

## ***Salacia pallescens* Oliv. (Celastraceae) scavenges free radicals and inhibits pro-inflammatory mediators in lipopolysaccharide-activated RAW cells 264.7 macrophages**

**Short Title: Antioxidant and anti-inflammatory activities of *S. pallescens***

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### **ABSTRACT**

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

**METHODS:** The Antioxidant property of methanol extract of *S. pallescens* leaf (SPL) was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) 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 MTT based assay. Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify SPL phytochemical components.

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

**CONCLUSION:** Methanol extract of *Salacia pallescens* leaf showed antioxidant and anti-inflammatory activities via free radicals and cytokines inhibition. The activity observed may be related to the polyphenolic compounds present in the plant.

**Keywords:** *Salacia pallescens*, antioxidant, anti-inflammation

## Introduction

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

Drugs for the treatment of inflammation are effective but associated with serious side effects when used for prolong period of time. For this reason, it is crucial to search for new and safe anti-inflammatory agents. Medicinal plants are useful source of novel molecules and are regarded as an efficient alternative strategy for newer therapeutics development.<sup>9</sup> Many plants have been documented in traditional medicine to ameliorate various inflammatory disorders.<sup>10</sup> *Salacia palleescens* is a plant commonly known as Elewekan in the Yoruba ethnomedicine. It is commonly used in folkloric medicine as ingredient for a decoction given to treat ailments peculiar to children,<sup>11</sup> such as fever and pain. However, pharmacological activities of this plant is not readily available except for report of antioxidant activity.<sup>12</sup> Although anti-diabetic, anti-inflammatory, antioxidant, anti-cancer, 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.<sup>13-20</sup> Thus this is the first report on *S. palleescens* anti-inflammatory activity and chemical composition.

## Material and Methods

### Plant material

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

### Extraction of leaf of *S. palleescens*

The leaf of *S. palleescens* was air dried and coarsely grinded. Two hundred and six grams of leaf of *S. palleescens* was macerated in 50% methanol for 72 hours. Thereafter, the methanol extract was concentrated at reduced temperature and pressure. The extraction process was done thrice to increase the yield. SPL methanol extract was stored at 4°C.

### Determination of total flavonoid content (TFC)

The method previously reported was used to determine TFC.<sup>21</sup> Briefly, 0.6 mL of SPL (1 mg/mL), 6.8 mL of methanol (30%), 0.30 mL of Sodium nitrite (0.5 M) and 0.30 mL of Aluminium chloride hexahydrate (0.3 M) were mixed. Five minutes later, 2 mL of 1 M Sodium

hydroxide was dispensed into the mixture and the optical density determined at 506 nm. TFC was reported as milligrams of rutin equivalents per gram of dried plant sample.

#### **Determination of total phenolic content (TPC)**

The TPC of SPL was estimated by spectrometric method.<sup>22</sup> Briefly, equal volume (0.1 mL) of SPL (1 mg/mL) and Folin & Ciocalteu's phenol reagent were added together. Five minutes later, 1.3 mL distilled water and 1 mL of 7% Na<sub>2</sub>CO<sub>3</sub> were dispensed into the mixture. Absorbance after 90 min at 750 nm was read. TPC was reported as milligrams of Gallic acid equivalents (GAE) per g of the dried sample.

#### **Antioxidant assays**

##### **DPPH scavenging activity of SPL**

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

##### **Nitric oxide scavenging activity of SPL**

Antioxidant activity of SPL in the nitric oxide inhibition assay was determined following a modified method of Panda and co-workers.<sup>24</sup> Briefly, 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 hours in the dark. Subsequently, equal amount of the incubated test solution and Griess reagent (1% sulphanilamide and 0.1% N-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) were dispensed into a 96-well plate in duplicate and kept for additional 15 minutes at 29°C in the dark. Absorbance at 550 nm was taken. The amount of nitric oxide generated was extrapolated from 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% FBS supplemented, 2 mM L-glutamine and 100 IU/mL of penicillin-100 µg/mL streptomycin, at 37°C in 5% CO<sub>2</sub> incubator was used. SPL effect on cell viability was determined following a previous method.<sup>25</sup> RAW 264.7 cells (5 x 10<sup>5</sup> cells/mL) were seeded in a 96 microtiter well plate for 18 hrs before exposure to graded concentration of SPL for 2 hours. 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. The plate was read at 540 nm.

##### **Anti-inflammatory testing**

The SPL effect on nitrite and interleukin-6 produced in liposaccharide (LPS) stimulated RAW 264.7 cell was determined. RAW 264.7 cells were seeded at 5 x 10<sup>5</sup> cells per well in a 24-well plate and permitted to grow into confluence before exposure to graded concentration of SPL (50 - 400 µg/mL) for 2 hours. The cells were subsequently stimulated with 100 ng/mL LPS and plate was incubated for 24 hours. The amount of nitrite in the supernatant was evaluated using the

Griess reagent.<sup>26</sup> The interleukin-6 level in the supernatant determined using mouse IL-6 ELISA MAX™ Deluxe kit according to the manufacturer instruction.

### **Brine shrimp lethality assay (BSL)**

The SPL cytotoxicity was evaluated in brine shrimp lethality assay according to method previously reported.<sup>27</sup> *Artemia salina* (brine shrimp eggs) was purchased at an Aquarium shop in UK. The nauplii were hatched by placing the eggs of *A. salina* in a tank containing sea water at 29°C. One portion of the tank was exposed to light and the other portion was covered with aluminium foil. The eggs were hatched after 48 hours to nauplii (larvae) and attracted to the tank side where there was light source. Ten nauplii each were transferred into graded concentrations of SPL extract (1.0 - 1,000 µg/mL) in plain test tubes. Cyclophosphamide drug was the positive control.

### **Chemical composition of SPL**

The SPL phytochemical components were identified using Agilent technologies 7890 Gas Chromatography system with a 5975 Mass Spectrometry following a method described previously.<sup>28</sup> Helium, 99.99% purity was the mobile phase while the column (HP5 MS) had thickness 0.25 µm, internal diameter 0.320 mm and length 30 m. Identification of compounds were done by comparing retention time and fragmentation pattern against the NIST mass spectra library.

### **Statistical analysis**

The antioxidants, anti-inflammatory and BSL assays were done in duplicates and repeated in three independent experiments. The IC<sub>50</sub> or LC<sub>50</sub> values were expressed as mean ± standard error of three independent data. The mean IC<sub>50</sub> or LC<sub>50</sub> comparison of the SPL with that of the standard drug was done with Mann-Whitney U test. P-value < 0.05 was taken as significant.

### **Results**

*S. palleescens* leaf (SPL) percentage yield after extraction in 50% methanol was 29.3%. The TFC and TPC of SPL extract were 190.29 ± 1.43 mg rutin equivalent/g and 609.5 ± 0.42 mg gallic acid equivalent/g respectively.

### **Antioxidant activity and Brine shrimp lethality (BSL) of SPL**

The SPL antioxidant activity was concentration dependent. Fifty percent inhibitory concentration (IC<sub>50</sub>) 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<sub>50</sub> 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<sub>50</sub> of SPL was 455.58 ± 82.35 µg/mL while cyclophosphamide gave LC<sub>50</sub> of 16.3 ± 0.15 µg/m (Table 1).

### **Anti-inflammatory activity**

The graded concentration effect of SPL on viability of cell in LPS stimulated RAW 264.7 cells was assessed using MTT based assay. Concentration of SPL ranging from 50 - 400 µg/mL show no effect on cell viability (fig. 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 in

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

### Phytochemical Constituents of SPL

The retention time, abundance as well as m+1 values of SPL are presented on (Table 2). Sixteen compounds were identified from SPL. 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 depicting the fragmentation patterns and structures of the compounds in SPL are represented in Fig.4a-e.

### Discussion

Findings in this study revealed that *S. palleescens* leaf (SPL) possess antioxidant activity. It scavenged free radicals that was generated by DPPH and nitric oxide (NO) in the DPPH and sodium nitroprusside based chemical assays. High antioxidant property in plants is linked to its flavonoids and phenolic constituents especially polyphenols<sup>29,30</sup> which was found to be present in SPL in this study. Previous studies have reported *S. palleescens* antioxidant activity and other species such *S. chinensis* and *S. Oblonga*.<sup>12, 31-32</sup> Oxidative stress occurs due to high production of free radicals like alkoxy, hydroxyl, superoxide and nitric oxide and non-radical species such as peroxy nitrite, hydrogen peroxide and singlet oxygen. These free radical and non-radical species cause redox system alteration, induction of DNA damage, procarcinogens activation are linked to pathological conditions' development and progression.<sup>33</sup> Interestingly, the nitric oxide scavenging activity of SPL was comparable to ascorbic acid, a standard antioxidant drug, suggesting the usefulness of the plant in diseases associated with oxidative stress.

Liposaccharide stimulated RAW 264.7 cell is an anti-inflammatory model for screening agents with anti-inflammatory property. Overproduction of nitrite and accumulation of cytokines which amplifies the inflammation cascade are the main characteristics of this model.<sup>34</sup> Interestingly, SPL significantly inhibited the over production of nitrite caused by liposaccharide and remarkably reduced the interleukin-6 (IL-6) level measured in this study. With the significant reduction of nitrite and IL-6 production in this study, the leaf of *S. palleescens* demonstrated anti-inflammatory activity. This appears to be the first anti-inflammatory activity report of *S. palleescens* to the best of our knowledge. However, species of Salacia with *in vivo* anti-inflammatory activity include; *S. Oblonga* and *S. lehmbachii*.<sup>13,35</sup>

Furthermore, the cytotoxicity of SPL was evaluated so as to determine its safety profile. The SPL cytotoxicity was evaluated in brine shrimp lethality test. SPL appears non-toxic on *A. salina* nauplii. LC<sub>50</sub> value of less than 100 µg/mL was said to be toxic,<sup>36</sup> and SPL produced LC<sub>50</sub> > 400 µg/mL. Also, SPL was twenty-eight times less toxic than cyclophosphamide, the standard drug. The BSL assay is used as model for primary screening of toxicity,<sup>37</sup> additional complementing tests might be needed before drawing safety conclusion. We also, observed that SPL has no effect on the RAW 264.7 cells viability.

In addition, 13 compounds were identified in SPL. Of these compounds, thymol had the highest relative abundance. Thymol, a monoterpene possess antifungal, antidepressant, as well as anti-inflammatory and cicatrizing properties.<sup>38-40</sup> 3-Carene a bicyclic monoterpene is the next most abundant compound in SPL. 3-carene is one the major components of *Bupleurum gibraltarium* essential oil with anti-inflammatory activity in carrageenan-induced paw edema in rats.<sup>41</sup> Antibacterial activity of 3-carene against *B. thermosphacta* and *P. fluorescens* which resulted in morphological, genomic damages and eventual cell death to the bacterial has been reported.<sup>42</sup> Also, 3-Carene exhibited hypnotic effect in mice.<sup>43</sup>

Anti-inflammatory activities, anti-nociceptive, antioxidant and anti-inflammatory activities of p-Cymene a monoterpene, the 3<sup>rd</sup> most abundant in SPL has been reported.<sup>44-46</sup> Caffeine, a CNS and metabolic stimulant is the 4<sup>th</sup> most abundant in SPL. Caffeine is a xanthine alkaloid present in 60 plant species.<sup>47</sup> It has effects on smooth muscle, mood, memory, alertness, and physical and cognitive performance.<sup>47</sup> Hexadecanoic acid, methyl ester is also known as palmitic acid. *Annona muricata* L. seeds fixed oil contains palmitic acid and other fatty acids that have shown free radical scavenging activity.<sup>48</sup> The antioxidant, anticancer and anti-inflammatory activities of palmitic acid have been documented.<sup>49-51</sup>

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 antifungi activities.<sup>52</sup> 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.<sup>53</sup> 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.<sup>54</sup> Other pharmacological activities of caryophyllene include; neuro-protective, anti-apoptosis, antioxidant and analgesia.<sup>54-56</sup> Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR (4a.alpha.7.alpha, 8a.beta.)] also known as  $\beta$ -selinene, is another component in SPL.  $\beta$ -selinene a component of essential oils in *Artemisia annua* had antioxidant activity.<sup>57,58</sup> Also, *Callicarpa Macrophylla*  $\beta$ -selinene rich essential oils showed antioxidant anti-inflammatory, antipyretic and analgesic properties.<sup>58</sup>

Methanol extract of *S. pallescens* leaf showed antioxidant and anti-inflammatory activities via free radicals and cytokines inhibition. The extract appears to be non-toxic. The activity observed may be associated to the polyphenolic compounds seen in the plant.

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**Authors' contributions:** OOA contributed to conception, design of the study and data interpretation. TAO, FOA, AOO and AOA contributed to acquisition of data, analysis and interpretation. TAO was involved in drafting the manuscript. OOA revised the manuscript critically for important intellectual content.

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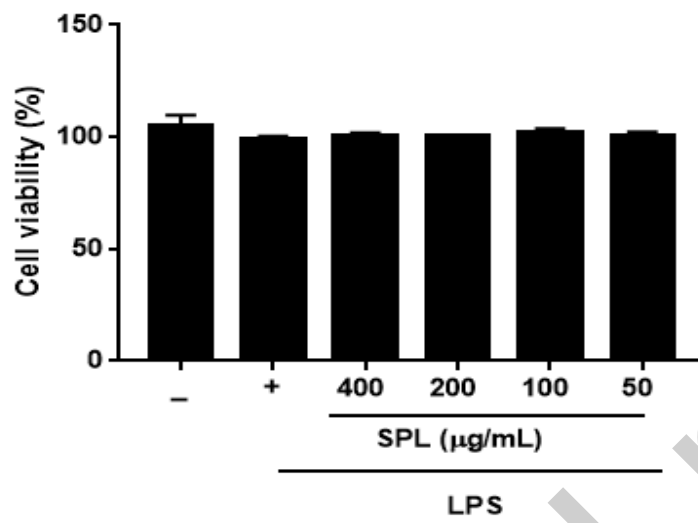
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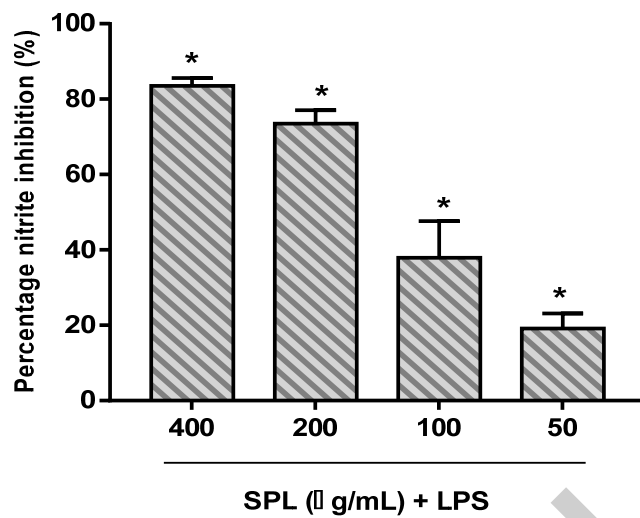
**Table 1: Fifty Percent Inhibition and Fifty Percent Lethal Concentrations of *Salacia pallescens* (SPL)**

IC <sub>50</sub> or LC <sub>50</sub> (µg/mL)	<i>S. pallescens</i>	Standard drug
*IC <sub>50</sub> DPPH inhibition	21.47 ± 1.96 <sup>#</sup>	4.31 ± 0.26*
IC <sub>50</sub> Nitric Oxide inhibition	49.49 ± 1.24	48.74 ± 1.41*
LC <sub>50</sub> brine shrimp lethality test <sup>£</sup>	455.58 ± 82.35 <sup>#</sup>	16.3 ± 0.15**

\*Ascorbic acid, \*\*Cyclophosphamide, <sup>#</sup>SPL vs control p<0.05

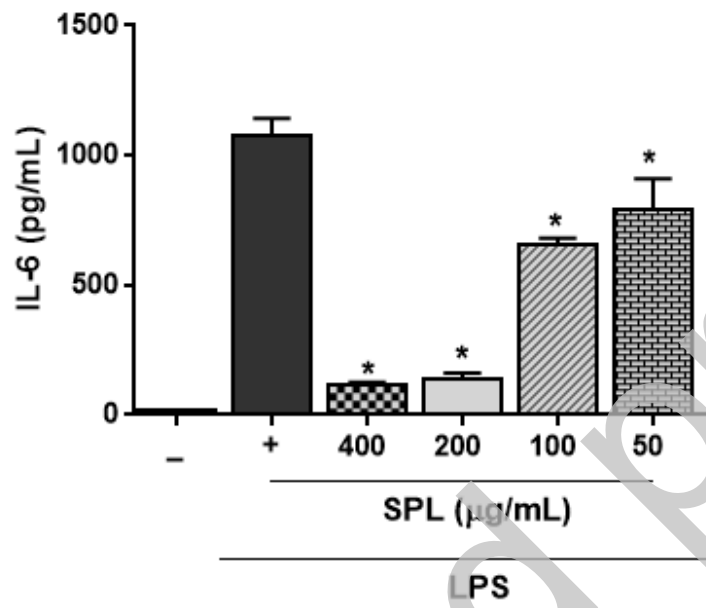


**Fig. 1** Effects of methanol extract of leaf of *S. pallescens* (SPL) on cell viability in LPS stimulated RAW 264.7 cells. Data are expressed as means  $\pm$  SEM



\*Significant percentage reduction in nitrite production

**Fig. 2 Inhibition of nitrite production in LPS stimulated RAW 264.7 cells by methanol extract of leaf of *S. pallescens* (SPL). Data are expressed as means  $\pm$  SEM**



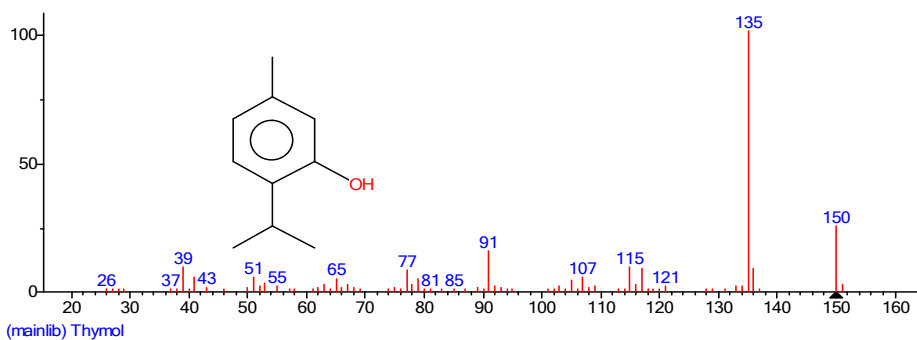
\*Significant percentage reduction in IL-6 production

**Fig. 3** Effects of methanol extract of the leaf of *S. palleescens* (SPL) on IL-6 production in LPS stimulated RAW264.7 cells. Data are expressed as means  $\pm$  SEM

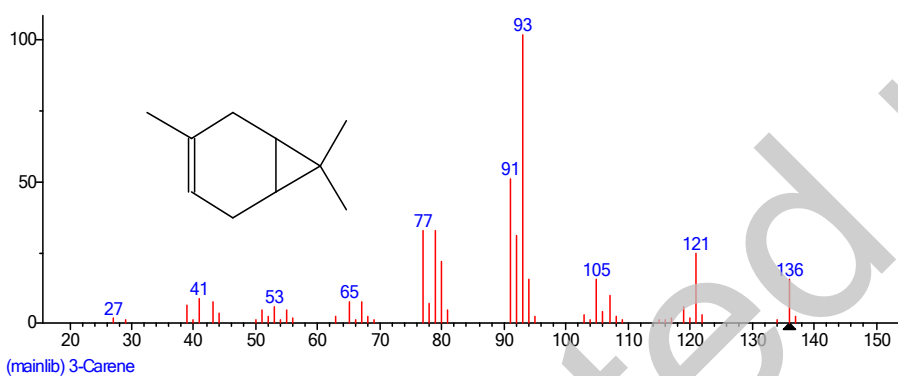
**Table 2: Chemical components of Ethyl acetate fraction of *Salacia pallescens* using GC-MS**

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-tetramethyl-	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

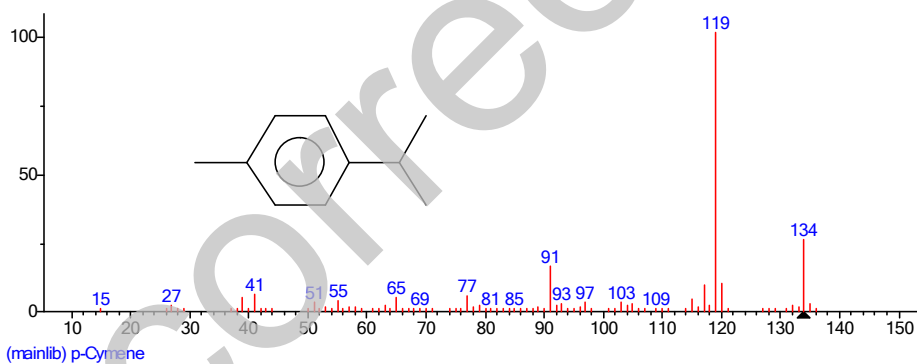




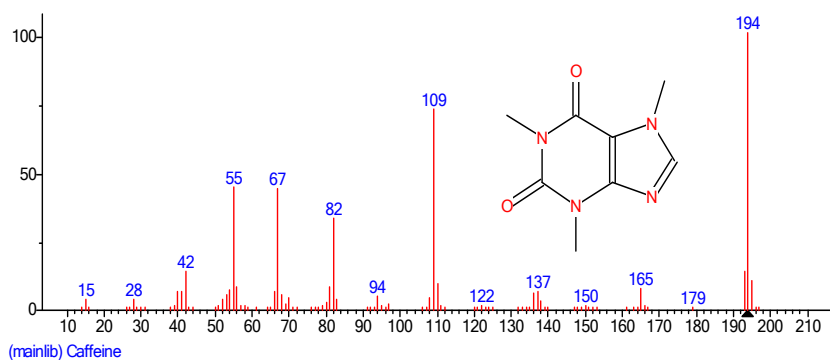
**Figure 4a Mass spectra and structure of thymol**



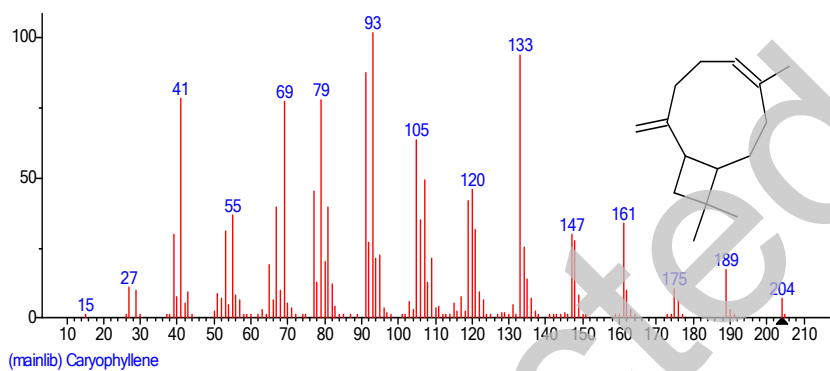
**Figure 4b Mass spectra and structure of 3-carene**



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



(mainlib) Caffeine  
**Figure 4d Mass spectra and structure of caffeine**



(mainlib) Caryophyllene  
**Figure 4e Mass spectra and structure of caryophyllene**