Study of Antiphlogistic Effect of Saussurea lappa Roots' Ethanol Extract in Comparison to Paracetamol Effect

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Abstract

The current research was designed to investigate the anti-inflammatory action of 100% *Saussurea lappa* roots' ethanol extract compared to paracetamol through carrageenan induced hind paw edema model with using of ibuprofen 40 mg/kg as a positive control. Prior to testing, it was important to check the acute toxicity effects of the plant, thus the evaluation test processed by the fixed-dose procedure (FDP). Statistical analysis was attempted to check the mean differences via one-way ANOVA, at that point followed by post-Tukey and Dunnett two-sided tests. The FDP test revealed that extract was safe for additional *in-vivo* testing, and it was classified as category 5/unclassified on globally harmonized system (GHS) for the classification of chemicals which cause acute toxicity. The extract showed a significant antiphlogistic potential of 15.11% at 500 mg/kg compared to 13.23% of paracetamol 100 mg/kg at $P< 0.01$. It is assumed that the fundamentally mindful phytoconstituents to share this impact are sesquiterpene lactones (costunolide and dehydrocostus lactone), in which numerous examinations uncovered that these mixtures which found in numerous plants have a powerful anti-inflammatory activity.

Key words: Anti-inflammatory effect, paracetamol, *Saussurea lappa*, Soxhlet extraction

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Introduction

Inflammation is a local response of living body tissues and cells to injury that is signed by capillary dilatation, leukocyte infiltration, redness, swelling, local raise in temperature and accompanied pain of several degree, this process acts as natural protective mechanism to get rid of noxious agent to help in repair process [1, 2].

There are numerous reasons for irritation and inflammation, for example, injury or different microbial infections. The body tissues and cells attempt to control the circumstance through explicated immunity responses which lead to association of specific immunity cells like neutrophils (essentially), basophils (phlogistic reaction), eosinophils (reaction to helminthes and parasites), mononuclear cells (monocytes and macrophages), primary mediators like vasoactive amines, and eicosanoids [3]. After its nearby beginning, the acute course proceed for not many days to end into one of these outcomes: it might resolve, or end in abscess formation, or progress to persistent aggravation and chronic course, thusly, there is a need of intercession to forestall such end and to alleviate related agony, thereupon the utilizing of engineered anti-inflammatory drugs [4].

The inflammation interaction is partitioned into two phases; a beginning phase begins few moments after irritation, at one hour point it starts to diminish and goes on for two hours, both of histamine and serotonin are ruling mediators of this stage. Their missions show up plainly in engendering of hyperemia at the site of aggravation. Then the last stage begins soon after initial one and goes on for five hours. This stage shows up as a postponed edema and leukocytes collection because of arrival of prostaglandins and bradykinin which are capable to increment vascular permeability, subsequently granuloma development. Both of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are significant medicines to influence edema at second stage by various activities [5]. Pharmacological examinations announced that larger part of NSAIDs influences mostly second stage. Steroidal drugs lessen the vasodilation which happens during irritation, and NSAIDs block prostaglandin and thromboxane formation by repressing cyclooxygenase potential [6].

Albeit the achievement of human preliminaries towards creating numerous intense anti-inflammatory therapies, be that as it may, the generally produced and utilized medications need to be liberated from side effects. NSAIDs and other antipyretics with less efficiency anti-inflammatory action like paracetamol, which applies a feeble mitigating impact, stay the most broadly utilized medicines to catch aggravation. They apply powerful pharmacological impacts to diminish pain, decline went with fever, forestall blood coagulation and lessen inflammation, and however the issue they instigate unwanted side effects and complications, for example, gastrointestinal ulcers, kidney sickness and numerous others added to drug interactions, for instance, individuals who consume NSAIDs and treated constantly by anticonvulsants showed an expanded pace of hepatotoxicity, likewise, it is accounted for that the utilization of NSAIDs with quinolones antibiotics like ciprofloxacin increment the danger of quinolones' unfavorable central nervous system impacts [7, 8]. Subsequently, advancing of new intense, safe therapy that can substitutes paracetamol or NSAIDs is of extraordinary significance and brings responsibility on concerned specialists in the field to fix the issue. Phytotherapy sounds to be a fitting settlement.

Herbal drugs and phytotherapy have been a principal part of human health care for a long period due to their therapeutic properties [9]. Traditional medicinal plants have been used for thousands of years [10], and different human civilizations have their medicinal plant library [11, 12]. According to the world health organization, the majority of the world's population relies on traditional medicine for their primary healthcare needs [13]. Ancient medical literature of our humanity disclosed that people used to deal with medicinal plants due to their obvious potent effects in both humans and animals. Traditional herbal medicine continued to gain its importance due to its wide therapeutic applications, it earned this prominence from plants' natural chemical composition, i.e. secondary metabolites. Some relative knowledge about many medicinal plants and their mechanism of action enables clinicians to use them as natural remedies [14].

Medicinal plants have powerful experienced efficacy with no or seldom encountered side effects, for that higher majority of people tend to depend on them, and about eighty percent of worldwide people mainly Asians are on use of these plants for their primary healthcare needs according to world health organization [15]. Approximately twenty five percent of modern drugs used in the United States have been made from plants, which may indicates a few fully developed clinical trials when compared to manufactured chemical pharmaceuticals [16]. Furthermore, one of the main factors that increased using of phytotherapy is the poverty and natural widespread of plants on the earth, this availability reflects the easy ability of patients to get them. A related scientific studies mentioned some pros of these medicinal plants such as their low cost and ease of use away from complicated health systems and hard transportation facilities that obstruct the way [17, 18].

Nowadays, there is an increase in consumers' interest and trust in natural and organic products, particularly medicines from natural sources [19, 20] due to their diversity of chemical components which make them enormous therapies without chemical side effects [21]. At the end of 19th century, many laboratories' trials begin experiments of investigation on medicinal plants. In fact, many phytoconstituents were the origin of medicinal treatments and man-made drugs. *Saussurea lappa* (*S. lappa*) is one of the important medicinal plants that experienced to shows many beneficial medical activities, including anti-inflammatory effects [22].

S. lappa considered as one of the important medicinal plants that played a prominent role in phytotherapy field and widely used in various medical systems mainly Ayurveda, but still there is a dearth of ultimate knowledge about medicinal use of this plant [23], for which reason, this study was destined to seek for anti-phlogistic effects of 100% *S. lappa* roots' ethanol extract for preclinical investigation, and to compare it to paracetamol anti-inflammatory action compared to ibuprofen as positive control.

Testing methodology

The proposal, study design and testing methodology were checked and permitted by the research and management center (RMC) of Management and Science University (MSU), Malaysia. Ethical approval was issued under the number: MSU-RMC-02/FR01/05/L3/014.

S. lappa dry roots were bought by the Kuth domestic name from G-M herbal shop, Shorkot city, Punjab, Pakistan. 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*.

Plant extract preparation

The roots were dried well by normal air drying at room temperature to preserve heat labile chemicals. First and foremost, they crushed by pestle into little pieces as they are extremely intense, at that point squashed and ground into fine powder by utilizing a blender to guarantee the best reaction with the solvent. Then 50 gm of roots powder poured in the cellulose thimble, and Soxhlet flask filled by 300 ml of 100% ethanol solution. After the obsession of the device, the warmer began running at 70°C. The extract was filtered with No. 1 Whatman paper, at that point putted into the rotary evaporator to dispose of the pre-owned solvent. The rotatory water bath adjusted to heat on 45°C and on pressure of 670 hpa for few hours until the dark brown sticky material was encouraged on the dividers of the rotary flask. The accompanying material was taken by spatula, gauged and saved in dark container at 2-8°C fridge.

Experimental animals

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 gm and 200-300 gm 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. Mice were collected in two groups for studying of acute toxicity, and rats were divided into four groups of six rats in each group, lived in separate sterilized polyethylene cages (MSU standardized cages).

Acute toxicity evaluation study

Acute toxicity study processed according to the organization for economic cooperation and development (OECD) guidelines-420 by fixed single-dose procedure (FDP). These guidelines comprise four important steps; half lethal dose (LD_{50}) estimation, body weight measurement, observation and monitoring of physical signs of toxicity, and evaluation of gross pathological changes [24].

Carrageenan induced hind paw edema method

This model based on the reduction of edema caused by phlogistic agents such as 1.0% carrageenan. It is commonly used as *in-vivo* model to investigate the anti-inflammatory properties of chemical agents and plant extracts [25, 26].

Rats separated in four groups, with six of either sex in each group and organized as follows: group 1: control group (negative control, was on feeding by vehicle only), group 2: fed by 100% *S. lappa* roots' ethanol extract orally (500 mg/kg), group 3: was on feeding by paracetamol 100

mg/kg (comparison test group), and group 4: positive control, treated by ibuprofen at a dose of 40 mg/kg.

After oral feeding of plant extract and test substances, all the groups let for absorption for thirty minutes to one hour before subcutaneous injection of 0.1 ml carrageenan (1% w/v in normal saline) in the right hind paw planter surface; left hind paw was 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 of 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, i.e., the difference between initial and subsequent paw volume readings is the actual edema volume and the percentage of inhibition of inflammation calculated as follow:

$$\text{Inhibition } \% = (Vc - Vt) / Vc * 100 \quad [1]$$

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

Finally, all results were expressed as the mean ± SEM. The results were analyzed for statistical significance ($P < 0.01$-0.05) by one way analysis of variance (ANOVA), then followed by Tukey and Dunnett post hoc tests to specify the exact mean differences magnitude and to show a comparison between them [27].

Results

Acute toxicity study findings

S. lappa roots ethanol extract was considered a safe plant as its evaluation presented in category five on the globally harmonized system for the classification of chemicals that cause acute toxicity according to the commission of the European communities, council directive 83/467/EEC [28]. The limit dose was higher than 2000 mg/kg upon being given orally to mice. Therefore, the conclusion of the testing dose will be 2000 mg/kg < LD_{50} < 5000 mg/kg. Moreover, the plant extract did not affect the daily life and 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, neurological reflexes, and vital signs. Thus, this plant considered to be safe for consumption.

Carrageenan-induced hind paw edema

As the paracetamol is highly used as a sedative anti-inflammatory treatment for acute tonsillitis cases, therefore, the upcoming part presented and discussed results of assessment of

plant roots' extract at 500 mg/kg dose and paracetamol 100 mg/kg. Next Table I provided results of related experiment.

Table I: The rat's paw edema size (cm) mean after one, three and five hours from carrageenan injection to test anti-phlogistic effect of 100% *S. lappa* roots' ethanol extract at 500 mg/kg dose compared to paracetamol 100 mg/kg and other controls

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 Group 1	1ml/100gm	3.35 ± 0.07	% of inhibition ➔-24.07 %	3.60 ± 0.07	% of inhibition ➔ -7.46 %	3.86 ± 0.04	% of inhibition ➔ -7.22 %
Plant extract Group 2	500 mg/kg	3.55 ± 0.07	-5.97 % ➔ -31.48 %	4.63 ± 0.10	-28.61 % ➔ -30.42 %	3.93 ± 0.08	-1.81 % ➔ **15.11 %**
Paracetamol Group 3	100 mg/kg	3.55 ± 0.05	-5.97 % ➔ -31.48%	3.55 ± 0.05	+1.38 % ➔ 0.00 %	3.08 ± 0.04	+20.20 % ➔ **13.23%**
Ibuprofen (+ve) control Group 4	40 mg/kg	3.45 ± 0.09	-2.98 % ➔ -27.77 %	3.33 ± 0.06	+7.50 % ➔ 3.47 %	2.98 ± 0.09	+22.79 % ➔ **10.51 %**

Values were expressed in means ± SEM, n=6, group differences determined by one-way ANOVA at p-value <0.01and <0.05 followed by post hoc Tukey and Dunnett tests. (➔) Sign denoted to percentage of enhancement of edema inhibition compared to previous reading, (-) sign meant negative effect (increased paw edema size), (+) sign meant positive effect (decreased paw edema size)

From listed results, *S. lappa* extract at 500 mg/kg dose showed no effect to decrease paw edema all the experimenting time when compared to both paracetamol and ibuprofen as percentage of inhibition was 1.81%, while percentage of inhibition for paracetamol group was 20.2% and for ibuprofen it was 22.79%. These results encouraged researcher to look for time to time effect by percentage of enhancement of edema inhibition compared to previous reading as percentage of inhibition was not useful, it calculates overall progress related to negative control, therefore, these values were calculated and listed in previous table.

Among all of these predictions, we needed to depend on statistical analysis of variance. ANOVA analysis revealed that there was a significant difference (*0.000*) between means of checked groups at *F-value* of *17.664*. Thus, it was preferred to check post Hoc tests to diagnose where the mean difference between groups were located and their relation to positive and negative controls. HSD test disclosed the significant difference (at $p < 0.01$) between plant test group to all other groups, which means the higher effect when compared to other groups. Dunnett-t test showed that difference between plant test group to negative control was more prominent (at $p < 0.01$) than difference between positive control ibuprofen to negative control (at $p < 0.05$). The paracetamol group showed no marked differences to other tested groups. To clarify these outcomes, next Figure 1 illustrated clustered bar mean of edema size reduction effect by hour.

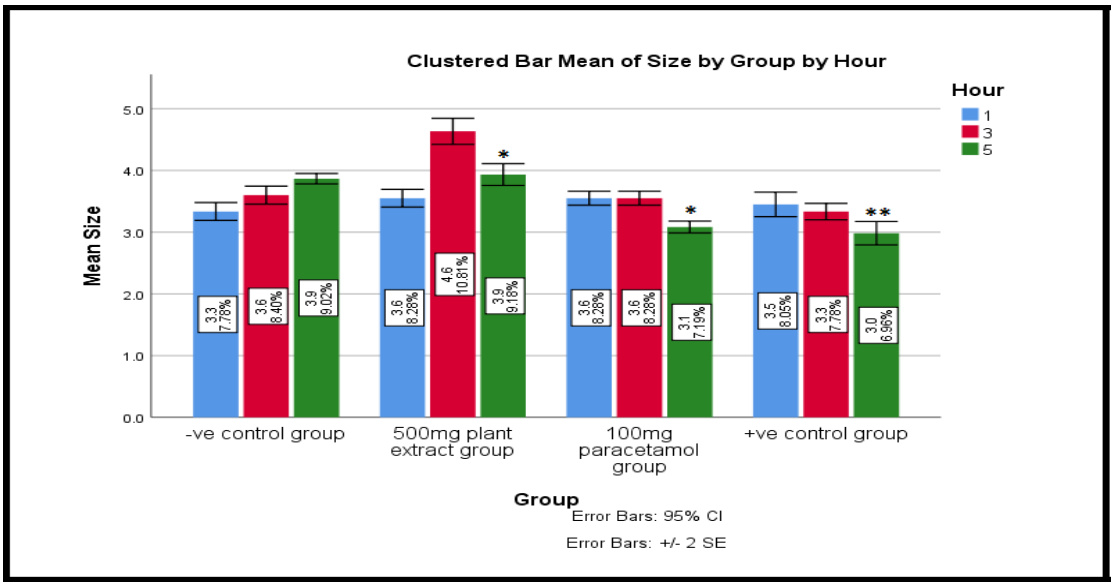

Figure 1: Clustered bar of the rat's paw edema size reduction (cm) mean after one, three and five hours from carrageenan injection to test anti-inflammatory effect of 100% *S. lappa* roots' ethanol extracts at 500 mg/kg dose compared to 100 mg/kg paracetamol, negative and positive controls. Values were expressed in means ± SEM, n=6, and group differences determined by one-way ANOVA at *p-value <0.01* and <*0.05* followed by post hoc Tukey and Dunnett tests. * *Sig*. level = *0.001* and *0.000* at *p-value < 0.01*, and ** *sig*. level = *0.01* at *p-value < 0.05*

It was clear from last figure that *S. lappa* extract exhibited its action after three hours from carrageenan injection with a good percentage of inhibition when compared to paracetamol. Plant extract group which treated by 500 mg/kg dose showed marked increase in paw edema size until three hours and this was a strange phenomenon, then the edema started to decrease in size rapidly at higher extent more than that of other groups. Ibuprofen 40 mg/kg positive control group revealed potent effect to minimize edema steadily over time not like that of paracetamol. All tested groups revealed some overlapping of error bars, except plant group which indicated the significant differences among data of this group. The present study clearly indicated *S. lappa* roots' ethanol extracts at 500 mg/kg dose possessed potent anti-inflammatory properties.

These findings suggested that there was a higher effect of plant extract at 500 mg/kg dose when recorded readings combined to percentage of enhancement of edema inhibition. In a comparison to ibuprofen and paracetamol, the plant extract submitted a 1.81% of inhibition with 15.11% enhancement of inhibition at dose of 500 mg/kg during five hours, while paracetamol exhibited a 20.2% of inhibition with 13.23% enhancement of inhibition at dose of 100 mg/kg during the same time, and ibuprofen exhibited a 22.79% of inhibition with 10.51% enhancement of inhibition at dose of 40 mg/kg during the same time. This indicated that *S. lappa* roots' ethanol extract at dose of 500 mg/kg had a role to decrease edema with more potency than that of ibuprofen and paracetamol apparently at the last stage.

Discussion

As explained above, the inflammation process is divided into two stages after carrageenan injection to induce local edema, pain, and fever resulted mainly as a consequence of the release of inflammatory mediators such as prostaglandins (PG-E mainly), cytokinin, bradykinins, histamines, leukotrienes, and serotonin, these were responsible to increases vascular permeability and thus enhances the flow of leukocytes at the site of inflammation. Therefore, any interruption at this pathway will results in the provoked release of mediators, thus, decreased inflammation response [29].

The 100% *S. lappa* roots' ethanol extract of 500 mg/kg presented a weak percentage (1.81%) of edema inhibition during five hours and its action appeared at the late stage of inflammation, and as known, the cut off process due to affection on PGs and BK happen at late phase. In a comparison to ibuprofen and paracetamol, the plant extract illustrated a 15.11% of inhibition at dose of 500 mg/kg during five hours, while paracetamol exhibited a 13.23% of inhibition at dose of 100 mg/kg during the same time, and ibuprofen presented a 10.51% of inhibition at dose of 40 mg/kg during the same time calculated over all time of experiment, this denoted to *S. lappa* ethanol extract has somehow similar potency to that of paracetamol and ibuprofen. The present study indicated that *S. lappa* roots' ethanol extract possessed potent anti-inflammatory properties seen after statistical analysis. Despite the proven anti-inflammatory effect of paracetamol compared to that of ibuprofen, however, its role in decreasing inflammation still not clear as this study looked just for its anti-inflammatory effect not for related mechanism of action.

Both steroidal and non-steroidal anti-inflammatory drugs are important treatments to affect edema in the second phase by different actions. Pharmacological studies reported that the vast majority of NSAIDs affects mainly the second stage. Steroidal anti-inflammatory drugs reduce the vasodilation which occurs during inflammation. The NSAIDs block prostaglandin and thromboxane formation by inhibiting cyclooxygenase activity [30].

Hypothetically, for the plant to have an anti-inflammatory effect, it ought to have secondary metabolites that intervene the activity. *S. lappa* has numerous metabolites which apply anti-phlogistic effect through interference of production of inflammatory mediators. As listed in previous studies about phytoconstituents of the plant roots, the significant mixtures are sesquiterpenes lactones (costunolide and dehydrocostus lactone). Numerous researches uncover that these compounds which found in numerous plants that utilized customarily for anti-inflammatory and anti-cancer purposes [31]. A study held and focused on determination of essential oils content from some species of the genus *Saussurea* DC. They reported sixty two different chemical compounds using a gas chromatography–mass spectrometry method. The essential oils included linalool, eudesmol, caryophyllene oxide, and spathulenol. They notify that the presence of essential oils can be candidates for new anti-inflammatory, analgesic and anti-tumor drugs due to relatively high concentration of caryophyllene oxide [32]. Another evidence that *S. lappa* revealed potent anti-inflammatory effect against ulcerative colitis, its defensive mechanism of dihydrocostus lactone may include in lessening aggravation and improving colorectal barrier function by means of down-regulating the IL-6/STAT3 signaling [33].

Scientific investigations revealed that *Edagajadi yoga (kwatha)* treatment which prepared from seven medicinal plants, namely: Edagaja (*Cassia tora* seeds), Vidanga (*Embelia ribes* fruit), Haridra (*Curcuma longa* rhizome), Daruharidra (*Berberis aristata* root), Amlatasa (*Cassia fistula* root), Kushta (*S. lappa* root) and Pippali (*Piper longum* fruit), has an anti-trypanosomal activity and helpful in treatment of pityriasis versicolor and some diseases related to excessive activation of the complement system, like rheumatoid arthritis, the clear indication of potent anti-fungal and antiphlogistic effect [34]. Another study held to investigate anti-phlogistic effects of ethanol extract of *S. lappa* revealed that it had a remarkable protective effect via its anti-inflammatory, anti-apoptotic, and antioxidant capacity. *S. lappa* oral administration could efficiently rescue triamcinolone acetonide-induced immunosuppression. In addition, *S. lappa* could moderately augment triamcinolone acetonide anti-inflammatory activity [35]. Additionally, ethanol extract of *S. lappa* assured to exert sturdy anti-inflammatory effect in a study seeked for determination of anti-arthritic potential. *S. lappa* conditionally lightened the seriousness of rheumatoid arthritis. Histopathological assessment demonstrated that it diminished the penetration of inflammatory cells. Also, it diminished the serum levels of CRP, TNF-α, IL-1β, and IL-6, decreased the total oxidative capacity, and improved the total antioxidant capacity. The *S. lappa* evidenced to help in joint action and improves repair, with character of damage-resistant and cancer-prevention monoarthritis agent working well on rodents [36].

An experiment aimed at testing of hemolytic activity of methanol extract of *S. lappa* at 100 μg/ml, it found that extract was potent to inhibit heat induced hemolysis and protects the membrane against hemolysis. Moreover, the extract significantly reduced the wound size and helped in tissue healing. The study suggested it is better to use it as anti-inflammatory agent for long time use due to its effect beside bare from reported side effects [37]. Also there was a role of alantolactone compounds mainly caryophyllene, costic acid, and costunolide, and dehydrocostuslactone partially isolated from *S. lappa* to suppress skin inflammation through suppression of tumor necrosis factor (TNF-α) and interferon (IFN-γ) by blocking phosphorylation of signal transducer and activator of transcription in HaCaT cells [38]. Noted that alantolactone and isoalantolactone sesquiterpenes compounds were detected by nuclear magnetic resonance (NMR) spectroscopy to present in roots of *S. lappa* harvested from Lahaul and Barot regions of Western Himalaya, the area inside Jammu and Kashmir region, it is worth to be mentioned that *S. lappa* used in current research inferred to be rich in these compounds [39].

A study attempted to screen santamarin, a sesquiterpene lactone isolated from *S. lappa* for anti-inflammatory properties. The outcome was astonishingly, santamarin suppressed cyclooxygenase (COX-2) and its derivative (PG-E_2), reduced tumor necrosis factor-α (TNF-α) and diminished interleukin-1β (IL-1β) production, inhibited inducible nitric oxide synthase protein and nitric oxide, induced heme oxygenase-1 mRNA expression which plays a cytoprotective role against inflammation, and suppressed the phosphorylation and degradation of IκB-α as well as the nuclear translocation of p65 in response to lipopolysaccharides [40]. On the basis of recorded findings, previous literature reports and scientific speculation, *S. lappa* roots' ethanol extract seems to follow the inhibition of cyclooxygenase pathway and inhibition of PGs formation, and as indicated by these outcomes, this plant can be considered as a compelling therapeutic source to fix in intense provocative problems. Be that as it may, the specific component of plant removes calming activity stays indistinct and need more examination.

Conclusion

Phytotherapy as a science-based medical practice lays a footprint on the map of medical field and its importance shined as it proved its influence. Many developed drugs were originated from medicinal plants, and many pharmaceutical companies striven to take responsibility for research and development of new drugs from secondary metabolites, these chemical compounds gained their prominence from their potent action. *S. lappa* is one of medicinal plants that widely used for its antimicrobial, antiseptic, anti-tumor and anti-inflammatory properties. This study confirmed the anti-inflammatory activity of 100% *S. lappa* roots' ethanol extract. The results revealed a better efficiency compared to ibuprofen and paracetamol and disclosed the dose of plant extract of 500 mg/kg showed its highest inhibition of edema (15.11%) between three and five hours, whereas reference drug ibuprofen exhibited 10.51% of inhibition, and paracetamol showed a 13.23% of inhibition at last stage. It is supposed that *S. lappa* contains many chemical compounds that participated its anti-phlogistic effects, mainly sesquiterpene lactones (costunolide and dehydrocostus lactone), alkaloids, tannins and flavonoids; however, the mechanism (s) of anti-inflammatory activities remains unclear and need further investigation.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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