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ORIGINAL ARTICLE

Salacia pallescens Oliv. (Celastraceae) Scavenges Free Radicals and Inhibits Pro-inflammatory Mediators in Lipopolysaccharide-activated RAW Cells 264.7 Macrophages

Lipopolisakkarit ile Aktive Olan RAW Hücreleri 264.7 Makrofajlarda *Salacia pallescens* Oliv. (Celastraceae) Serbest Radikalleri Uzaklaştırması ve Pro-inflamatuvar Mediatörlerini İnhibe Etmesi

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ABSTRACT

Objectives: *Salacia pallescens* has folkloric anti-inflammatory claims, with little scientific investigation. Hence, the antioxidant and anti-inflammatory effects along with phytochemical components of the plant were investigated.

Materials and Methods: The antioxidant property of *S. pallescens* leaf (SPL) methanol extract was evaluated using 1,1-diphenyl-2-picrylhydrazyl and nitric oxide inhibition assays. The anti-inflammatory property of SPL in lipopolysaccharide-stimulated RAW 264.7 macrophages was determined. The cytotoxicity of SPL was assessed in brine shrimp lethality assay (BSL) and against RAW 264.7 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide based assay. Gas chromatography-mass spectrometry was employed to identify SPL phytochemical compounds.

Results: SPL significantly scavenged free radical generated in the antioxidant assays and inhibited nitrite production in stimulated RAW 264.7 cells. Similarly, there was a 9-fold reduction in interleukin-6 produced in RAW 264.7 cells when exposed to the highest concentration of SPL. In addition, 50% lethal concentration of SPL was 455.58±82.35 µg/mL while cyclophosphamide gave 16.3±0.15 µg/mL in BSL test. Moreover, cell viability was not affected by SPL. Sixteen compounds were identified from SPL where thymol (29.79%), 3-carene (15.97%), and p-cymene (12.19%) are the most abundant.

Conclusion: Methanol extract of SPL showed antioxidant and anti-inflammatory activities by free radicals and cytokines inhibition. The activity observed may be related to the polyphenolic compounds in the plant.

Key words: Anti-inflammation, antioxidant, *Salacia pallescens*

ÖZ

Amaç: *Salacia pallescens*, çok az bilimsel araştırma ile folklorik anti-inflamatuvar iddialara sahiptir. Bu nedenle bitkinin fitokimyasal bileşenleri ile birlikte antioksidan ve anti-inflamatuvar etkileri araştırılmıştır.

Gereç ve Yöntemler: *S. pallescens* yaprağı (SPL) metanol ekstresinin antioksidan özelliği, 1,1-difenil-2-pikrilhidrazil ve nitrik oksit inhibisyon deneyleri kullanılarak değerlendirilmiştir. Lipopolisakkarit ile uyarılan RAW 264,7 makrofajlarda SPL'nin anti-inflamatuvar özelliği belirlenmiştir. SPL'nin sitotoksisitesi, tuzlu su karides ölümcül tahlilinde (BSL) ve 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür bazlı bir tahlilde RAW 264,7 hücrelerine karşı değerlendirilmiştir. SPL fitokimyasal bileşiklerini tanımlamak için gaz kromatografisi-kütle spektrometrisi kullanılmıştır.

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Bulgular: SPL, antioksidan deneylerde üretilen serbest radikali önemli ölçüde uzaklaştırmış ve uyarılmış RAW 264,7 hücrelerinde nitrit üretimini inhibe etmiştir. Benzer şekilde, en yüksek SPL konsantrasyonuna maruz bırakıldığında RAW 264,7 hücrelerinde üretilen interlökin-6'da 9 kat azalma olmuştur. Ek olarak, BSL testinde SPL'nin lethal konsantrasyon 50'si 455,58±82,35 µg/mL iken, siklofosfamid için bu değer 16,3±0,15 µg/mL olarak belirlenmiştir. Ayrıca, hücre canlılığı SPL'den etkilenmemiştir. SPL'den on altı bileşik tanımlanırken, bunların aralarında en çok olanları timol (%29,79), 3-karen (%15,97) ve p-simenindi (%12,19).

Sonuç: SPL'nin methanol ekstresi, serbest radikaller ve sitokinlerin inhibisyonu ile antioksidan ve anti-inflamatuvar aktiviteler göstermiştir. Gözlenen aktivite, bitkideki polifenolik bileşiklerle ilgili olabilir.

Anahtar kelimeler: Anti-inflamatuvar, antioksidan, *Salacia pallescens*

INTRODUCTION

Inflammation is the host defensive immune response to tissue damage or infection.[1] Inflammation aims at localizing, eliminating, removing infecting agent, or repairing the injured tissue. In order to achieve these goals, the innate immune system tissue-resident cells discover the toxic insult and alert circulating neutrophils, which then proceed to the inflamed tissue.[2] Thus, promoting inflammatory monocyte recruitment and potentiating pro-inflammatory mediators such as cytokines and chemokine to handle the situation appropriately.[3] This process can cause reactive oxygen species (ROS) formation, vital signaling molecules that play a crucial part in the initiation, progression, and resolution of inflammatory response.[4] An increased ROS production by polymorphonuclear neutrophils at the inflammation area leads to tissue injury and endothelial dysfunction.[1] However, neutrophils undergo apoptosis under normal conditions after executing their roles.[5] The removal of apoptotic neutrophils prompts a change from a pro- to an anti-inflammatory macrophage phenotype.[6,7] Nonetheless, when inflammation is unresolved, it can progress to chronic inflammation. The persistence of chronic inflammation can result in cardiovascular, neurodegenerative, and respiratory diseases, including cancer.[8]

Drugs for inflammation treatment are effective but have serious side effects when used for prolonged time. For this reason, it is crucial to search for new and safe anti-inflammatory agents. Medicinal plants are valuable source of novel molecules and efficient alternative strategy for newer therapeutics development.[9] Many plants have been documented in traditional medicine to ameliorate various inflammatory disorders.[10] *Salacia pallescens* is a plant commonly known as Elewekan in the Yoruba ethnomedicine. It is used in folk medicine as an ingredient for a decoction to treat children,[11] particularly fever and pain. However, pharmacological activities of this plant are not readily available except for antioxidant activity.[12] Antidiabetic, anti-inflammatory, antioxidant, anticancer, nephroprotective, or hepatoprotective activities of other species of *Salacia* such as *S. chinensis, S. oblonga, S. reticulata, S. reticulate, Salacia parviflora, S. lehmbachii, S. senegalensis*, and *S. crassifolia* have been reported.[13-20] Therefore, this is the first report on *S. pallescens* anti-inflammatory activity and chemical composition.

MATERIALS AND METHODS

Plant material

Salacia pallescens leaf (SPL) was collected from Idi-Ayunre, Ibadan. A sample was taken for identification and authentication at the Forestry Research Institute of Nigeria (110527).

Extraction of the leaf of S. pallescens

The leaf of *S. pallescens* was air-dried and coarsely ground. Then, 206 g of SPL was macerated in 50% methanol for 72 h. Subsequently, the methanol extract was concentrated at reduced temperature and pressure. The extraction process was done thrice to increase the yield. The SPL methanol extract was stored at 4°C for further use.

Determination of total flavonoid content (TFC)

The method previously reported was used to determine TFC.[21] Briefly, 0.6 mL of SPL (1 mg/mL), 6.8 mL of 30% methanol, 0.30 mL of 0.5 M sodium nitrite, and 0.30 mL of 0.3 M aluminum chloride hexahydrate were mixed. Five minutes later, 2 mL of 1 M sodium hydroxide was added into the mixture, and the absorbance was read at 506 nm. TFC was reported as milligrams of rutin equivalents (RE) per gram of dried plant sample.

Determination of total phenolic content (TPC)

The TPC of SPL was estimated by a spectrometric method.[22] An equal volume (0.1 mL) of SPL (1 mg/mL) and Folin and Folin-Ciocalteuphenol reagent were mixed. After 5 min incubation, 1.3 mL of distilled water and 1 mL of 7% Na_2CO_3 were added into the mixture. Absorbance at 750 nm was read after 90 min. TPC was reported as milligrams of gallic acid equivalents (GAE) per g of the dried sample.

Antioxidant assays

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of SPL

SPL antioxidant activity by DPPH assay was estimated using a method previously reported.[23] Briefly, gradient concentrations of SPL (6.25-400 µg/mL) or standard ascorbic acid (0.25-16 µg/mL) were prepared in a 96 microtiter well plate, incubated for 30 min at 29°C in the dark after the addition of a freshly prepared solution of DPPH (0.04 mg/mL). The absorbance at 517 nm was read against the blank, and values obtained were expressed as the percentage of the control.

Nitric oxide scavenging activity of SPL

Antioxidant activity of SPL by nitric oxide inhibition assay was determined following a modified method of Panda et al.[24] Sodium nitroprusside solution (40 mM) was mixed with graded concentrations (50-800 µg/mL) of SPL (1:4 v/v) and incubated at 29°C for 2 h in the dark. Then, an equal volume of the incubated test solution and Griess reagent (1% sulphanilamide and 0.1% N-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) were added into a 96-well plate in duplicate and kept for an additional 15 min at 29°C in the dark. Absorbance at 550 nm was read. The amount of nitric oxide generated was extrapolated from the sodium nitrite curve.

Cell viability testing

RAW 264.7 cell line from the American Type Culture Collection (TIB-71; Rockville, MD, USA) maintained in cultured Dulbecco's modified eagle medium, 10% fetal bovine serum supplemented, 2 mM L-glutamine, and 100 IU/mL of penicillin-100 µg/mL streptomycin, at 37°C in 5% carbon dioxide incubator was used. SPL effect on cell viability was determined following a previously published method.[25] RAW 264.7 cells (5×10^5 cells/mL) were placed in a 96 microtiter well plate for 18 h prior to exposure to a graded concentration of SPL for 2 h. The cells were subsequently stimulated with 100 ng/mL liposaccharide from *Escherichia coli* 055: B5 for 24 h. Thereafter, the cultured medium was substituted with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in culture medium and further incubated for 2 h. The formazan blue formed due to the addition of MTT was dissolved with DMSO. Absorbance was read at 540 nm.

Anti-inflammatory testing

The SPL effect on nitrite and interleukin-6 (IL-6) produced in liposacharride (LPS) stimulated RAW 264.7 cell was determined. RAW 264.7 cells were seeded at 5×10^5 cells per well in a 24-well plate and permitted to grow into confluence before exposure to a graded concentration of SPL (50-400 µg/mL) for 2 h. The cells were subsequently stimulated with 100 ng/mL LPS, and the plate was incubated for 24 h. The nitrite content in the supernatant was estimated using the Griess reagent.[26] The IL-6 level in the supernatant was determined using m*ouse IL-6* ELISA MAX™ deluxe kit according to the manufacturer's instruction.

Brine shrimp lethality assay (BSL)

The SPL cytotoxicity was evaluated using BSL according to the method previously reported.[27] *Artemia salina* (brine shrimp eggs) was purchased at an Aquarium shop in the UK. The nauplii (larvae) were hatched by placing the eggs of *A. salina* in a tank containing seawater at 29°C. One part of the tank was exposed to light, and the other was covered with aluminum foil. The eggs were hatched after 48 h to nauplii which were attracted to light. Ten nauplii were transferred into graded concentrations of SPL extract (1.0-1.000 µg/mL) in plain test tubes. Cyclophosphamide drug was a positive control.

Chemical composition of SPL

The SPL phytochemical compounds were identified using an Agilent technologies 7890 gas chromatography system with a 5975 mass spectrometry (MS) following a previously described method.[28] Helium, 99.99% purity, was the mobile phase. The column (HP5 MS) had a thickness of 0.25 µm, an internal diameter of 0.320 mm, and length of 30 m. Compounds were identified by comparing retention time and fragmentation pattern against the NIST mass spectra library.

Statistical analysis

The antioxidants, anti-inflammatory, and BSL assays were performed in duplicates and repeated in three independent experiments. The 50% inhibitory concentration (IC_{50}) or lethal concentration (LC_{50}) values were expressed as mean ± standard error of three independent data. The mean IC_{50} or LC_{50} comparison of the SPL with the standard drug was made with the Mann-Whitney U test. P value <0.05 was taken as significant.

RESULTS

SPL percentage yield extracted with 50% methanol was 29.3%. The TFC and TPC of SPL extract were 190.29±1.43 mg RE/g and 609.5±0.42 mg GAE/g, respectively.

Antioxidant activity and BSL of SPL

The SPL antioxidant activity was concentration-dependent. Fifty percent IC_{50} in the DPPH and nitric oxide scavenging assays were 21.47±1.96 and 49.49±1.24 µg/mL, respectively (Table 1). Ascorbic acid gave an IC_{50} of 4.31±0.26 and 48.74±1.41 in DPPH and nitric oxide scavenging assays, respectively (Table 1). In the BSL test, LC_{50} of SPL was 455.58±82.35 µg/mL while cyclophosphamide was 16.3±0.15 mg/mL (Table 1).

Anti-inflammatory activity

The graded concentration effect of SPL on cell viability in LPS stimulated RAW 264.7 cells was assessed using MTT based assay. The concentration of SPL ranging from 50 to 400 µg/mL showed no effect on cell viability (Figure 1). Since SPL appeared non-toxic to RAW 264.7 cells at the tested concentration, its effect on nitrite production in LPS stimulated RAW 264.7 cells were assessed. SPL pre-treated LPS stimulated cells released a lower level of nitrite in the medium than the untreated control.

Table 1. IC_{50} and LC_{50} of *Salacia pallescens* leaf (SPL)

IC_{50} or LC_{50} (µg/mL)	*S. pallescens*	Standard drug
*IC_{50} DPPH inhibition	21.47±1.96#	4.31±0.26*
IC_{50} nitric oxide inhibition	49.49±1.24	48.74±1.41*
LC_{50} brine shrimp lethality test	455.58±82.35#	16.3±0.15**

*Ascorbic acid, **Cyclophosphamide, #SPL vs. control p<0.05, IC_{50}: 50% inhibitory concentration, LC_{50}: 50%$_{lethal}$ concentration, DPPH: 1,1-diphenyl-2-picrylhydrazyl

The SPL graded concentration (50-400 µg/mL) significantly inhibited nitrite production caused in LPS stimulated macrophages with inhibition ranging from 19.2 to 83.5% (Figure 2). Similarly, there was a 9-fold reduction in LPS induced IL-6 production in RAW 264.7 cells pre-treated with 400 µg/mL of SPL while pre-treatment with 50 µg/mL SPL resulted in a 1.4-fold decrease in IL-6 production (Figure 3).

Phytochemical constituents of SPL

The retention time, abundance, and m+1 values of SPL are presented in Table 2. Sixteen compounds were identified from the SPL extract. The most abundant compound is thymol (29.79%), followed by 3-carene (15.97%), p-cymene (12.19%), caffeine (8.28%), hexadecanoic acid (6.19%), bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl) (5.92%), and caryophyllene (5.17%). The mass spectra data depicted the fragmentation patterns and structures of the compounds in SPL are shown in Figure 4a-e.

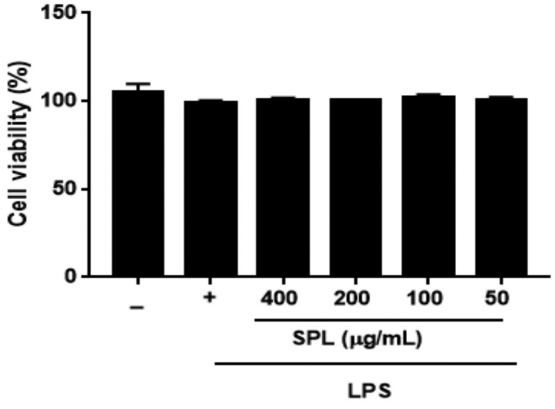

Figure 1. Effects of methanol extract of leaf of *S. pallescens* on cell viability in LPS stimulated RAW 264.7 cells. Data are expressed as means ± SEM

SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean

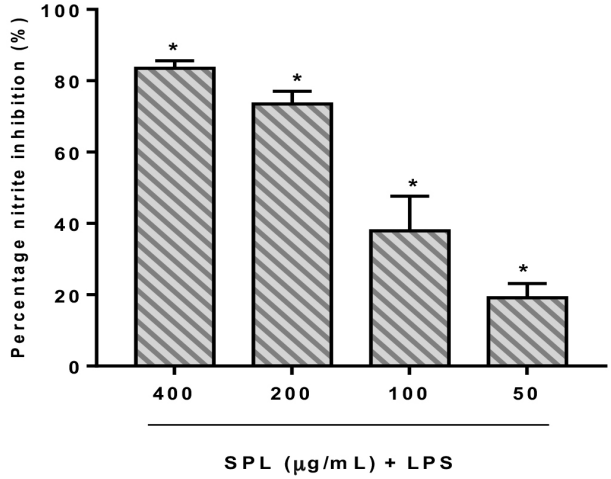

Figure 2. Inhibition of nitrite production in LPS stimulated RAW 264.7 cells by methanol extract of leaf of *S. pallescens*. Data are expressed as means ± SEM

*Significant percentage reduction in nitrite production, SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean

Table 2. Chemical compounds of ethyl acetate fraction of *Salacia pallescens* using GC-MS

GC peak	Compound names	GC-MS-RT (min)	Relative abundance %	M+1
1	p-Cymene	6.58	12.19	134
2	1R)-2,6,6-Trimethylbicyclo[3.1.1] hept-2-ene	7.10	1.82	136
3	3-carene	7.83	15.97	136
4	Methyl m-tolyl carbinol	9.12	1.99	136
5	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	9.91	1.44	164
6	Thymol	10.88	29.79	150
7	2-(Thiazolylazo)-p-cresol	12.25	1.06	219
8	Caryophyllene	12.70	5.17	204
9	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	12.83	5.92	204
10	Humulene	13.17	0.97	204
11	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]	13.62	4.08	204
12	3,6-Nonadien-5-one, 2,2,8,8-tetram ethyl-	13.72	1.43	190
13	Formamide, N-(4-benzofurazanyl)-	17.93	2.24	101
14	Caffeine	19.21	8.28	194
15	Hexadecanoic acid, methyl ester	19.70	6.19	270
16	9-Hexadecenoic acid	22.59	1.47	254

GC-MS: Gas chromatography-mass spectrometry, RT: Retention time

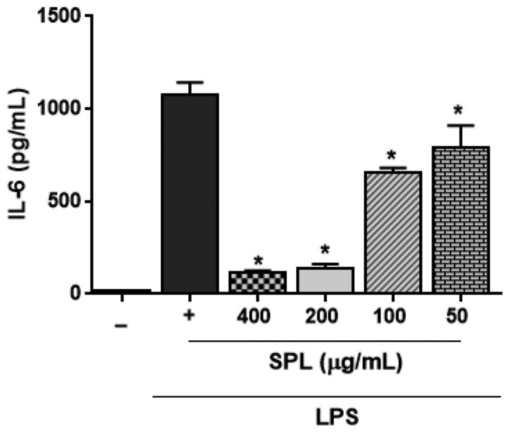

Figure 3. Effects of methanol extract of the leaf of *S. pallescens* on IL-6 production in LPS stimulated RAW264.7 cells. Data are expressed as means ± SEM

*Significant percentage reduction in IL-6 production, SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean, IL-6: Interleukin-6

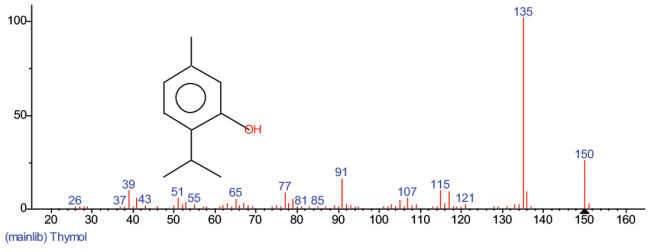

Figure 4a. Mass spectra and structure of thymol

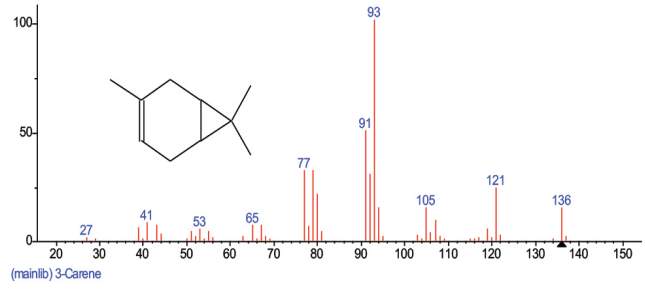

Figure 4b. Mass spectra and structure of 3-carene

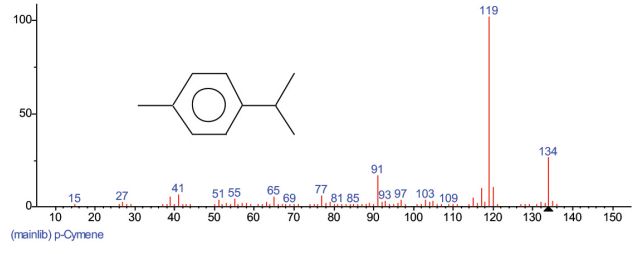

Figure 4c. Mass spectra and structure of p-cymene

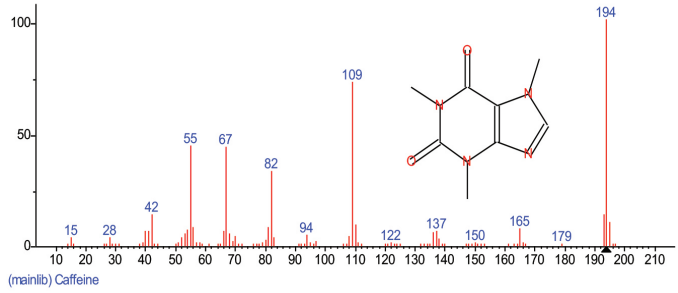

Figure 4d. Mass spectra and structure of caffeine

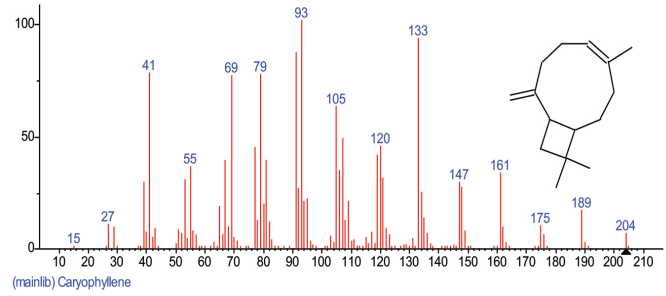

Figure 4e. Mass spectra and structure of caryophyllene

DISCUSSION

Findings in this study revealed that SPL possesses significant antioxidant activity. It scavenged free radicals generated by DPPH and nitric oxide in the DPPH- and sodium nitroprusside-based chemical assays. The high antioxidant property in plants is linked to its flavonoids and phenolic constituents, especially polyphenols,[29,30] found in SPL. Previous studies have reported antioxidant activity of *S. pallescens* and other species such *S. chinensis* and *S. oblonga*.[12,31,32] Oxidative stress occurs due to the high production of free radicals like alkoxy, hydroxyl, superoxide, and nitric oxide and non-radical species such as peroxynitrite, hydrogen peroxide, and singlet oxygen. These free radical and non-radical species cause redox system alteration, induction of DNA damage, and procarcinogens activation, which are linked to pathological conditions' development and progression.[33] Interestingly, the nitric oxide scavenging activity of SPL was comparable to ascorbic acid, a standard antioxidant drug, suggesting the benefit of the plant in controlling diseases associated with oxidative stress.

Liposaccharide stimulated RAW 264.7 cell is an anti-inflammatory model for screening agents with anti-inflammatory properties. Overproduction of nitrite and accumulation of cytokines which amplifies the inflammation cascade are the main characteristics of this model.[34] SPL significantly inhibited the overproduction of nitrite caused by liposaccharide and remarkably reduced the IL-6 level measured in this study. With the significant reduction of nitrite and IL-6 production, the leaf of *S. pallescens* demonstrated anti-inflammatory activity. To the best of our knowledge, this is the first anti-inflammatory activity report of *S. pallescens*. However, species of Salacia

with *in vivo* anti-inflammatory activity include *S. oblonga* and *S. lehmbachii*.[13,35]

Furthermore, the cytotoxicity of SPL was evaluated to determine its safety profile. The SPL cytotoxicity was evaluated in the BSL test. SPL was not toxic on *A. salina* nauplii. LC_{50} value of less than 100 µg/mL was toxic,[36] and SPL produced LC_{50} >400 µg/mL. Also, SPL was 28 times less toxic than cyclophosphamide, the standard drug. The BSL assay is used as a model for primary toxicity screening;[37] additional complementing tests might be needed before drawing a safety conclusion. Additionally, it was observed that SPL does not affect the RAW 264.7 cells viability.

Besides that, 13 compounds were identified in SPL, where thymol had the highest relative abundance. Thymol, a monoterpene, possesses antifungal, antidepressant, and anti-inflammatory, and cicatrizing properties.[38-40] The second most abundant compound was 3-Carene, a bicyclic monoterpene. 3-carene is one the major components of *Bupleurum gibraltaricum* essential oil with anti-inflammatory activity in carrageenan-induced paw edema in rats.[41] Antibacterial activity of 3-carene against *B. thermosphacta* and *P. fluorescens* resulted in morphological, genomic damages, and eventual cell death to the bacterial has been reported.[42] Also, 3-Carene exhibited a hypnotic effect in mice.[43]

Anti-inflammatory, anti-nociceptive, and antioxidant of p-Cymene, a monoterpene, the third most abundant in SPL, has been reported.[44-46] Caffeine, a CNS and metabolic stimulant, is the fourth most abundant in SPL. Caffeine is a xanthine alkaloid present in 60 plant species.[47] It has effects on smooth muscle, mood, memory, alertness, and physical and cognitive performance.[47] Hexadecanoic acid, methyl ester, is also known as palmitic acid. *Annona muricata* L. seeds fixed oil contains palmitic acid and other fatty acids showing free radical scavenging activity.[48] The antioxidant, anticancer, and anti-inflammatory activities of palmitic acid have been documented.[49-51]

Similarly, bicyclo [3.1.1] hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- found in SPL has been reported to be present in *Cinnamomum zeylanicum* with antibacterial and anti-fungi activities.[52] Likewise, antioxidant, anti-obesity, and antidiabetic activities of bicyclo [3.1.1] hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl) in *Ocimum basilicum* has been documented.[53] A bicyclic sesquiterpene, caryophyllene, also identified in SPL possesses anti-inflammatory activity by inhibiting pro-inflammatory cytokines, cyclooxygenase 1 and 2, and inducible nitric oxide synthase.[54] Other pharmacological activities of caryophyllene include neuro-protective, anti-apoptosis, antioxidant, and analgesia.[54-56] Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR (4a.alpha.7. alpha, 8a.beta.)] also known as β-selinene, is another compound found in SPL. β-selinene a component of essential oils in *Artemisia annua* has antioxidant activity.[57,58] Also, *Callicarpa macrophylla* β-selinene rich essential oils showed antioxidant anti-inflammatory, antipyretic, and analgesic properties.[58]

CONCLUSION

Methanol extract of SPL showed antioxidant and anti-inflammatory activities by free radicals and cytokines inhibition. The extract appears to be non-toxic. The activities observed may be related to the polyphenolic compounds in the plant.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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