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SHORT COMMUNICATION

Inducible Nitric Oxide Synthase Downregulation and Cellular Antioxidant Enzyme Enhancing Potential of Oleanolic Acid from *Isodon wightii* (Bentham) H. Hara against Lipopolysaccharide-induced Liver Damage in Bagg Albino Strain C Mice

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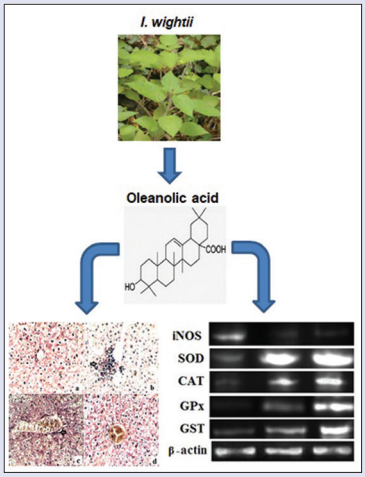
ABSTRACT

Background: *Isodon wightii* (Bentham) H. Hara is an aromatic medicinal herb belongs to *Lamiaceae* which has been considered as a prolific source of diterpenoids with diverse structural and medicinal values. **Objective:** The objective of the study is to evaluate *in vivo* anti-inflammatory activity of oleanolic acid from *I. wightii* against lipopolysaccharide-induced liver inflammation in Bagg albino strain C mice. **Materials and Methods:** Fractions were obtained using silica gel 60–120 mesh column chromatography, and structural elucidation was done using spectroscopic studies. Hepatoprotection, inducible nitric oxide synthase (iNOS) gene downregulation and the levels of cellular antioxidant enzymes with their respective gene expression were analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR). **Results:** White amorphous powder (58 mg) was isolated from petroleum ether extract (15 g) eluted with petroleum ether: ethyl acetate mixture (90.5:9.5), and structure has been elucidated as oleanolic acid. 50 µg of oleanolic acid showed effective hepatoprotection and multiple fold increase in the level of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase. RT-PCR analysis revealed that oleanolic acid had a remarkable effect on iNOS downregulation and antioxidant genes upregulation at 50 µg concentration. **Conclusion:** The present study proves that oleanolic acid possesses liver protecting activity with a positive effect on increased antioxidant enzymes production. The triterpenoid acid and oleanolic acid could be a suitable natural source for preparing hepatoprotective tonics.

Key words: Antioxidant enzymes, hepatoprotection, inducible nitric oxide synthase, *Isodon wightii*, oleanolic acid

SUMMARY

- Oleanolic acid isolated from *Isodon wightii* showed a remarkable downregulation of inducible nitric oxide synthase gene expression and protects liver damage induced by lipopolysaccharide
- Hepatoprotection of oleanolic acid was found to be highly associated with the increased production of cellular antioxidant enzymes and their respective genes upregulation.



Abbreviations used: iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; BALB/c: Bagg albino strain C mice; Nrf2: Nuclear factor erythroid 2-related factor 2; NF-κB: Nuclear factor kappa B.

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INTRODUCTION

Lipopolysaccharide (LPS) is the major cause of the liver injury which initiates inflammation followed by liver damage and cancer induction. The mechanism of liver damage induced by LPS is either by activation of nuclear factor Kappa B (NF-κB) or producing reactive oxygen species.[1] Therefore, the drug which has the capacity of inhibiting NF-κB and reducing oxidative stress may act as an effective hepatoprotectant.

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Isodon wightii is a tall perennial herb with white flowers and serrated leaves with numerous trichomes. *I. wightii* is a rich source of bioactive terpenoids including melissoidesin,[2] abietic acid,[3] mono, and sesquiterpenes.[4] Oleanolic acid, a pentacyclic triterpenoid widely distributed in leaves, fruits, and vegetables, has wide spectrum of medicinal properties.[5] To the best of our knowledge, no previous reports are available on anti-inflammatory property of oleanolic acid from natural source. Hence, oleanolic acid isolated from the leaves of *I. wightii* was used in this study.

MATERIALS AND METHODS

Plant material

I. wightii (Bentham) H. Hara leaves were collected from Coonoor, Tamil Nadu. Authenticated voucher specimen (BSI/SC/5/23/06-07-Tech. 881) has been deposited in the herbarium of Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India.

Extraction and isolation

Petroleum ether extract of leaf powder (715 g) was obtained using Soxhlet apparatus at room temperature. After evaporating the solvent *in vacuo* at 45°C, 15 g of crude extract was obtained and subjected to silica gel (60–120 mesh size, Ranbaxy Fine Chemicals Limited, New Delhi, India) column chromatography (15:45) and eluted with petroleum ether: ethylacetate mixture in the ratio of 90.5:9.5 v/v (Ranbaxy Fine Chemicals Limited, New Delhi, India). Further, the fractions were analyzed by thin-layer chromatography (TLC) coated with silica gel G-60 (Himedia Laboratories Pvt. Ltd., Mumbai, India). The eluted fraction was white amorphous powder. Further, structural elucidation of isolated compound was carried using spectroscopic studies.

General

^{1}H (400MHz), ^{13}C (100MHz) (Bruker Avance-400), and 2D NMR (Bruker Avance-400MHz) spectra including ^{1}H=H correlated spectroscopy, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) were recorded on Bruker Avance-400 spectrometer in $CDCl_3$. ^{13}C NMR chemical shifts are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard. Infrared (IR) spectrum was recorded on a Nicolet Avatar Model FT-IR (4000–400 cm^{-1}) spectrometer using KBr pellets. Shimadzu LC-MS was used for recording EI-MS. Data are reported as follows: chemical shift, multiplicity (s¼singlet, brs¼broad singlet, d¼doublet, t¼triplet, q¼quartet, and m¼multiplet), coupling constants (Hertz), and integration.

Animals and experimental design

Based on the regulations of Council Directive CPCSEA no: 08/2016/IAEC/KASC regarding good laboratory practice on animal experimentation, animals were maintained. Adult Bagg albino strain C (BALB/c) mice were purchased from Small Animal Breeding Centre, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. Micro isolators with autoclaved bedding, cages, food pellets, and deionized water were used for animal maintenance. Standard conditions of humidity, temperature (25 ± 2°C), and light (12 h light/dark) were also provided. Aseptic condition was set up to avoid the entry of harmful microbes. Adult BALB/c mice weighing 30 g were used and randomly assigned into three groups normal group (n = 3), LPS treated (n = 3) and experimental group (n = 6) (oleanolic acid + LPS). Mice were placed on restricted, once a day diet and given 25 and 50 μg concentrations of oleanolic acid/30 g body weight followed by LPS (1.5 μg/30 g body weight). After treatment, animals were sacrificed and liver was excised.[6] A part of liver fixed in 10% formalin was used for histopathological analysis, and another part of the liver was taken for antioxidant enzyme and gene expression studies.

Histopathological analysis

The liver tissue was impregnated with histology grade paraffin wax (melting point 58–60°C) at 60°C for two changes of 1 h each. The wax impregnated tissues were embedded in paraffin blocks, mounted, and cut with rotary microtome at 3 μM thickness. The sections were stained for 8 min with Ehrlich's hematoxylin (0.75%). Further, they were counterstained in 1% aqueous eosin (1 g in 100 mL water) for 1 min, and the excess stain was washed in tap water and allowed to dry. The dried sections were mounted in Distrene, Plasticiser, and Xylene. The sections were wetted in xylene, and the mount was inverted to place over a coverslip. Low-power and high-power dry objective lens with × 10 magnification were used for observing the hepatocyte architecture and injury.

Extraction and estimation of cellular antioxidant enzymes

Excised liver was homogenized using 0.1M ice cold sodium phosphate buffer (10% w/v) and then centrifuged at 10,000 rpm for 15–20 min at 4°C, and finally, the supernatant was collected and used for subsequent enzyme assays. The total protein was assayed with bovine serum albumin as the standard.[7] Antioxidant enzymes, namely superoxide dismutase (SOD),[8] catalase (CAT),[9] glutathione peroxidase (GPx),[10] and glutathione S-transferase (GST)[11] were estimated.

Gene expression profiling

Total RNA extraction and cDNA synthesis

Total RNA was extracted from excised liver tissue using Trizol reagent (GeNei, Bengaluru). Synthesis of cDNA from isolated total RNA was performed using a cDNA kit (HELINI, Chennai). Briefly, 20 μL reaction mixture was prepared through the addition of 6 μL of cDNA reaction mix, 1 μL of oligodT, 1 μL of random hexamer, 2 μL of enzyme mix, 2 μL of isolated RNA, and 8 μL of nuclease-free water in a 0.2 mL polymerase chain reaction (PCR) tube. Reverse transcription was carried out for one cycle at 42°C for 30 min and inactivation at 95°C for 2 min.

Gene amplification

The PCR analysis was performed on the aliquots of prepared cDNA for detecting the expression patterns of genes such as inducible nitric oxide synthase (iNOS), SOD, CAT, GPx, and GST. The sequence of oligonucleotide primers for iNOS-F (5'-ATG GAC CAG TAT AAG GCA AGC-3'), iNOS-R (5'-GCT CTG GAT GAG CCT ATA TTG-3'), SOD-F (5'-GAC CTG CCT TAC GAC TAT GG-3'), SOD-R (5'-GAC CTT GCT CCT TAT TGA AGC-3'), CAT-F (5'-GAA CGA GGA GGA GAG GAA AC-3'), CAT-R (5'-TGA AAT TCT TGA CCG CTT TC-3'), GPx-F (5'-CCT CAA GTA CGT CCG ACC TG-3'), GPx-R (5'-CAA TGT CGT TGC GGC ACA CC-3'), GST-F (5'-TGG CCG GAA GCA CAA CCT G-3'), GST-R (5'-CCC CAC CAA CAC CGG CAC-3'), β-actin-F (5'-TGG AAT CCT GTG GCA TCC ATG AAA-3'), and β-actin-R (5'-TAA AAC GAG CTC AGT AAC ATC CG-3'). Amplification reactions were performed in a gradient thermocycler (Applied Biosystems, California) as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 92°C for 30s, annealing at 58°C (iNOS and SOD), 55°C (CAT), 50°C (GPx), and 54°C (GST) for 1 min, and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. PCR reactions were carried out in a volume of 10 μL containing 5 μL of PCR master mix (2×) (Merck Specialties, Mumbai) solution, 1 μL of forward and reverse primer, 1.5 μL of cDNA, and 1.5 μL of nuclease-free water.

Statistical analysis

Mean ± standard deviation (n = 3) was calculated to analyze the data obtained from the antioxidant enzyme study.

RESULTS

Compound isolation and structure elucidation

Compound isolated as white amorphous powder and observed on TLC as a light-yellow fluorescent spot under ultraviolet (UV) light (366 nm) and showed UV absorption maxima at 281 and 232 nm. The IR spectrum displayed intense absorption at 3415.31 (COOH), 1715.37 (C = O), 1621.00 (C = C), and 1453.1 cm^{-1} (Ar). A combination of ^{13}C-NMR and HSQC spectral data suggested that seven methyl, five methane, and ten methylene groups. The proton and ^{13}C-NMR spectrum showed signals at δ5.21 (1H, s, H-12), 3.80 (1H, s, H-3), 1.57 (1H, d, H-18) along with seven singlets at δ1.08, 0.76, 0.95, 0.93, 1.14, 0.99, and 1.50 and another one signal at δ9.80 (1H, br, s) denoted the presence of an carboxylic group. The interpretation of HMBC correlations showed the correlations of H-29 (δ0.99) to C-21 (δ31.50), H-27 (δ1.14) to C-27 (22.06), C-8 (39.00), C-14 (43.00), and C-16 (20.27). The HMBC also shows a correlation between methyl protons with carbon signals, H-23 (δ1.08) to C-23 (24.54), H-24 (δ0.76) to C-24 (19.51), H-25 (δ0.95) to C-25 (16.06), and H-26 (δ0.93) to C-26 (17.40) [Table 1]. Based on mass spectral data, molecular weight has been determined as 457.73. The spectral studies support the compound as oleanolic acid ($C_{30}H_{48}O_3$) [Figure 1].

Table 1: Nuclear magnetic resonance spectrum data of oleanolic acid (δppm)[a]

Carbon position	^{13}C shift	HSQC	HMBC[b]
1	38.38	1.61	
2	29.50	1.30	
3	75.00	3.8	
4	40.00	-	
5	53.30	1.50	
6	19.50	1.48	
7	30.00	1.40	
8	39.00	-	
9	52.91	1.57	C-9
10	33.28	-	
11	22.63	1.32	C-2, C-10
12	119.55	5.21	
13	139.11	-	
14	43.00	-	
15	27.00	1.58	
16	20.27	1.26	
17	45.51	-	
18	46.47	1.14	C-18
19	46.16	1.57	
20	32.06	-	
21	35.19	1.52	
22	27.98	1.51	C-18, C-20, C-16, C-21
23	19.51	1.08 (3H, s)	
24	24.54	0.76 (3H, s)	
25	16.06	0.95	C-24
26	17.40	0.93	C-25
27	22.06	1.14	C-26
28	27.98	1.50	C-27, C-8, C14, C16
29	31.50	0.99	
30	182.00	9.80	C-21

[a]Data were recorded in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C);
[b]HMBC correlations are from proton (s) stated to the indicated carbon.
HSQC: Heteronuclear single quantum coherence; HMBC: Heteronuclear multiple bond correlation

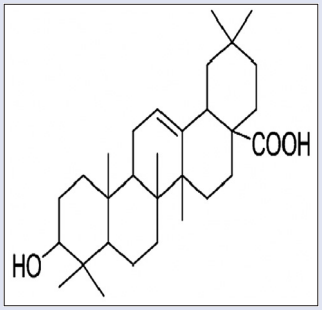

Figure 1: Structure of oleanolic acid

Hepatoprotective studies

1.5 µg LPS induced the liver inflammation with characteristic lymphocytic infiltration near the central vein and severe necrosis [Figure 2b]. Mildly affected hepatic lobule toward the central vein was seen in 25 µg of oleanolic acid-treated hepatocytes [Figure 2c]. Complete reduction in inflammation and revived liver architecture was noticed in 50 µg of oleanolic acid [Figure 2d] similar to normal liver [Figure 2a] and it is considered to be the optimum dose to have high rate of liver protection.

Cellular antioxidant enhancement

A reducible amount of antioxidant enzymes was recorded in LPS (1.5 µg)-induced liver whereas fourfold increase in SOD (12.36 ± 0.16 µM/min/mg protein) and fifteen-fold (141.98 ± 2.34 µM/min/mg protein) increase in CAT were observed with 50 µg of oleanolic acid administration. GPx and GST levels were also increased considerably (1.10 ± 0.02 and 0.40 ± 0.07 µM/min/mg protein, respectively) after administering with 50 µg of oleanolic acid. Overall, it is observed that the level of antioxidant enzymes was increased with oleanolic acid treatment [Figure 3A].

Inducible nitric oxide synthase downregulation and antioxidant genes upregulation

iNOS mRNA production was high in 1.5 µg LPS-induced hepatocytes [Figure 3B Lane a]. 50 µg of oleanolic acid showed good inhibitory effect on iNOS mRNA production [Figure 3B Lane c]. LPS inhibited the expression of SOD, CAT, GPx, and GST genes at transcriptional

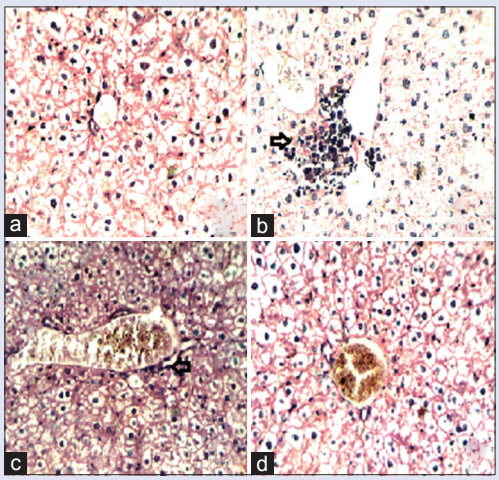

Figure 2: Hepatoprotective effect of oleanolic acid on lipopolysaccharide-induced Bagg albino strain C mice: (a) normal liver, (b) lipopolysaccharide-induced liver, (c and d) LPS-induced liver treated with 25 and 50 µg of oleanolic acid, respectively

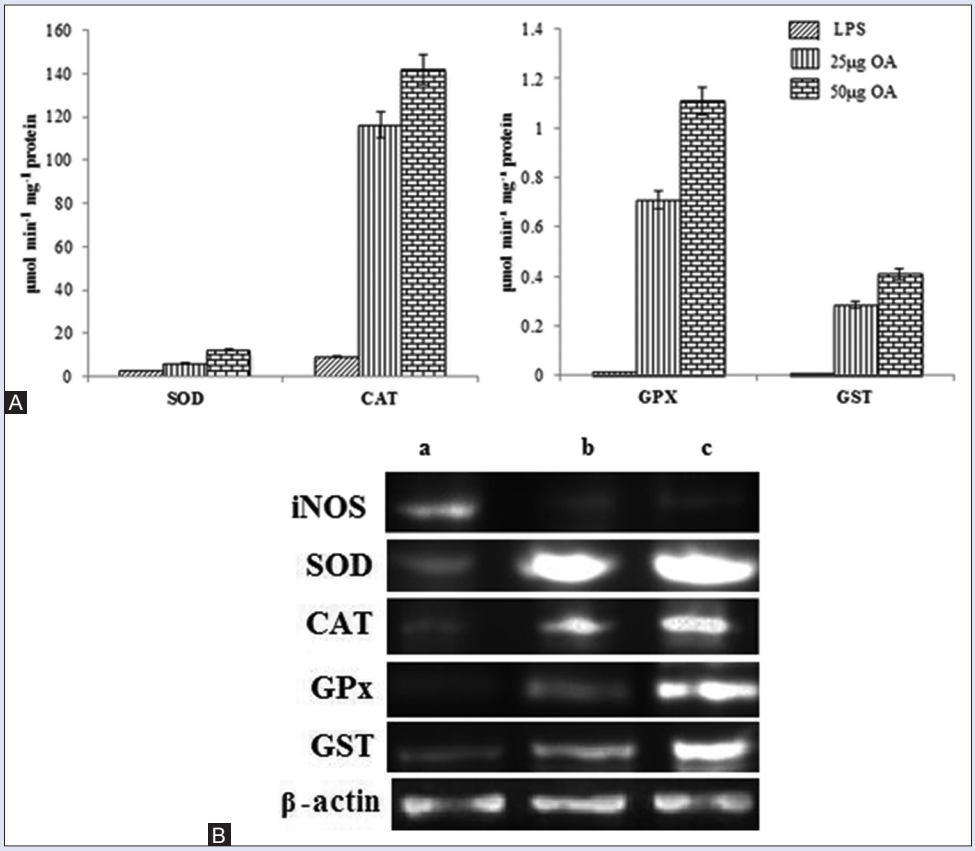

Figure 3: (A) Effect of oleanolic acid on antioxidant enzymes level in lipopolysaccharide-induced Bagg albino strain C mice liver. Error bar represents mean±standard deviation (*n*=3), (B) Downregulation of inducible nitric oxide synthase and upregulation of superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase genes by oleanolic acid in lipopolysaccharide-induced Bagg albino mice strain C mice liver: (a) lipopolysaccharide-induced liver and (b and c) lipopolysaccharide-induced liver treated with 25 and 50 µg of oleanolic acid, respectively. β-actin-Internal marker

level [Figure 3B Lane a]. On treatment with 25 µg of oleanolic acid, the respective antioxidant genes were upregulated; however, 50 µg concentration was found to be highly efficient for upregulating antioxidant genes expression to overcome the oxidative stress caused by LPS [Figure 3B Lanes b and c].

DISCUSSION

Oleanolic acid is a pentacyclic triterpenoid widespread in many food and medicinal plants, and it is also reported from *Isodon loxothyrsus, Isodon phyllostachys,* and *Isodon xerophilus*.[12-14] Oleanolic acid isolated from hexane extract of *Olea europaea* using petroleum ether: ethyl acetate solvent system[15] supports the present study where petroleum ether: ethyl acetate was used for oleanolic acid extraction.

LPS acts on NF-κB/IκB complex by attaching with CD14 receptor and induces phosphorylation and ubiquitination of IκB. Further, NF-κB gets released and upregulates nitric oxide synthase gene.[16] LPS-induced nitric oxide synthase (iNOS) produces nitric oxide that mediates signal transduction to induce inflammatory response and leads to hepatic injury.[17] In this study, it is assumed that iNOS downregulation followed by hepatoprotection effect of oleanolic acid might be associated with the inhibition of NF-κB or prevention of binding of LPS with NF-κB/IκB complex, but the exact mechanism of action remains obscure.

Reactive oxygen species induced by LPS is thought to reduce the level of antioxidant enzymes, namely, SOD, CAT, GPx, and GST in the liver. Results of the present study clearly showed the increased production of antioxidant enzymes in oleanolic acid-treated liver. It is assumed that oleanolic acid could have increased the nuclear accumulation of nuclear factor erythroid 2-related factor 2-dependent genes which play an important role in the protection of liver by regulating antioxidant and detoxifying enzymes.[18] In addition, it is taken into consideration that antioxidant potential of oleanolic acid is also one of the reasons for observed hepatoprotection. This is the first report on *in vivo* anti-inflammatory activity of oleanolic acid isolated from *I. wightii*.

CONCLUSION

The pharmacological investigation of oleanolic acid isolated from *I. wightii* may act as a promising phytocompound which could protect the liver from oxidative stress and other damages.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Liu Y, Li F, Zhang L, Wu J, Wang Y, Yu H, *et al*. Taurine alleviates lipopolysaccharideinduced liver injury by antiinflammation and antioxidants in rats. Mol Med Rep 2017;16:6512-7.
2. Thirugnanasampandan R, Jayakumar R, Narmatha Bai V, Martin E, Rajendra Prasad KJ. Antiacetylcholinesterase and antioxidant ent-kaurene diterpenoid, melissoidesin from *Isodon wightii* (Bentham) H. Hara. Nat Prod Res 2008;22:681-8.
3. Gogul Ramnath M, Thirugnanasampandan R, Sadasivam M, Mohan PS. Antioxidant, antibacterial and antiacetylcholinesterase activities of abietic acid from *Isodon wightii* (Bentham) H. Hara. Free Rad Antioxid 2015;5:1-5.
4. Gogul Ramnath M, Thirugnanasampandan R. Chemical composition analysis of essential oil of *Isodon wightii* (Bentham) H. Hara. J Essent Oil Bear Plants 2016;19:1553-6.
5. Sultana N, Ata A. Oleanolic acid and related derivatives as medicinally important compounds. J Enzyme Inhib Med Chem 2008;23:739-56.
6. Chan MM, Huang HI, Fenton MR, Fong D. *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. Biochem Pharmacol 1998;55:1955-62.
7. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
8. Kono Y. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Arch Biochem Biophys 1978;186:189-95.
9. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. Weinheim, Deerfield Beach, Florida: Verlag Chemie; 1983. p. 273.
10. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG, *et al*. Selenium: Biochemical role as a component of glutathione peroxidase. Science 1973;179:588-90.
11. Mannervik B, Danielson UH. Glutathione transferases – Structure and catalytic activity. CRC Crit Rev Biochem 1988;23:283-337.
12. Hao H, Dong SH, Xun ZS. Triterpenoids of *Isodon loxothyrsus*. Phytochemistry 1996;42:1665-6.
13. Li X, Xiao W, Pu J, Ban L, Shen Y, Weng Z, *et al*. Cytotoxic ent-kaurene diterpenoids from *Isodon phyllostachys*. Phytochemistry 2006;67:1336-40.
14. Li LM, Xin PJ, Lie XW, Sun HD. Triterpenes from *Isodon xerophilus*. Chin J Nat Med 2012;10:307-10.
15. Chouaïb K, Hichri F, Nguir A, Daami-Remadi M, Elie N, Touboul D, *et al*. Semi-synthesis of new antimicrobial esters from the natural oleanolic and maslinic acids. Food Chem 2015;183:8-17.
16. Shi Q, Cao J, Fang L, Zhao H, Liu Z, Ran J, *et al*. Geniposide suppresses LPS-induced nitric oxide, PGE2 and inflammatory cytokine by downregulating NF-κB, MAPK and AP-1 signaling pathways in macrophages. Int Immunopharmacol 2014;20:298-306.
17. Su GL. Lipopolysaccharides in liver injury: Molecular mechanisms of kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 2002;283:G256-65.
18. Klaassen CD, Reisman SA. Nrf2 the rescue: Effects of the antioxidative/electrophilic response on the liver. Toxicol Appl Pharmacol 2010;244:57-65.