YUGATO

ISSN: 0387-5695, eISSN: 0387-5695
Vol. 76 No. 1 (2024)

Received: November 16, 2023 / Revised: December 11, 2023 / Accepted: January 09, 2024 / Published: January 30, 2024

ACTIVE FRACTION ISOLATED FROM *ORTHOSIPHON STAMINEUS* LEAF EXTRACT HAS AN ANTI-NEPHRITIC EFFECT IN STZ INDUCED DIABETIC RATS

Venkatesan Natarajan[1]*, Pradeepraj Devarasu[2], Aravinth Velmurugan[3], Thamizh Sendhamaraikannan[4]
[1*,2,3,4]School of Pharmacy, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry-607402, India
***Corresponding Author:-**Venkatesan Natarajan
*School of Pharmacy, Sri BalajiVidyapeeth (Deemed to be University), Pondicherry-607402, India

ABSTRACT

AIM: To evaluate the effect of an active fraction from o*rthosiphon stamineus* leaves on anti-nephritic effect in STZ induced diabetic rats

METHOD: Nephritis was induced by streptozotocin in Wistar rats. Petroleum ether, ethyl acetate, and ethanol extracts of *Orthosiphon stamineus* leaves were administered orally at a dose of 200 mg/kg, p.o.An extract showing higher antinephritic activity was subjected to column chromatography and led to the isolation of an active fraction, which was given trivial name OS-1. OS-1 (100 mg/kg, p.o.) was studied for its anti-nephritic potential.

RESULTS: The ethanol extract was found to lower the creatinine level significantly ($P< 0.05$) in diabetic rats. OS-1 caused a significant ($P< 0.05$) reduction in Urea and Creatinine level, and additionally it caused reduction in blood glucose in diabetic rats.

CONCLUSION: Reduction in the Urea and Creatinine levels and an improvement in the electrolytes maintenance by OS-1 indicates that OS-1 has anti-nephritic activity, along with anti diabetic potential, and provides a scientific rationale for the use as an anti-nephritic agent.

KEY WORDS: *Orthosiphon stamineus*; Creatinne; Urea; GC-MS.

INTRODUCTION

Almost one-third of hyperglycemic and hypertensive individuals have glomeruli nephritis, one of the most serious consequences linked to diabetes and high blood pressure. If the condition worsens and develops into end-stage renal disease, the patients' mortality and morbidity will rise. The main pathological alterations associated with nephritis are thickening of the tubular and capillary basement membranes, expansion of the mesangial matrix, loss of podocytes, glomerulosclerosis, and tubulointerstitial fibrosis [1]. According to certain research, apoptosis and an increase in reactive oxygen species (ROS) in renal tissues may cause a drop in the quantity of renal cells. Thus, lowering oxidative stress and inhibiting apoptosis would be advantageous for treating glomeruli nephritis. Although there isn't a medication that explicitly treats kidney failure, there are medications that can assist manage many of the issues that lead to the illness and its potential complications. India continues to employ medicines for healthcare for a variety of reasons, including as cultural acceptance, ease of acceptance and affordability, and in certain cases, the prohibitive cost and non-availability of allopathic drugs [2]. Southeast Asian herbal tea containing *Orthosiphonstamineus* (Lamiaceae family) has long been used to cure a variety of illnesses, such as diabetes, gastrointestinal tract issues, renal problems, and obesity [3]. Based on previous phytochemical analyses

of *O. stamineus*, the leaves exhibit significant potassium content as well as significant amounts of flavones, polyphenols, bioactive proteins, glycosides, and essential oils [4]. Twenty phenolic compounds, comprising two flavanol glycosides, nine caffeic acid derivatives, and nine lipophilic flavones, have been identified. Phenolic compounds are highly concentrated in the leaves. Thus, for the purpose of standardising various O. stamineus leaf extracts, rosmarinic acid (RA), 3-hydroxy-5,6,7,4-tetramethoxyflavone, sinensetin, and eupatorin were considered as marker compounds [5]. Recent research indicates that *Orthosiphonstamineus* (OS) may have minimal or no negative effects when used as a medication due to its biological activity [6]. A biologically active ingredient with anti-apototic and anti-oxidative properties is different extract of OS [7]. Investigating the OS's protective impact against type 2 diabetic mellitus (T2DM) nephropathy caused by a high-fat diet (HFD) and streptozotocin (STZ) is difficult, though. Thus, the purpose of this experiment was to investigate the impact of an *Orthosiphonstamineus* on nephritis associated with hyperglycemia. However, the anti-nephritic activities of *Orthosiphonstamineus* and their underlying mechanisms have not been reported.

MATERIAL AND METHODS

Drugs and chemicals

Streptozotocin (STZ) was purchased from Sigma Aldrich, Bangalore, India. Analytical grade chemicals, including various organic solvents (petroleum ether, ethyl acetate, chloroform, ethanol and methanol) from S.D. Fine Chemicals, India, were used for the extraction and phytochemical studies of the constituents.

Preparation of plant extracts

*Orthosiphonstamineus*leaves were collected from the forest of kalakatu, Tirunelveli District, India. Taxonomic identification was made from botanical survey of medicinal plants, Siddha Unit, Government of India, Palayamkottai and authenticated by TaxonomistDr. S. Mutheeswaran with the voucher specimen No (XCH-40490). Voucher specimen has been prepared and preserved in the School of Pharmacy, Sri BalajiVidyapeeth University for the future reference. Fresh plant leaves were shade dried at room temperature, ground into fine powder and stored in airtight containers. Then extracted (amount 500 g) with solvents of increasing polarity such as petroleum ether, ethyl acetate, and ethanol, for 72 hours with each solvent, by continuous hot extraction using the soxhlet apparatus at a temperature of 60 °C [8]. The extracts were concentrated under reduced pressure using a rotary evaporator to constant weight. The extracts were collected and preserved in a desiccator until used for further studies.

Acute toxicity study

An acute toxicity study was performed according to OECD-423 guidelines. Albino rats (n = 6) of either sex and selected by a random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water only. The various extracts of *Orthosiphon stamineus* were suspended in normal saline : Tween 80 (95 : 5) which was administered orally at a dose of 5 mg/kg initially and the incidence of mortality was observed for three days. The mortality was observed in 5/6 or 6/6 animals, and then the dose administered was considered as the toxic dose. However, if the mortality was observed in less than four rats out of six animals, then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher doses, such as 50, 300, 1000, and 2000 mg/kg [9].

Animals

Male Wistar rats each weighing 180–220 g was obtained from MGMCRI in Sri Balaji Vidyapeeth University, Pondicherry, India. The guidelines of the Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA) of the Government of India were followed, and prior permission was granted from the Institutional Animal Ethics Committee (No. 10/IAEC/MG/04/2023-I). Rodent laboratory chow and water were accessed *ad libitum*, and rats were maintained on a 12 h light/dark cycle in a temperature regulated room (20–25 °C) during the experimental procedures.

Induction of Nephritis

The fasted rats were injected intravenously with 50 mg/kg of STZ, and fed with high energy diet of 20% sucrose and 10% lard. The STZ was freshly dissolved in citrate buffer (0.01 mol/L, pH 4.5) and kept on ice prior to use. One week after STZ administration, the rats with blood creatinine concentrations of over 5mg/dL were considered to be nephritic and were used in the experiments.

Isolation of the active principle from the ethanolic extract of *Orthosiphon stamineus* leaf material

It decided to do further studies on an ethanolic extract of *Orthosiphon stamineus* as it had been found in previous studies to have swift pharmacological activity [8].The ethanolic extract of *Orthosiphon stamineus*(10 g) was subjected to chromatography over a column of silica gel (60- 120 mesh). The column was eluted successively with petroleum ether, petroleum ether–chloroform mixtures, and chloroform–methanol mixtures in different proportions in the order of increasing polarity. Fractions with same R_f values on TLC were combined and evaporated under reduced pressure. The major active fraction (2.5 g) was obtained after elution with chloroform : methanol (70 : 30), and was further purified by chromatography over a column of silica gel (100–200 mesh) resulting in a yellow amorphous solid (2.0 g) after elution with chloroform:methanol (75 : 25). The isolated fraction was examined by GC-MS analysis, and was given a trivial name OS-1.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis of isolated fraction of *Orthosiphon stamineus*was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused with a capillary column (30 × 0.25 µm × 0.25 µmdf). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 2 µL was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min to 200°C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectrum was taken at 70 eV; a scan interval of 0.5 sec and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC-MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass version-5.2.

Identification of the isolated fraction

Interpretation of the GC-MS was conducted using the database of the National Institute Standard and Technology (NIST) having more than 62 000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

Effect of OS-1 on renal profile in nephritic rats

Normal and nephritic rats were divided into four groups with six rats in each group. Group I-normal rats received distilled water (1 mL) only. Group II-nephritic rats received normal water (1 mL) only. Group III-nephritic rats received 100 mg/kg OS-1. Group IV-nephritic rats received 200 mg/kg of OS-1. All of the groups were treated orally for 21 days. At the end of the experimental period, the animals were fasted overnight for eight hours and blood samples were taken from the retro orbital plexus under mild ether anesthesia. Plasma was separated out and the urea and creatinine level measured by the method of Jaffe"s AlkalinePicrate-Kinetic.[10].

Histopathological examination

After sacrifice the animals, the kidney of each rat will remove quickly and store in a -80^{o} C refrigerator. The kidney tissue will be embedded in paraffin and then cut into 5µm transverse slices, which will be stained with different staining solutions and then observe through an optical microscope [11].

Statistical Analysis

Data are expressed as $\bar{x} \pm$ SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference test was used for mean comparisons and $P< 0.05$ was considered to be statistically significant.

RESULTS

Acute toxicity study

The acute toxicity study revealed that isolated fraction of *Orthosiphon stamineus* did not produce any toxic symptoms when administered orally to rats. The lethal dose (LD_{50} value) was 2000 mg/kg body weight.

Identification of the isolated fraction and dose establishment

OS-1 was found to be the active fraction of *Orthosiphon stamineus* leaf. GC-MS confirmed the complete structure of OS-1 which was identified to be a mixture of Ocimene, 3-Carene, Cyclohexanol and Limonene epoxide as shown in Fig. 1. The acute toxicity study revealed that OS-1 did not produce any toxic symptoms when administered orally to rats. The lethal dose (LD_{50} value) OS-1 was 1000 mg/kg body weight.

Effect of OS-1 on renal profile of nephritic rats

OS-1 produced a significant ($P< 0.05$) reduction in urea and creatinine in diabetic rats as summarized in Table-1. Additionally OS-1 also caused significant ($P< 0.05$) reduction in the level of blood glucose.

Histopathology of Kidney

Histopathological studies of the kidney slides of normal rats showed glomerulus in normal size and cellularity, no increase in mesangial matrix or thickening of the glomerulus basement membrane. Tubules were with in the normal limits.Kidney slides of nephritic induced rats showed increase in glomerulus size, increase in mesangial matrix and thickening of glomerulus basement membrane. Kidney slides of OS-1 (100 mg/kg) treated rats showed glomerulus in normal size and cellularity, moderate increase in mesangial matrix is seen. There is also mild thickening of glomerular basement membranes. Tubules were within the normal limits.Kidney slides of OS-1 (200 mg/kg) treated rats showed glomerulus in normal size and cellularity, moderate increase in mesangial matrixis seen. There is also mild thickening of glomerular basement membranes. Tubules were within the normal limits. The histopathologicsl results illustrated as Figure-2.

Table 1: Effect of OS -1 on the Serum Urea and Serum Creatinine Level

Treatment	Urea (mg/dl)	Creatinine (mg/dl)
Normal control	24.36 ± 4.32	1.38 ± 0.23
Nephrtitic control	31.13 ± 6.51	5.94 ± 0.50
OS-1 (100 mg/kg)	28.87 ± 1.38	3.48 ± 0.19
OS -1 (2000 mg/kg)	24.80 ± 6.51*	1.48 ± 0.15*

n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnett's multiple comparison tests. *$P < 0.05$, compared to normal control group

DISCUSSION

In experimental fields, STZ is commonly used as a diabetogenic agent to destroy pancreatic β-cells, resulting in the development of type 1 diabetes [12,13]. However, in analyses of renal phenotypes in STZ-induced type 1 diabetic mice, one potential concern is renal toxicity induced by the administration of STZ. In consideration of the increased usage of STZ to treat NETs, a more detailed understanding of the underlying mechanisms of renal toxicity by STZ is essential for preventing its adverse effects. Although experimental findings on STZ-induced nephrotoxicity have already been reported[14, 15] the underlying molecular mechanisms remain unclear. An ethanol extract was fractionated by column chromatography which led to the isolation of several specific compounds from the isolated fraction and identification by GC-MS. After performing a GC-MS study of the isolated fraction (OS-1), the GC chromatogram revealed four compounds at retention times of 6.65, 7.2, 8.43 and 8.93 min respectively. Further, the mass spectrum of the compound having RT 6.65 and 7.2 min confirmed the structure of ocimene and careen. The compound having RT 8.43 and 8.93 min confirmed the structure of cyclohexanol and limonene epoxidase from the mass spectrum. At present, the exact mechanism of action of the isolated fraction of OS-1 is not yet known and will be the subject of further studies.

ACKNOWLEDGEMENT

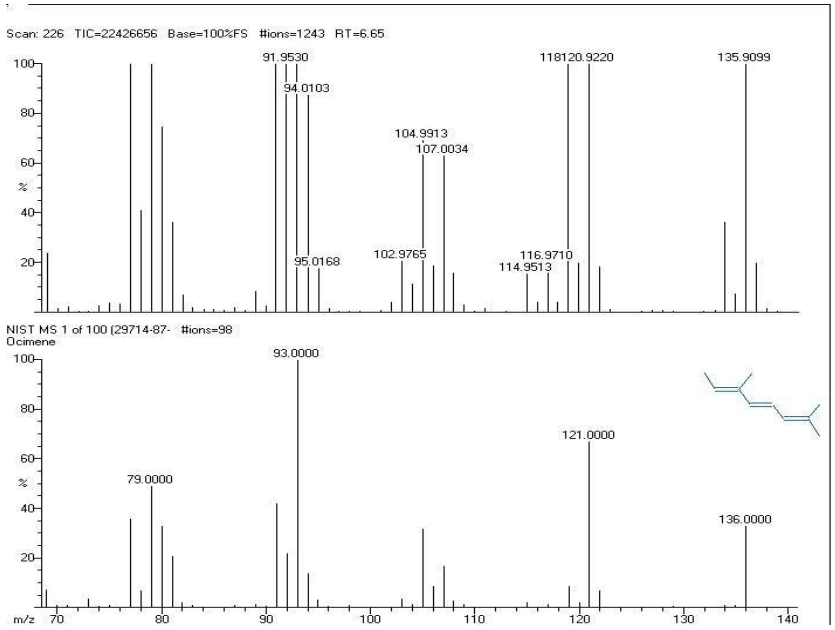
The authors express their sincere thanks to the Sri BalajiVidyapeeth (Deemed to be University) for financial support to carry out this research Intramural Seed Money Research Grant Number 150/2023.

CONFLICT OF INTERSEST

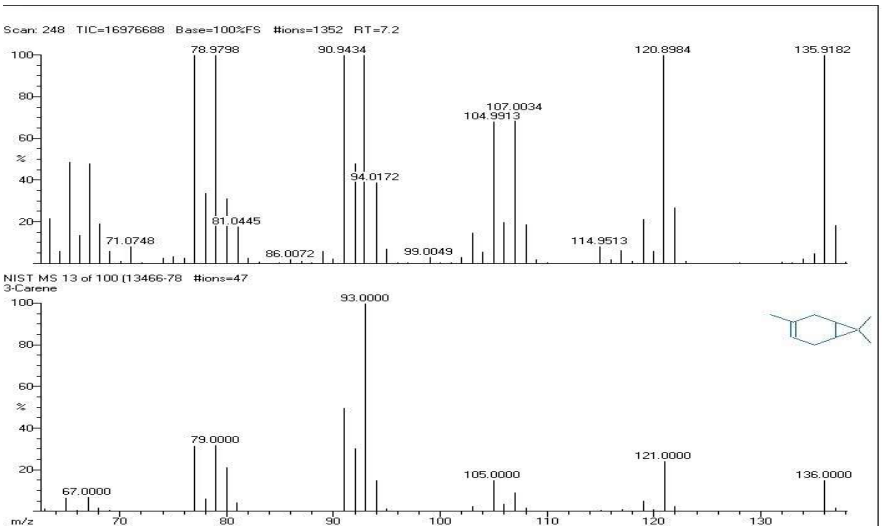
The authors declare no conflict of interest.

REFERENCE

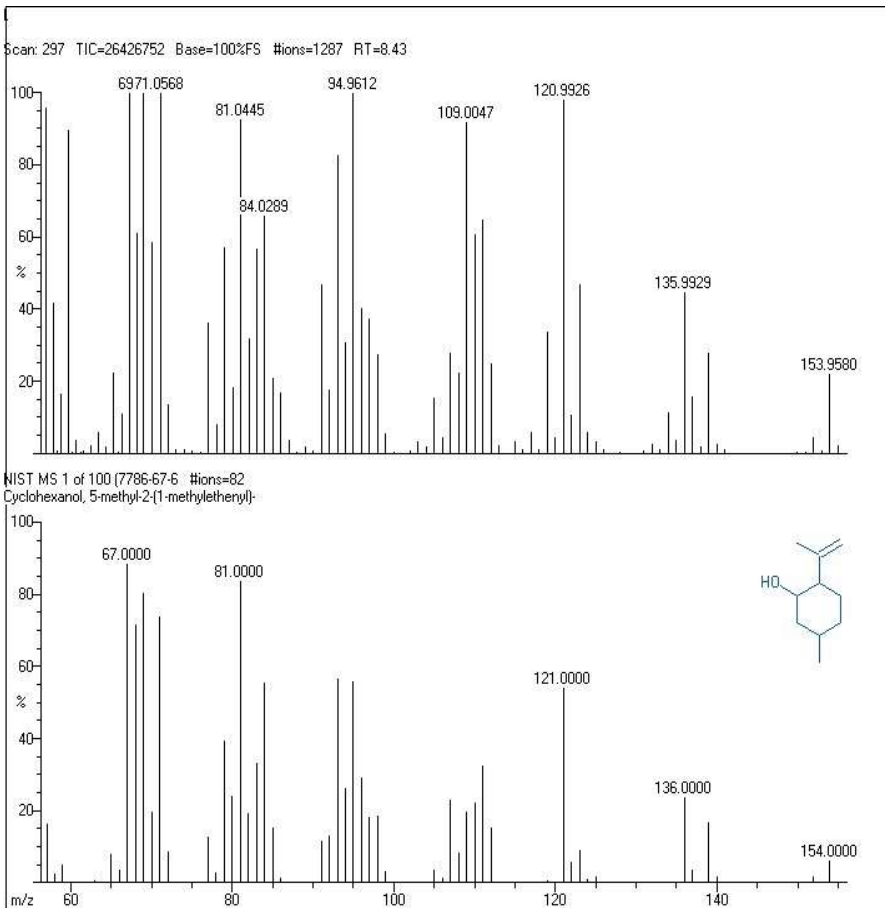
1. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care. 1998;**21**:1414–1431.
2. United States Renal Data System. USRDS 2007 Annual Data Report. Bethesda, MD: National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, U.S. Department of Health and Human Services. 2007.
3. Olennikov DN, Tankhaeva LM. Physicochemical characteristics and antioxidant activity of melanoidin pigment from the fermented leaves of *Orthosiphon stamineus*. *Brazilian J Pharmacog.* 2011;22:284–90.
4. Akowuah GA, Zhari I. Effect of extraction temperature on stability of major polyphenols and antioxidant activity of *Orthosiphon stamineus* leaf. *J Herbs Spices Med Plant.* 2010;16:160.
5. Masuda T, Masuda K, Shiragami S, Jitoe A, Nakatani N. Orthosiphol A and B, novel diterpenoid inhibitors of TPA (12-O-tetradecanoylphorbol–13–acetate)–induced inflammation, from *Orthosiphonstamineus*. *Tetrahedron.* 1992;48:6787–92.
6. Tezuka Y, Stampoulis P, Banskota AH, Awale S, Tran KQ, Saiki I, et al. Constituents of the Vietnamese medicinal plant *Orthosiphon stamineus*. *Chem Pharm Bull.* 2000;48:1711–9.
7. Mohamed EA, Mohamed AJ, Asmawi MZ, Sadikun A, Ebrika OS, Yam MF. Antihyperglycemic effect of *Orthosiphon stamineus* Benth leaves extract and its bioassay-guided fractions. *Molecules.* 2011;16:3787–801.
8. Venkatesan Natarajan, Anton smith A. Effect of an active fraction isolated from the leaf extract of ***Leptadenia reticulata***on plasma glucose concentration and lipid profile in streptozotocin-induced diabetic rats *Chinese Journal of Natural Medicines*. 2014;12(6): 463-68.
9. Martina Jonsson, Marika Jestoi, Alexis V *et al.*, Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. *Food and Chemical Toxicology*. 2013; 53: 27-32.
10. Kume T, Saglam B, Ergon C, Sisman AR. Evaluation and comparison of Abbott Jaffe and enzymatic creatinine methods: Could the old method meet the new requirements?.*J Clin Lab Anal*. 2018;32(1):e22168.
11. He X, Li G, Chen Y, Xiao Q, Yu X, Yu X, Lu X, Xiang Z. Pharmacokinetics and Pharmacodynamics of the Combination of Rhein and Curcumin in the Treatment of Chronic Kidney Disease in Rats. Front Pharmacol. 2020; 23(11):573118.
12. Elsner, M., Guldbakke, B., Tiedge, M., Munday, R. &Lenzen, S. Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. *Diabetologia.*(2000); 43: 1528–1533.
13. Umino, H. *et al.* High basolateral glucose increases sodium-glucose cotransporter 2 and reduces sirtuin-1 in renal tubules through glucose transporter-2 detection. *Sci. Rep.* (2018); 8: 6791.
14. Itagaki, S., Nishida, E., Lee, M. J. & Doi, K. Histopathology of subacute renal lesions in mice induced by streptozotocin. *Exp. Toxicol. Pathol.* 1995;47: 485–491.
15. Gezginci-Oktayoglu, S., Coskun, E., Ercin, M. &Bolkent, S. 4-Methylcatechol prevents streptozotocin-induced acute kidney injury through modulating NGF/TrkA and ROS-related Akt/GSK3beta/beta-catenin pathways. *Int. Immunopharmacol.* 2018; 64: 52–59.



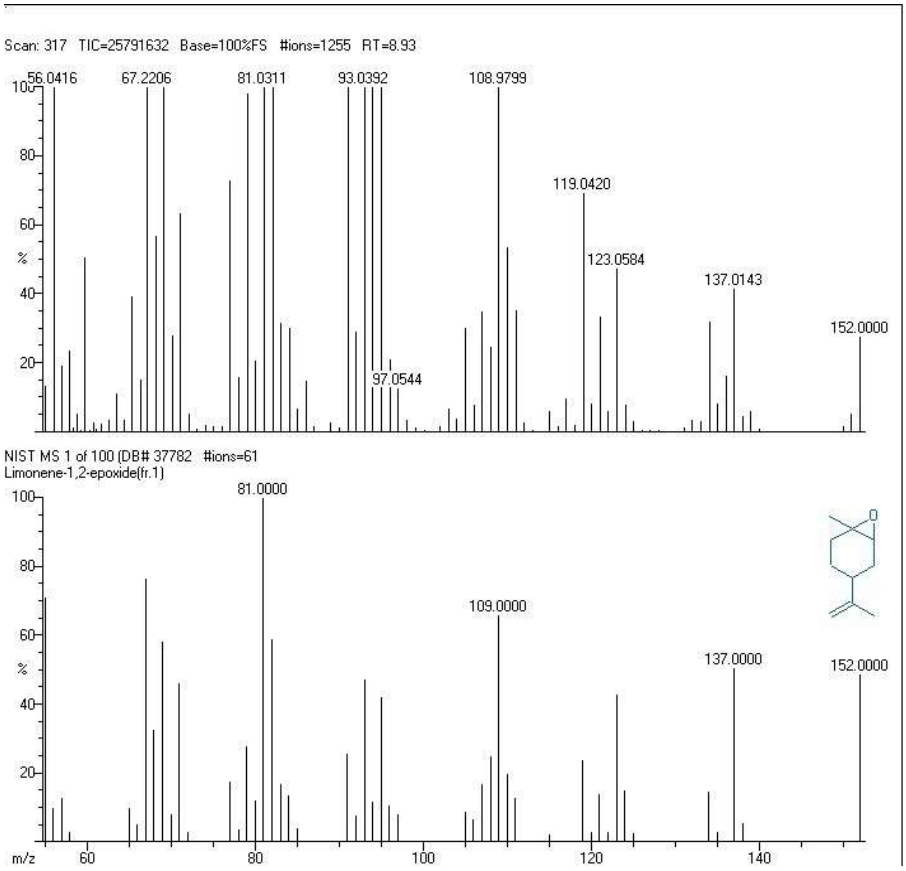
a) Mass spectrum of isolated fraction ocimene



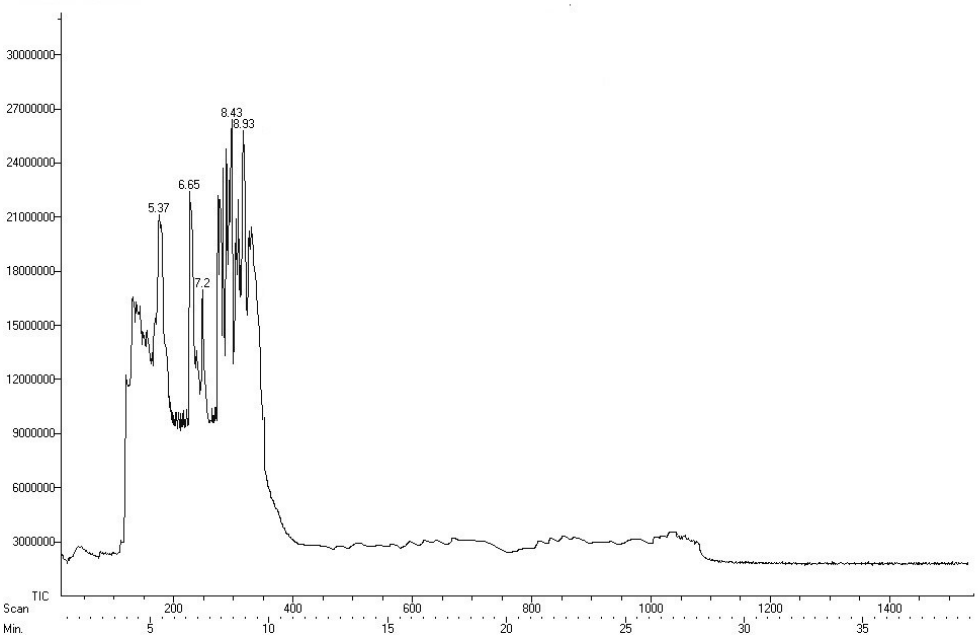
b) Mass spectrum of carene



c) Mass spectrum of cyclohexanol



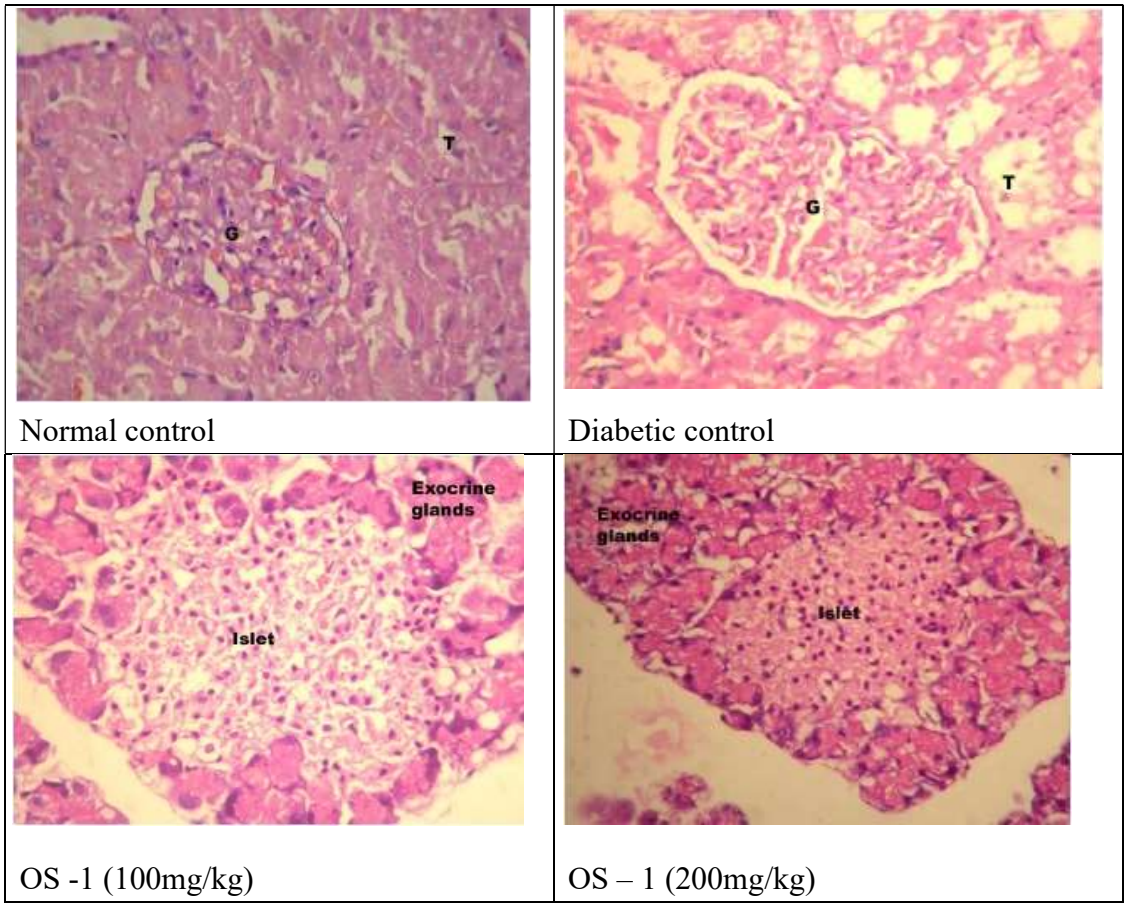
d) Mass spectrum of limonene epoxidase



e) Gas chromatogram of isolated fraction OS-1

Figure. 1: (a) Mass spectrum of isolated fraction ocimene; (b) Mass spectrum of isolated fraction carene；(c) Mass spectrum of isolated fraction cyclohexanol; (d) Mass spectrum of isolated fraction limonene epoxidase；

(e) Gas chromatogram of isolated fraction OS-1



Normal control | Diabetic control

OS -1 (100mg/kg) | OS – 1 (200mg/kg)

Figure.2: Histopathological study of Kidney