Immunosuppressive and hepatoprotective potential of *Sarcococca saligna* and its biomarker components


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**A B S T R A C T**

*Sarcococca saligna* methanolic extract, fractions and isolated pure compounds saracocine (1), saracodie (2), pachyximine-A (3) and terminaline (4) were found to possess potent immunosuppressive activities. The fractions and compounds were tested in-vitro for their effects on human T-cell proliferation, and cytokine (IL-2) production. All the fractions, sub-fractions and purified compounds showed significant suppressive effect on IL-2 production in a dose-dependent manner. They also exhibited a suppressive effect on the phytohemagglutinin-stimulated T-cell proliferation. None of the extracts and purified compounds showed any cytotoxicity effects on the 3T3 mice fibroblast cell line. The crude extract, DCM fraction (pH 9), DCM fractions (pH 7) and one of the steroidal alkaloids (terminaline) were checked in-vivo for their hepato-protective potential against CCl4-induced liver injury. In in-vivo experiments, the basic and neutral DCM fractions and terminaline (4) significantly reduced inflammation in the liver. DCM fraction (pH 9), DCM fractions (pH 7) and compound 4 reduced the serum enzyme levels (ALT, AST, and ALP) down to control levels despite CCl4 treatment. They also reduced the CCl4-induced damaged area to almost zero as assessed by histopathology. The pale necrotic areas and mixed inflammatory infiltrate which are seen after CCl4 treatment were absent in the cases of basic, neutral fractions and terminaline treatment. These hepato-protective effects were better than the positive control silymarin. Our results suggest the therapeutic effect of *S. saligna* extract, fractions and bioactive steroidal alkaloids against CCl4-induced liver injury in-vivo and their immunosuppressive function in vitro.

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**1. Introduction**

The liver plays a vital role in the innate immune response, providing the first line of defense against microbes and toxins [18]. Inflammatory cytokines (such as TNF-α) are produced from macrophages that lead to various conditions such as inflammatory, allergic, or autoimmune diseases of different organs [35]. Acute inflammation of the liver or acute hepatitis is characterized by infiltration of inflammatory cells mostly consisting of macrophages, neutrophils and T-cells [15; 21].

Interleukin-2 is produced mainly by CD4 + T-cells in response to different stimuli via T-cell activation of TCR and the major histocompatibility complexes (MHC) I and II molecules of the APCs [22]. The circulating level of IL-2 in normal healthy subjects is almost undetectable; however, when a person is exposed to infection, the level of circulating IL-2 rises dramatically.

Kupffer cells of the liver are critically involved in the rapid clearance of microorganisms from the systemic circulation [14,25]. Although Kupffer cells themselves are highly phagocytic and are able to remove microorganisms, they also facilitate the generation of the inflammatory response leading to the recruitment of inflammatory cells such as neutrophils, monocytes, T and B lymphocytes, as well as natural killer (NK) cells to the site of injury in the liver caused by viruses, bacteria or drug/chemicals. Upon infection, Kupffer cells generate neutrophilic inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukins (IL-1, and IL-6), chemokines and reactive oxygen species (ROS) [34]. If the liver injury is persistent then these inflammatory mediators will activate neutrophils in the hepatic microvasculature leading to a variety of oxidative stress events culminating in hepatocellular death.

For the treatment of such inflammatory diseases anti-inflammatory agents that would be harmless to the immune system are required. The development of effective anti-inflammatory and immunosuppressive agents over the last decades has made organ transplantation and the control of autoimmune diseases possible. Most of the immunosuppressive...
drugs such as cyclosporine and tacrolimus prevent graft rejection by suppressing T-cell proliferation, which in turn suppresses IL-2 production, resulting in the inhibition of the proliferation of T-cells.

Sarcococca saligna Muell.-Arg., which belong to the family Buxaceae with 16–20 species, is an evergreen aromatic shrub widely distributed throughout the hilly areas of District Swat, Hazara division of Khyber Pakhtunkhwa and Kashmir near northern Pakistan. It has been used extensively for the treatment of pain and rheumatic fever by the local population [26]. Steroidal alkaloids isolated from S. saligna [29] have shown antitumor, anticholinesterase [30] and antinflammatory activities. Ahmad et al. [1] investigated the antibacterial, antifungal, phytotoxic, insecticidal and haemagglutination activities of the crude extract and its different fractions. Several pregnane-type steroidal alkaloids with potential antitumor, antibacterial [28] and antinflammatory pharmacological properties have been isolated from this plant [17]. Some of them potentiate the action of naturally secreted acetylcholine in the isolated diaphragm of the rat and in the serum of the rabbit [20]. Some alkaloids of the Sarcococca plant exhibited ganglion-blocking activity by decreasing the effects of nicotine on blood pressure [21]. Crude extract of the Sarcococca plant caused a dose-dependent inhibitory effect on K+-induced contractions in the rat stomach fundus, guinea-pig ileum and rabbit jejunum, while calcium channel blocking activities were previously reported. In in-vivo studies, the extract exhibited antiinflammatory and antiseptic properties against causor oil-induced diarrhea and intestinal fluid accumulation in mice [13]. Characteristic steroidal compounds of the plant, saracocine, saracodine, saracorine and alkaloid-C exhibited a similar combination of acetylcholinesterase (AChE) inhibitory activity. The present study was undertaken to investigate the effects of S. saligna extract, its fractions and steroidal alkaloids isolated from these fractions on T-cell proliferation, IL-2 production and cytotoxicity. Additionally, the extract, its fractions and steroidal alkaloids (saracocine, saracodine, pachyinine-A and termenaline) were further evaluated in an in-vivo hepatoprotective study to determine their potential antiinflammatory role in the prevention of chemically induced acute hepatitis.

2. Materials and methods

2.1. Extraction, isolation and structural elucidation of pure compounds:

Whole plant of S. saligna (D.Don) Muell. was collected by Dr. Seraji—a botanist from Miandam area of Swat District, Khyber Pakhtunkhwa province, Pakistan. Plant material was identified in the Department of Botany, University of Karachi, Karachi, Pakistan. The herbarium number (85854) and voucher specimen number 01 were deposited in the Herbarium of Karachi University. Whole plant of S. saligna (about 35 kg) was dried in the shade and soaked in 80% MeOH for one week at room temperature. The solvent was evaporated with the help of a rotary evaporator and a crude extract (A, 6.5 kg) of S. saligna was obtained. Extract A (6.5 kg) was suspended in cold distilled water and defatted with total petroleum ether (3 x 20 L) producing “petroleum ether extract” (AP, 170 g). The “defatted aqueous fraction” that was left behind (B) was then extracted with dichloromethane (DCM, 3 x 20 L) to obtain another fraction in nearly neutral conditions called the “neutral fraction” (SSA, 195 g) and an aqueous fraction C. The fraction C was then made alkaline by adding ammonia solution (9-10). After that, it was extracted with DCM to obtain “alkaline fraction” (SSB, 183 g).

The alkaline fraction SSB was subjected to alumina gel (basic) vacuum liquid chromatography (VLC), by using hexane/acetone as an eluting agent which produced different fractions A1–A7. Fraction A3 (20.5 g) was further subjected to alumina column chromatography using hexane/acetone to give four sub fractions A31–A34. Subfraction A33 (3.20 g) was further subjected to alumina column chromatography by using hexane/acetone/diethyl amine (6:3:1) which yielded compounds 1 (358 mg), 2 (372.3 mg), 3 (431 mg). While the compound 4 (220 mg) was isolated from neutral DCM fraction.

Structures of the compounds were elucidated by using mass spectrometry, 1D- and 2D-NMR spectroscopy techniques. All the physical and spectral data of the compounds were unambiguously matched with the reported values in literature (Atta-ur-[30]).

3. Materials

Lithium-heparin vacutainers were obtained from BD Biosciences (Franklin Lakes, NJ, USA), RPMI-1640 from Mediatech Inc. (Herndon, VA, USA), fetal bovine serum (FBS) from Thermo Scientific Hyclone (South Logan, USA), L-glutamine and lymphocyte separation medium (LSM), and penicillin/streptomycin antibiotic from Invitrogen (Carlsbad, CA, USA), phytohemagglutinin (PHA) from Sigma-Aldrich (St. Louis, MO, USA), trypan blue from Amresco, (Solon, OH, USA), 3H-thymidine from Amersham (Little Chalfont, UK), and glass fiber filters from Connectorate AG (Dietikon, Switzerland). Phorbol-12-myristate-13-acetate (PMA) was obtained from MP Biomedicals (Illkirch, France), and the DuoSet ELISA development kit, ELISA Plate Sealers, clear high-binding, flat-bottom polystyrene microplates, Streptavidin-HRP, Substrate Reagent Pack (Reagent A, stabilized hydrogen peroxide and Reagent B, stabilized tetramethylbenzidine) from R & D systems, Inc., (Minneapolis, MN, USA).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Acros organics (NJ, USA), Dulbecco’s Modified Eagle’s Medium—high glucose (DMEM) from Sigma-Aldrich (St. Louis, MO, USA), dimethyl sulfoxide (DMSO) from Fisher Scientific (Loughborough, UK), and trypsin-EDTA from Gibco, (Grand Island, NY 14072, USA). Carbon tetrachloride (CCl4), olive oil and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.1. Radioactive 3H-thymidine-based lymphocyte proliferation assay

According to the method standardized by Froebel et al. [11], fresh peripheral blood mononuclear cells (PBMCs) were isolated and incubated with or without various stimuli in a 96-well plate and allowed to proliferate for three days at 37 °C in a 5% CO2 environment. The amount of proliferation was detected after 72 h by the addition of radioactive 3H-thymidine for an additional 18 h for incorporation into the newly synthesized DNA of the dividing cells. The proliferation of T-cells was estimated from the amount of radioactivity incorporated into the DNA in each well.

3.2. Isolation of lymphocytes

Fresh human peripheral blood was collected from healthy volunteers by vein puncture, placed into sterile vacutainers containing Lithium-heparin and thoroughly mixed. An equal volume of RPMI-1640 containing 2 mM L-glutamine was mixed with the blood sample in a 50 mL sterile centrifuge tube. Nine milliliters of the diluted blood was layered onto 5 mL of LSM in another sterile 15 mL centrifuge tubes. Care was taken not to disturb this interphase. The tubes were then centrifuged at 400 xg for 20 min at 25 °C. The buffy layer containing mononuclear cells at the interphase between the LSM and the blood plasma was carefully removed to a sterile 15 mL centrifuge tube containing incomplete RPMI-1640. The cells were washed by centrifugation at 300 xg for 10 min at 4 °C. The pellet of the peripheral blood mononuclear cells (PBMCs) was resuspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), and the cells were counted under a high power objective lens after 1:1 (v/v) dilution with trypan blue.

3.3. Preparation of [methyl-3H] thymidine and assay setup

[Methyl-3H] thymidine tracer (1.0 μCi/mL) was diluted with sterile RPMI-1640 to a concentration of 20 μCi/mL and stored in aliquots of 5 mL at −20 °C as working solutions.
Sterile 96-well round-bottomed plates were mapped and labeled for assaying the dose effect of the test compounds in triplicate. Fifty micro-liter of the PBMCs (1.2 x 10^6 cells) was cultured with 50 μL of PHA with a final concentration of 5 μg/mL, 50 μL FBS-supplemented RPMI-1640, and 50 μL of the test compounds at a final concentration of 10 μg/mL. The culture was kept at 37 °C in a humidified atmosphere of 5% CO2 in air for 72 h. To each well 0.5 μCi [methyl-3H] thymidine (25 μL) was added and the cells were further incubated for another 18 h.

3.4. Harvesting and counting

Cells were harvested on glass fiber filters using a cell harvester (Connectorate AG, Switzerland). The filters were dried by applying vacuum suction while the glass fiber filters were clamped in position. When filters were completely dried, their discs were carefully punched-out into scintillation vials. The discs were mixed with a scintillation fluid, CytoScint, and the vials were placed in a liquid beta scintillation counter (Beckman coulter, CA, USA) to measure the incorporation of radioactive thymidine by recording radioactivity as counts per minute (cpm).

3.5. Cell culture for determination of IL-2 production

Fresh human peripheral blood T-lymphocytes were used to study the effect of different compounds on the production of interleukin-2 (IL-2). The PBMCs were isolated from fresh venous blood as described above in the T-cell proliferation assay. In 96-well flat-bottomed plates, 50 μL of cell suspension (2.5 x 10^6 cell/mL), 50 μL of phytomenadnin (PHA, final dilution of 5 μg/mL), 50 μL of phorbol myristate acetate (PMA, final concentration of 20 ng/mL), and 50 μL of the test compounds (final concentration of 0.5, 5.0 or 20 μg/mL) were added. Plates were incubated in a 5% CO2 incubator at 37 °C for 18 h, and then the supernatants were collected for IL-2 determination using ELISA.

3.6. Interleukin 2 enzyme-linked immunosorbent assay

This assay was developed using the basic components of IL-2 ELISA from R & D Systems Inc. The recombinant anti-IL-2 capture antibody was diluted in PBS to give a concentration of 4 μg/mL, and then used at 100 μL/well to coat the high-binding, flat-bottom polystyrene micro-plates. Coated plates were sealed by the ELISA plate sealers and incubated at room temperature for 24 h. The antibody solution was aspirated and plates were washed three times with a 300 μL wash buffer (0.05% Tween® 20 in PBS, pH 7.2-7.4), followed by the addition of 100 μL blocking buffer (1% BSA in PBS with 0.05% NaN3) to each well. The wells were left for 1 h at room temperature. Following exposure to each test compound, cell viability was assessed using 0.5 μg/mL of MTS in complete media for 4 h followed by the removal of the supernatant and the addition of 100 μL of DMSO to each well to solubilize the formazan complex formed by the action of mitochondrial dehydrogenases. The plates were read at 540 nm after 1 min of gentle shaking. The optical density readings were recorded and, using MS Excel software, the results were analyzed and expressed as mean ± SD of triplicate readings.

3.8. Cytotoxicity using 3T3 cells and MTT assays

In-vitro cytotoxicity assays were performed as described by Scudiero et al. [32] using the 3T3 NIH mouse embryo fibroblast cell line. The 3T3 cells were maintained in DMEM formulated with 10% FBS. These are adherent cells and need to be detached from the culture flask surfaces using trypsin/EDTA treatment. The medium was removed and sterile PBS was added to each flask to wash cell debris from the cells. In each flask, 0.25% Trypsin/EDTA solution was added to the attached cells and incubated for 2–3 min at 37 °C. The flask were gently tapped and observed under the microscope to check for detachment of cells from flask surfaces followed by addition of media containing 10% FBS. Cells were collected in a 15 mL centrifuge tube and centrifuged at 1200 rpm. The pellet was resuspended in complete media and cells were enumerated using a microscope and Neubauer counting chamber.

The MTT assays on the 3T3 cells were performed using 6 x 10^3 cell/well in 100 μL complete media in a flat-bottomed 96-well plate. All plates were incubated for 24 h at 37 °C in a 5% CO2 incubator. After attachment of cells, the media was replaced by 200 μL of media containing the test compounds at variable concentrations (0.5, 5, and 50 μg/mL) in triplicate and the plates were further incubated for 48 h at 37 °C in a 5% CO2 incubator. Following exposure to each test compound, cell viability was assessed using 0.5 mg/mL of MTT in complete media for 4 h followed by the removal of the supernatant and the addition of 100 μL of DMSO to each well to solubilize the formazan complex formed by the action of mitochondrial dehydrogenases. The plates were read at 540 nm after 1 min of gentle shaking. The optical density readings were recorded and, using MS Excel software, the results were analyzed and expressed as mean ± SD of triplicate readings.

3.9. Animals

Male Wistar rats, 2–3 months old, were divided into 6 groups with 6 animals per group. Rats were fed a standard rodent diet. The animals were maintained in a 12 h light/dark cycle at a constant temperature (25 °C). All experimental procedures were approved (Animal Study Protocol #2013-0006) by the animal ethical committee of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

3.10. In-vivo experimental design

Male Wistar rats were treated with CCl4 intraperitoneally (i.p.) at a dose of 0.5 ml/kg, dissolved in olive oil, twice a day for 2 days (CCl4 group) while the normal control group received vehicle only. Other groups were pretreated with positive control silymarin [19] or crude extract or DCM fraction or terminaline (4) at doses of 200 mg/kg, 100 mg/kg, 100 mg/kg and 50 mg/kg, respectively, for three days prior to CCl4 injection [4] and the two days during the CCl4 injections.

3.11. Histology

Twenty four hours after the last CCl4 injection all the experimental groups were dissected and the liver was kept in isotonic saline and cut into pieces for different experimental protocols. The livers from all the experimental groups were fixed with buffered formalin: paraffin embedded, and cut into 6 μm thick sections. Sections for histopathological
examination were stained with hematoxylin and eosin (H&E) stain using standard histological procedure.

3.12. Blood biochemistry study

For determination of hepatic function and liver damage serum was obtained from blood collected from the heart and the serum levels of alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were measured using a dry chemistry analyzer (Roche Diagnostics, Mannheim, Germany).

3.13. Determination of MDA, SOD and GSH activity

To determine the antioxidant activity of the liver, it was excised immediately after the animals were sacrificed and one of the lobes of the liver was quickly frozen and stored at −80 °C. The liver tissue samples were thawed and homogenized in 9 volumes of ice cold 50 mM PBS (pH 7.4), centrifuged for 20 min and stored at 4 °C. For determination of MDA, SOD, GSH and protein determination commercially available kits were used (Sigma Aldrich, Saint Louis, USA). MDA, SOD and GSH levels were normalized with protein.

3.14. Statistical analysis

Data analysis was carried out using SPSS software. Statistically significant differences between the samples were evaluated by t-test. Differences at P < 0.05, or P < 0.01 were considered as significant.

4. Results

4.1. Effect of the test compounds on T-cell proliferation

Most of the drugs that are in clinical use for prevention of graft rejection act through suppressing the proliferation of T-cells, such as the immunophillin-binding agents (cyclosporine and tacrolimus), which suppress IL-2 production, resulting in the inhibition of T-cell proliferation. IL-2R antibodies also inhibit the proliferation of T-cells by blocking the downstream signaling of the IL-2 receptor. Therefore in this study, the effects of the compounds were tested on T-cell proliferation first. During the optimization of the T-cell proliferation assay, we tested four different mitogens; among them, PHA was found to be the best activator at a concentration of 5 μg/mL (Fig. 1). We then investigated the inhibitory effect of all the test compounds on PHA-activated T-cell proliferation. Most of the tested compounds isolated from the extract and fractions of S. saligna showed T-cell suppressive activity with an IC_{50} value of less than 10 μg/mL (Fig. 1), which indicated that they are promising drug candidates for graft rejection prevention.

The extract, the fractions of its isolated alkaloids, and compounds 1–4 showed variable activities against T-cell proliferation. The crude extract showed 96 ± 0.6, neutral DCM (at pH 7) showed 62 ± 3.0, and basic DCM (at pH 9) fraction showed 44 ± 9.5 inhibitory activities against the proliferation of T-cells when used at a concentration of 10 μg/mL. Of the pure compounds, pachyamine-A (3) showed 97 ± 0.4, saracocine (1) showed 55 ± 4.5, terminaline (4) showed 86 ± 0.6 and saracodine showed 81 ± 5.5 (2) T-cell inhibitory activities (Table 1). The treated extract, fractions and purified compounds showed a different range of inhibitory activities against the proliferation of T-cells, ranging from 44 to 97%; and the results are summarized in Fig. 1 and Table 1, which show suppressive activities with an IC_{50} of less than 10 μg/mL. These suppressive activities were comparable to those of the standard drugs, cyclosporine and tacrolimus.

4.2. Effect of the test compounds on IL-2 production

The extracts and compounds subjected to the T-cell proliferation assay were also tested for the production of the cytokine IL-2 by PHA-activated T-cells, as this cytokine is responsible for their proliferation as well as the proliferation of other immune cells which contribute in cellular and adaptive immune response. All of them showed significant suppressive effects on IL-2 production with an IC_{50} ≤ 5.0 μg/mL. The results are summarized in Fig. 2 and Table 1.

4.3. Cytotoxicity assay

Cytotoxicity of the pure compounds, fractions and extract that showed immunosuppressive activity was also evaluated using the mouse fibroblast cell-line (3T3) to ensure that the immune suppressive activity was not merely due to their cellular toxicities. Although the incubation time of the compounds with the 3T3 cells was far longer than their incubation with the immune cells, we found that the minimum
The effects of S. saligna extracts and compounds on CCl₄-induced oxidative stress.

### 4.4. Effect of S. saligna biomarker components on CCl₄-induced oxidative stress

In order to substantiate whether S. saligna crude extract, DCM fraction and terminaline (4) protected mice against CCl₄-induced oxidative stress, MDA production, GSH level and the activities of SOD enzymes in the liver were measured. Fig. 7 shows the effects of S. saligna biomarkers on CCl₄-induced oxidative stress. Upon CCl₄ intoxication the level of MDA was significantly increased (P < 0.01). However, treatment with crude extract, DCM fraction and terminaline significantly decreased the level of the hepatic MDA (P < 0.05 or 0.01). Intraperitoneal injection of CCl₄ dramatically reduced the level of GSH in the liver of rats compared with the normal control group (P < 0.01). However, GSH level was increased significantly by pretreatment with S. saligna biomarkers (P < 0.05). The activities of the hepatic antioxidant enzyme SOD were decreased significantly in rats treated with CCl₄ alone (P < 0.01); however, co-administration of crude extract, DCM fraction and terminaline (4) avoided the CCl₄-induced decrease in the activities of hepatic antioxidant enzymes significantly (P < 0.05).

### Table 1

The effect of S. salinga extracts and compounds on T-cells proliferation, IL-2 production and cytotoxicity.

<table>
<thead>
<tr>
<th>Name and codes of analyte and standards</th>
<th>T-cell proliferation, % inhibited (mean ± SD) at 10 μg/mL</th>
<th>Inhibition of IL-2 production, IC₅₀ in μg/mL</th>
<th>3T3, CC₅₀ μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic DCM</td>
<td>44 ± 9.5</td>
<td>4.3 ± 1.4</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Saracocine</td>
<td>55 ± 4.5</td>
<td>2.1 ± 0.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Neutral DCM</td>
<td>62 ± 3.0</td>
<td>2.97 ± 0.3</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Saracedine</td>
<td>81 ± 5.5</td>
<td>2.47 ± 0.2</td>
<td>14.9 ± 1.5</td>
</tr>
<tr>
<td>Pachyxmine-A</td>
<td>97 ± 0.4</td>
<td>1.82 ± 0.17</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Crude extract</td>
<td>96 ± 0.6</td>
<td>5.1 ± 0.70</td>
<td>16.3 ± 1.0</td>
</tr>
<tr>
<td>Terminaline</td>
<td>86 ± 0.6</td>
<td>0.7 ± 0.02</td>
<td>23.4 ± 2.1</td>
</tr>
<tr>
<td>Cycloheximide (Std1)</td>
<td>--</td>
<td>&lt;0.05</td>
<td>--</td>
</tr>
<tr>
<td>Cyclosporine (Std2)</td>
<td>98 ± 1.3</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

IC₅₀ among the examined compounds was around 8.5 μg/mL except for pachyximine-A (3). The other compounds, extracts and fractions were found to be very safe having no effect up to 50 μg/mL (basic DCM) (Table. 1 and Fig. 3). Of the pure compounds terminaline (4) was found to be the least toxic and therefore was selected for testing in-vivo.

### 4.5. Hepatoprotective activity of S. saligna: histopathological studies

The liver sections from normal control rats that were stained with hematoxylin and eosin (H&E) showed normal hepatic architecture, that is, hepatic cords of cells lined with endothelial cells with distinct sinusoidal spaces (Fig. 4A). In contrast, liver sections from the CCl₄ control group showed degenerated and necrotic hepatocytes containing hyaline bodies (Fig. 4B). Extensive mixed inflammatory cell infiltrate was present around the central vein areas. In the vicinity of the injured region, hepatic damage was less extensive, particularly immediately alongside the lesion border (Fig. 4B). Treatment with positive control silymarin at a dose of 200 mg/kg reduced the pathological changes of CCl₄ and maintained the structural integrity of the perportal and non-parenchymal cells as shown in Fig. 4C. However, few inflammatory cells were still present around the central vein in the injured area. Interestingly, MeOH extract of S. saligna, at a dose of 100 mg/kg, completely protected the hepatic membrane integrity from CCl₄-induced oxidative damage and made it appear normal as shown in Fig. 4D. The DCM fraction at a dose of 100 mg/kg, reduced the pathological changes induced by CCl₄ as the sinusoidal spaces lined with endothelial cells which is one of the morphological hallmarks of a normal liver was observed (Fig. 4E). Similarly, terminaline (4) at a dose of 50 mg/kg body weight also showed hepatoprotective morphology compared to the CCl₄ control group (Fig. 4F). However, some hydropic degeneration which is indicative of mild oxidative stress was also present in the livers of the terminaline treated group. Therefore, the extract, fractions and terminaline (4) significantly reduced the pathological changes produced by oxidative injury to the liver compared to the positive control group.

### 4.6. Hepatoprotective activity of S. saligna: biochemical studies

Serum levels of ALT, AST, and ALP activities were determined in all of the experimental groups. The levels of ALT, AST, and ALP were significantly elevated after receiving CCl₄ indicating membrane damage and necrosis of hepatocytes (Fig. 5). Pretreatment with the positive control silymarin reduced the ALT, AST, and ALP levels but not to control levels indicating that some hepatocyte necrosis persisted. In contrast, the MeOH extract of S. saligna and one of its DCM fractions, showed excellent hepatoprotection as the levels of ALT, AST, and ALP were similar to the control despite the CCl₄ treatment. Moreover, terminaline (4)
Fig. 3. Cytotoxicity effects of the test materials on 3T3 mice fibroblast cell line. Cells were incubated with compounds for 48 h then MTT was added for 4 h followed by addition of DMSO and absorbance was read at 540 nm using a 96-well plate reader. Results are expressed as mean ± SD of three replicates. Positive control is normal fibroblasts without any treatment.

Fig. 4. Histopathological profile of the effects of the test materials on hepatitis in-vivo. Normal control group showed the characteristic normal hepatocytes arranged in chords and lined with sinusoidal spaces (A); liver tissue from CCl₄-treated group revealed the presence of centrilobular necrosis (B); liver tissue from silymarin-treated group revealed reduced inflammation but accumulation of some mononuclear cells around the central vein region (C); liver tissue from rats pretreated with MeOH extract showed lower intensity of mononuclear accumulations around central vein (D); liver tissue from rats pretreated with DCM fraction completely protected from CCl₄ induced injury (E); terminaline treated group reduced CCl₄ injured oxidative liver injury to a large extent (F). Green arrows indicate areas of normal tissue whereas black arrows indicate areas of damaged liver tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
isolated from the DCM fraction also exhibited protective role by significantly reducing the elevated levels of ALT, AST, ALP more than the positive control silymarin as shown in Fig. 5.

5. Discussion

The immune system is constantly on the guard against any invasion of bacteria, viruses, or other potentially dangerous biological agents. When tissue from a donor is transplanted, or during autoimmune diseases, the immune system recognizes the transplanted tissue or one’s own tissue as a foreign invader and goes into battle against it [36]. White blood cells in general attack and destroy the unknown tissue in a process known as the immune response [3]. T-lymphocytes play a critical role in all types of immune response [5].

The objective of the present study was to discover different classes of compounds that could be lead agents for inhibiting T-cell proliferation without causing cytotoxicity and which would have good hepatoprotective properties, as it is well known that in viral hepatitis the destruction of hepatocytes is due to the immune response and not due to the virus itself. We investigated compounds for their effect on PHA-activated T-cells, as PHA is known to specifically activate CD4+ T-cells [37]. Compounds that show inhibitory activity against the proliferation of T-cells were investigated for their effect on the production of IL-2 by PHA-activated T-cells, as this cytokine is responsible for their proliferation as well as for the proliferation of other immune cells. IL-2 is produced mainly by CD4+ T-cells in response to different stimuli via T-cell activation through its TCR and the major histocompatibility complexes (MHC) I and II molecules of the APCs. The circulating level of IL-2 in normal healthy subjects is almost undetectable; however, when a person is exposed to infection, the level of circulating IL-2 rises dramatically. The combined stimulation of PHA + PMA was found to be very efficient for the production of IL-2, and is consistent with a previously reported optimization protocol [33]. The PHA binds to the sugars on glycosylated T-cell surface proteins, including the TCR, and thereby causes their crosslinking. This PHA and TCR crosslinking activates signal 1 (and possibly also signal 2 via crosslinking of co-stimulatory molecules). These

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Fig. 5. Biochemical profile of the effects of S. saligna extracts, fraction and compound on CCl4-induced liver injury: Serum levels of ALT, AST, and ALP as markers of liver injury under various conditions. Note that the methanolic extract (MeOH), DCM fraction, and terminaline showed complete protection against CCl4-induced liver injury as compared to CCl4 (P < 0.001) or CCl4 + silymarin (P < 0.001).

Fig. 6. The structures of the steroidal alkaloid compounds used in the present study: saracocine (1), saracodine (2), pachyximine-A (3) and terminaline (4).
processes occur on the cell surface and might, therefore, involve a variety of signaling pathways. Phorbol 12-myristate 13-acetate (PMA), on the other hand, is an ester of a natural, plant-derived organic compound phorbol, which was first isolated from Croton tiglium. PMA diffuses through the cell membrane into the cytoplasm, where it directly activates protein kinase C (PKC) as it is structurally similar to the natural PKC activator diacylglycerol. Activation of PKC is required for the production of IL-2 by the stimulated T-cells. The examined steroidal alkaloid compounds can be considered for further investigations as immunosuppressive agents since they showed promising immunosuppressive properties (Fig. 6).

We further determined the cytotoxicity of the tested compounds, which showed that the suppressive activity against T-cell proliferation and IL-2 production is not merely due to their cytotoxicity. The steroidal alkaloid class of compounds was investigated for their anti-inflammatory effects, as plants having these compounds have been used as a source of medicine throughout history and continues to serve as the basis for many pharmaceuticals used today [24].

During acute hepatitis although innate immune cell activation after cell death/necrosis aggravates the initial tissue injury, the sterile inflammatory response does not necessarily cause liver damage. The main purpose of activating the Kupffer cells and recruiting monocytes, neutrophils and natural killer cells into the liver after CCl4-induced hepatic injury is to remove dead or dying hepatocytes. This removal of cellular debris is vital for the regeneration of lost tissue. Typical examples are CCl4-induced hepatic injury, concanavalin-A [27], lipo-polysaccharide [8] or acetaminophen hepatotoxicity, which trigger the inflammatory response, with neutrophils appearing within an hour after early liver injury and the recruitment of monocytes and macrophages after 24–48 h [6,16]. In our in-vivo experiments, the crude extract and its fractions, as well as the isolated steroidal alkaloids, showed excellent protective results by suppressing the damage to the hepatocytes without causing any side effects on other tissues. CCl4 is accumulated in the hepatocytes—the hepatic parenchymal cells and metabolically activated by cytochrome P450-dependent monoxygenases to form highly reactive free radical metabolites, such as trichloromethyl radical (CCl3−) and trichloromethyl peroxide radical (CCl4OO−) [31] causing hepatotoxic effects such as hepatocellular death, degeneration and fibrosis [23,38]. These overproduced free radicals could overwhelm the cellular antioxidant defense systems causing lipid oxidation. Among the complex micro-environmental factors, the resident hepatic macrophages known as the Kupffer cells carry out a very important function in modulating the severity of liver inflammation [7,10]. It has been suggested that a variety of proinflammatory cytokines, monocyte chemo attractant protein (MCP), and tumor necrosis factor-α (TNF-α) are produced by the Kupffer cells upon liver injury provoking the hepatic stellate cells to enhance expression of extracellular matrix proteins in prolonged liver inflammation, and this subsequently contributes to hepatic injury [10,12].

Our results suggest that the steroidal alkaloids may decrease liver inflammation in two possible ways: 1) by decreasing the proliferation of T-cells and IL-2 levels, and thereby muting the whole inflammatory response and 2) by acting as antioxidants and scavenging the free radicals generated by the hepatocytes.

In short, the major in-vitro finding of this study is that *S. saligna* extract, fractions and isolated steroidal alkaloids are non-cytotoxic but inhibit T-cell proliferation. When further explored in an in-vivo study they significantly reduced CCl4-induced liver injury and mixed inflammatory infiltrate. Thus, we are suggesting that *S. saligna* and its bioactive alkaloids may be excellent anti-inflammatory and hepatoprotective agents with very good therapeutic potential.

**References**


