, in turn, tissue causes liberation of certain chemical substances that result in a full picture of inflammation, this reaction considered as a mechanism of innate immunity [4].

There are many causes of acute inflammation, such as injury or bacterial infections like acute tonsillitis [2]. The body tissues and cells try to control the situation through specific immunity reactions which lead to involvement of major cells such as neutrophils (primarily), basophils (inflammatory response), eosinophils (response to helminthes and parasites), and mononuclear cells (monocytes and macrophages), with participation of primary mediators such as vasoactive amines and eicosanoids [5]. After its immediate onset, the acute course continue for few days to end into one of these consequences: it may resolve, fate in abscess formation, or progress to chronic inflammation, therefore, in many cases there is a need of intervention to prevent such consequences and to mitigate associated pain, thence the using of synthetic anti-inflammatory drugs [6].

Although the success of human trials towards developing many potent anti-inflammatory treatments, the already manufactured and used drugs lack to be free of undesirable side effects [7]. Non-steroidal anti-inflammatory drugs (NSAIDs) and some of analgesic and antipyretic drugs like paracetamol which exerts a weak anti-

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inflammatory effect, remain the most widely used treatments to grapple inflammation [8]. They exert potent pharmacological effects to lessen the pain, fade accompanied fever, block blood coagulation and constringe inflammation, but the issue they induce undesirable side effects, for instance, gastrointestinal tract conditions, heart attacks, and kidney disease and many others [9]. Furthermore, paracetamol and NSAIDs can lead to drug interactions [10]. For instance, people who treated chronically by anticonvulsants showed an increased rate of hepatotoxicity [11]. Also, it is reported that the use of NSAIDs with quinolones antibiotics like ciprofloxacin initiates some perils upon central nervous system and increases side effects of quinolones [12]. Thus, evolving of new potent, safe anti-phlogistic agent is of great importance. Phytotherapy sounds to be a cogent settlement.

Phytotherapy embraces the use of medicinal plants as a treatment for various health conditions in traditional medicine [13]. According to the World Health Organization, nearly eighty percent of Asian and African countries depending on using medicinal herbs in many health conditions [14]. For its prominent potency that recorded over a long time without noticed side effects, it shows successful spread all over the world [15]. Many evidence-based pharmaceutical drugs are manufactured and used in modern medicine are derived from beneficial plants [16]. Despite enormous beneficial roles of phytomedicine, however, approximately twenty-five percent of modern drugs used in the US have been made from plants, which indicates a few fully developed clinical trials when compared to chemical manufactured pharmaceuticals [17]. Medicinal plants contain many phytoconstituents that can be extracted, purified, and used as natural chemical treatments, for instance, clinicians used to deal with plants for many health conditions, such as using of ginger and rosemary for inflammatory conditions [18]. Saussurea lappa (S. lappa) is one of the important medicinal plants that supposed to shows many beneficial medical activities, including anti-inflammatory effects [19].

Previously, S. lappa utilized by Asian herbalists to treat numerous conditions, for example, peptic ulcer, asthma, hack, and pharyngitis [20]. Indeed, even it is utilized as pain calming, septic agent, and anti-tumor treatment [14]. This plant broadly utilized among ethno-pharmacological plants in Shopian backwoods of Kashmir. S. lappa family is generally spread in the Indian subcontinent [21]. It is valuable in the treatment of numerous ailments as indicated by Ayurveda clinicians [22]. Previous studies revealed that S. lappa has an incredible collection of secondary metabolites, like costus oil which comprised primarily of sesquiterpene lactones (dihydrocostus lactone [15 %] and costus lactone [10 %]), sesquiterpenes lactones has to share its role as anti-asthmatic, pain reliving and anti-tumor agent [23]. Despite robust research on activities of medicinal plants, the absolute profile of comprehensive effects of plants still incomplete, such thing makes researchers necessarily to be wrapped up for working hard to scout more, thus, the current research aimed to detect anti-inflammatory properties of S. lappa for clinical applications.

II. MATERIALS AND METHODS

The experimentation and testing methodology were affirmed and supported by the research and management center (RMC) of Management and Science University (MSU), Malaysia. Ethical approval was granted under the number: MSU-RMC-02/FR01/05/L3/014.

S. lappa dry roots were bought from G-M herbal shop, Shorkot city, Punjab, Pakistan. According to the seller, it was cultivated from Jammu and Kashmir area after the rainy season, enclosed well and transported in the contained jar and preserved naturally at room temperature. It is known domestically as Kuth.

Specimen of the plant was sent to the forest research institute of Malaysia (FRIM) to be identified and authenticated for plant taxonomy under report number FRIM (S).600-5/6/1 Klt. 2 (40). The institute confirmed the sample of the plant (HSID 006/19) through high-performance thin-layer chromatography (HP-TLC), as reported in the 17th edition of Japanese Pharmacopoeia.

2.1. Plant extract preparation

Mainly, the extraction followed conventional Soxhlet extraction protocol without any modification. The roots were clean and dried well at ambient room temperature as the air drying does not force sample to dry, thus it can preserve heat-labile compounds. Firstly, they smashed into small pieces as they are very tough, then crushed and grounded into fine powder by using a blender to ensure the best treatment with the solvent. 50 g of roots powder poured in the cellulose thimble which placed inside Soxhlet apparatus, and the flask filled up by 300 ml of 100 % ethanol solvent. This study was originally contrived to evaluate effect of S. lappa extract on acute tonsillitis' causative organism, associated inflammation and pain, hence the main cause of acute tonsillitis is streptococcus pyogenes the ethanol was selected as it exhibits greater activity against bacteria than other solvents with no reported cellular toxicity [40].

After the fixation of the apparatus, the heater started running at 70 °C. The extract inside the flask was filtered with No. 1 Whatman filter paper, then placed into the rotary evaporator to get rid of the used solvent. The rotary bath heating was adjusted on 45 °C, and the pressure settled on 670 hpa for few hours until the dark brown

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sticky material was precipitated on the walls of the rotary flask. The extracted material was taken by spatula, weighed and preserved in dark container at 2-8 °C refrigerator. For experimentation purposes, the extract dissolved in distilled water as it is found to be highly soluble.

2.2. Experimental animals

As stated above, this project was initially designed to test plant extract on acute tonsillitis' anti-phlogistic, toxic and anti-nociceptive properties, therefore, throughout experimentation the mice were selected, except for determination of anti-inflammatory effect, in which rats were selected as they are preferred for reliability and validity purposes. Twelve female ICR albino mice of Swiss strain and twenty four young Wistar albino rats of either sex randomly selected. All mice and rats obtained from KRK SERI ENTERPRISE under MSU lab supervision, weighing between 26-30 g and 200-300 g respectively.

The animals lived at standard living conditions and allowed to accommodate to the environment for seven days before experimentation. Animals fasted on a diet only overnight before the experiments. All mice collected in two groups for studying of acute toxicity, one group served as weight control, and rats were grouped into four sets accordingly, in separate sterilized polyethylene cages (MSU standardized cages).

2.3. Fixed-dose procedure (FDP)

Acute toxicity study subjected to rules of Organisation for Economic Cooperation and Development (OECD) guidelines 420 (Fixed single-Dose Procedure-FDP). These guidelines comprise four important steps; half lethal dose (LD50) estimation, body weight measurement, observation and monitoring of physical signs of toxicity, and evaluation of gross pathological changes for both sighting and main studies [24]. Routinely, this experiment was processed to check safety of target extract according to performed extraction under adjusted parameters despite that S. lappa is generally recognized as safe (GRAS) by United States food and drug administration (FDA) [25]. The experimentation was done following FDP protocols without any changes, and the mice was selected as they are more sensitive than other rodents, beside that rats lack tonsils and gall bladder which constitutes main segment in detection of toxicity particularly for testing of extract that planned to be used for treating of acute tonsillitis cases.

2.4. Carrageenan induced hind paw edema method

This model used to assess the anti-inflammatory activity in-vivo by the principle based on the determining of reduction of edema size diminished by antiphlogistic agents. This method employs using of 1.0 % carrageenan as a phlogistic agent. It is the most common used paradigm to investigate the anti-inflammatory properties of chemical agents and plant extracts [26, 27]. The experiment started by preparation of carrageenan 1%. 1 g of powder form of carrageenan (lambda or sigma type) poured slowly to 100 ml distilled water inside 200 ml Schott bottle and not mixed (pour the powder slowly on the water surface). The bottle kept for seven days before test; then, it used within three days.

Preparing of four groups of rats, with six of either sex in each group and organized as follows, Group 1: control (called negative control group, was on feeding by vehicle only which is distilled water), group 2 and 3: fed by ethanol extract of plant roots (100 and 200 mg/kg respectively), and group 4: positive control, treated by ibuprofen 40 mg/kg. All the last groups fed orally and let for absorption for thirty minutes to one hour before subcutaneous (s/c) injection of 0.1 ml carrageenan (1% w/v in normal saline) in the right paw planter surface; left hind paw is considered as control. Note that each hind paw of all animals should be marked at the level of tibio-tarsal junction, hence during measuring, all values controlled and read at the same level. Measuring the extent of paw edema was done at certain time intervals of one, three, and five hours after carrageenan injection, followed by recording and comparison of results of treated groups compared to the control group to estimate extent of percentage of inhibition.

The percentage of inhibition of inflammation as follow:

$$\text{Percentage of inhibition (\%)} = (Vc - Vt) / Vc * 100 \quad (1)$$

Where Vc = edema volume in control, Vt = edema volume in groups treated with test substance and NSAIDs.

Finally, all results expressed as the mean ± standard error of mean, then analyzed for statistical variance at significance level ($P < 0.01$) by one way ANOVA using SPSS version 25, followed by post hoc tests to specify the exact mean differences magnitude and to show a comparison between them [28].

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III. RESULTS

3.1. Acute toxicity experiments results

As per the Commission of the European Communities (1983), Council Directive 83/467/EEC, the classification of chemical lethality is classified and termed 'unclassified' when the half lethal dose is more than 2000 mg/kg [29]. S. lappa roots ethanol extract is considered a safe plant as it falls in category five on the globally harmonized system (GHS) for the classification of chemicals that place acute toxicity.

The limit dose was found to be unharmed for > 2000 mg/kg upon being given orally to mice. Therefore, the conclusion of the testing dose will be 2000 mg/kg < LD50 < 5000 mg/kg. In any case, it isn't viewed as a point gauge as the FDP-used protocol was not initially intended to decide a point value of LD50, and also as confidence limits are not assessed. Be that as it may, a dependable guideline was built up that allows a rough LD50 estimate to be induced from the classification that outcomes from FDP.

Moreover, the plant extract did not affect the weights of tested mice, did not exhibit any toxic signs, and did not cause any abnormalities upon gross necropsy. Upon following-ups of the weights of experimental animals, which considered as a main part of the study, all animals showed normal weights and normal gaining of weight during testing time. All mice showed normal routine of life upon eating, drinking, micturition, defecation, life activities and weight gain, neurological reflexes, and vital signs, and the plant dose did not affect the animals upon last parameters. Thus, this plant considered to be safe for consumption.

3.2. Results of carrageenan-induced hind paw edema method

Outcomes of testing of anti-inflammatory effects are presented by mean ± SEM on *Table* 1. The results of performed experiments on S. lappa 100% ethanol extract in comparison to ibuprofen (standard drug) disclosed that the dose of plant extract of 200 mg/kg showed its highest inhibition (13.73 %) when compared to that of standard (22.79 %) despite that other tested concentrations of 100 mg/kg showed effects pattern nearly similar to that of 200 mg dose.

Table 1: The mean effects of 100% ethanol extract of *S. lappa* roots against carrageenan paw edema (cm) in rats.

Treatment tested	Dose (mg/kg.bw)	Edema size (cm) after carrageenan injection					
		After one hour		After three hours		After five hours	
Distilled water (-ve) control Group1	1ml/100gm	3.35 ± 0.07	% of inhibition	3.60 ± 0.07	% of inhibition	3.86 ± 0.04	% of inhibition
Plant extract 1 Group2	100 mg/kg	3.51 ± 0.08	4.77 %	3.45 ± 0.04	4.16 %	3.40 ± 0.05	11.91 %
Plant extract 2 Group3	200 mg/kg	3.48 ± 0.06	3.88 %	3.35 ± 0.04	6.94 %	3.33 ± 0.03	13.73 %
Ibuprofen (+ve) control Group4	40 mg/kg	3.45 ± 0.09	2.98 %	3.33 ± 0.06	7.5 %	2.98 ± 0.09	22.79 %

Values are expressed in means ± SEM, n=6, group differences determined by one-way ANOVA at p-value <0.01, followed by post hoc Tukey and Dunnett tests.

From one to three hours after carrageenan injection, it seems that there is somehow effect anti-inflammatory effect for both tested plant groups, then after five hours, a clear response starts to appear especially for the second plant group.

Statistically, checked significant values with their comparison through analysis of variance by ANOVA, it found the significant value was < 0.0005 with F-value of 7.626, which means there is significant difference between the tested groups. By inspecting the post hoc tests, the significance (Sig.) value was ‘0.040‘ between plant group two and positive control, in which it is lower than our significance threshold of P < 0.05. Hence, we should dismiss the invalid theory and acknowledge the alternative speculation. Therefore, after administration of plant

extract to rats, there was a significant difference in reduction of rats paw edema between two experimental groups (F (3, 71) = 7.626, P < 0.01), specifically, the negative control (3.6 ± 0,267), plant extract (3.456 ± 0.154) and positive control (3.25 ± 0.287) groups were significantly different from each other (P < 0.01 and 0.05).

The Tukey HSD and Dunnett two-sided tests used to evaluate the significant differences magnitude and direction between four groups. According to Tukey HSD test, there is differences between plant extract of 200 mg dose and negative control group, plant extract of 100 mg dose to positive control groups, and there was no significant difference between 200 mg extract group and positive control group. Two-sided Dunnett t-test showed the significant differences between plant extract test groups as stated with Tukey HSD test. Important to know differences in the checked parameters and criteria reveals that the extract of 200 mg/kg dose resembles the same affectivity of the positive control group as both of them showed no significant differences to each other. To elucidate these results, next *figure* 1 revealed Clustered bar means of tested groups.

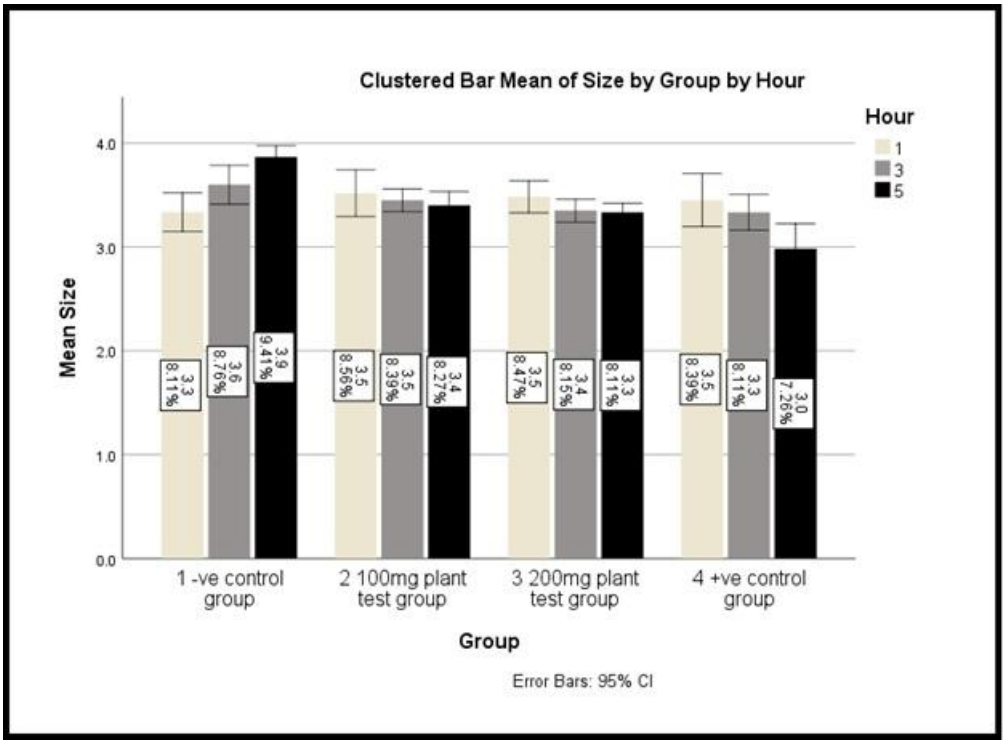

Figure 1: Clustered bar means of paw edema size reduction effect of two concentrations of S. lappa extract (100 and 200 mg) recorded in three-time intervals after one, three and five hours as it compared to negative control (distilled water) and positive control (Ibuprofen 40 mg).

From the diagram, there is an instant increase in paw edema size constantly with time for negative control group which was on distilled water feeding, this suggests the inflammation was progressed with time in the absence of treatment, both of 100 and 200 mg/kg test plant groups revealed decrease in edema size upon time of experiment similar to way of ibuprofen, with the more prominent decrease in edema size of ibuprofen group after five hours. This effect indicates that S. lappa extract was effective to decrease paw edema size and to reduce inflammation process.

IV. DISCUSSION

Carrageenan-incited paw edema model is a typical critically predictive technique utilized in pharmacology labs for examination of the mitigating action of a specific plant extract on inflammation [30]. To understand the effect of anti-inflammatory treatments in real-time experiments, we need to understand the process of inflammation, which is a local response of tissue and cells to injury that is signed by capillary dilatation, leukocyte infiltration, redness, swelling, a local increase in temperature, and pain of several degrees, this process acts as a natural protective mechanism to get rid of the noxious agent to help in the repair process.

The inflammation process is divided into two stages; an early stage starts few minutes after inflammation induction, remains a time, then begins to decrease after one hour and lasts for two hours; both histamine and serotonin are dominating mediators of this stage. Their activities appear clearly in the propagation of hyperemia at the site of inflammation. The last stage starts shortly after the first one and lasts for five hours. The later stage

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appears as a delayed edema and leukocytes accumulation due to the release of prostaglandins (PGs) and bradykinin (BK), which are responsible for increased vascular permeability, thus granuloma formation [31]. Thus, to initiate inflammation, we need a unique material that can demonstrate inflammation as a real one. Carrageenan is a potent phlogistic substance that exhibits a clear picture of inflammation even to a lesser extent at minimum concentration, hence the carrageenan model considered as a very sensitive model to estimate provoked inflammation by herbal substances. Carrageenan-induced edema, pain, and fever resulted as a consequence of the release of inflammatory mediators such as PGs (PG-E mainly), cytokinin, bradykinins, histamines, leukotrienes, and serotonin [32]. Carrageenan induces the release of inflammatory mediators, which in turn increases vascular permeability and thus enhances the flow of leukocytes at the site of inflammation. Therefore any interruption of this pathway will results in the provoked release of mediators, thus decreased inflammation response [33].

From tabulated results, it was clear that the extract decreased the size of edema at both stages of the experiment, more notable between three and five hours, which indicates that the cut off process due to affection on PGs and BK happen at late phase. The plant extract exhibited a good antiinflammatory effect at a dose of 200 mg. In a comparison to ibuprofen, the plant extract illustrated a 11.91 % of inhibition at dose of 100 mg/kg during five hours, while it exhibited a 13.73 % of inhibition at dose of 200 mg/kg during the same time, and ibuprofen exhibited a 22.79 % of inhibition at dose of 40 mg/kg during the same time, these outcomes indicates that S. lappa ethanol extract has somehow similar potency to that of ibuprofen. Both steroidal and non-steroidal antiphlogistic drugs are important treatments to influence edema in the second phase by various activities. Pharmacology studies reported that the vast majority of NSAIDs affects mainly the second stage [34]. Steroidal medicaments reduce the vasodilation which happens during aggravation, while non-steroidal ones block prostaglandin and thromboxane makeup by repressing of cyclooxygenase activity [35].

Theoretically, for the plant to possess an anti-inflammatory effect, it should have phytoconstituents that mediate the action. S. lappa has many secondary metabolites which exert anti-inflammatory activity through interruption of production of inflammatory mediators [36]. According to phytochemical studies about phytoconstituents of the plant roots, the major compounds are sesquiterpenes lactones (costunolide and dehydrocostus lactone). Preceding studies disclosed that these compounds which found in many plants were used traditionally for anti-inflammatory and anti-cancer purposes [37]. Moreover, another study suggested that S. lappa had ability to show significant dose-dependent inhibition of transudative phase dosed between 25–100 mg/kg, hence the anti-inflammatory activity of the sesquiterpenes lactone fraction of S. lappa suggested to be due to stabilization of lysosomal membranes and an antiproliferative effect [38]. Also it is noted that anti-inflammatory effect of the extract increased by progress of time. Not sesquiterpenes alone, many secondary metabolites reported to reveal anti-inflammatory effects, such as flavonoids, alkaloids, glycosides and tannins. These constituents were found in S. lappa phytoconstituents studies [39].

Based on recorded findings, previous literature reports, and scientific speculation, S. lappa ethanol extract seems to follow the inhibition of cyclooxygenase pathway and inhibition of PGs formation, and according to these results, this plant can be considered as a compelling therapeutic source to fix in intense fiery phlogistic issues.

V. CONCLUSION

Nowadays, phytotherapy attests to wide turnouts around the world as the people experienced and believes in its potent natural desired effects from too-old eras until now. Humankind used to depend on many plants to treat many conditions without the aforementioned side effects. Therefore, many pharmaceutical companies took upon their selves to research and develop medicines from plant phytoconstituents, and they got successful trials which encourage researchers to work hard and seek for more applications of medicinal plants in related fields. This study converged on studying the anti-inflammatory effectiveness of S. lappa 100 % ethanol extract. The roots extract showed antiphlogistic leverage at 200 mg/kg dose of 13.73 % of inhibition of growth of inflammatory edema during the late stage of experiment, while ibuprofen exhibited a 22.79 % of inhibition at 40 mg/kg dose for the same experimentation time. These findings emphasize that S. lappa plants contain particular secondary metabolites that were responsible for exerting this action. These feedbacks promote and motivate investigators to seriously taking this plant for further studies to investigate potency and mechanisms of action at clinical studies.

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