In-vitro Anti-inflammatory Activity of the Fractions Isolated from *Walsura trifoliata* (A.Juss.)Harms. Root

J. Josphin Mini[1], I.VS. Nimal Christhudas[2], Natarajan Gajendran[3]

[1, 3]*Dept of Plant Biology and Plant Biotechnology, Presidency College, Chennai-600 005*
[2]*R & D Senior Scientist, Jayagen Biologics, Kottur,Chennai -600085.*
josphinmini@gmail.com, nimalchristudoss@gmail.com, indjst@gmail.com

Abstract

Background: Inflammation is a complex process of various mediators. When huge amount of nitric oxide is produced by inducible nitric oxide synthase (iNOS) which leads to the vasolidation and hypotension that usually observed during septic shock and inflammation. So the inhibitors of iNOS is useful for the treatment of inflammatory diseases.
Methods: *Walsura trifoliata*, known for antimicrobial and antioxidant activities was tested for anti-inflammatory activity to isolate its bioactive compound. The major fractions obtained from the methanol extract of the root of *Walsura trifoliate* using column chromatography was screened for their inhibitory activity against NO production in lipopolysaccharide (LPS) activated macrophages.
Findings:A total of 79 fractions were isolated and based on TLC, similar fractions were combined together to form 8 major fractions. Among the eight major frcations, fractions 4 and 7 showed potent anti-inflammatory activity. Significant cytotoxicity was also observed in fractions 4 and 7. Using Infrared spectroscopy and Mass spectroscopy analysis fraction 4 was identified as 3,4,-5 trihydroxy benzoic acid and fraction 7 was identified as β-sitosterol.
Application: Further *in vivo* studies should be undergone for the development of drug from the active fractions of *Walsura trifoliata* for its phytotherapeutical uses in different ailments such as antimicrobial, antioxidant and anti inflammatory diseases.

Key words: *Walsura trifoliata*, anti-inflammatory, fractions, nitric oxide.

1. Introduction

Most of the commonly used drugs are derived from plants which forms the ingredients in traditional system of medicines and also it constitute the lead compounds in synthetic drugs [1]. Plant based drugs are biodegradable safe, less expensive, efficient and rarely have side effects [2,3]. Many drugs which are available to relieve pain and reduce inflammation act by interfering with various chemical mediators which cause side effects. So that it is necessary to screen medicinal plants for novel bioactive compounds [4].Inflammation symptoms occur when the human body attempts to counteract potentially injurious agents such as invading bacteria, virus and other pathogens [5,6]. Inflammation can also be induced by biochemical and pharmacological agents from the environment in addition to a diverse and potentially huge array of cell types and soluble mediators including cytokines [7, 8].

Nitric oxide (NO) is involved in several inflammatory disorders. Indeed, virtually every cell and many immunological parameters are modulated by NO. It has been shown that NO can be pro-inflammatory (immunostimulatory, anti-apoptotic) or anti-inflammatory (immunosuppressive, pro-apoptotic), host-protective or host-damaging during infections [9].The nitric oxide synthase (NOS) which synthesize NO from L-arginine using NADPH and molecular oxygen, is a free radical which is short-lived and an intercellular messenger produced by a variety of mammalian cells, that include macrophages, neutrophils, platelets, fibroblasts, endothelium, neuronal, and smooth muscle cells.[10, 11]. Infected macrophages produce NO to mediate host defence functions including anti microbial and anti tumor activities. However, its excess production cause tissue damage associated with acute and chronic inflammation [12].

Walsura trifoliata (Meliaceae) is well reputed in traditional system of medicine [13]. It is also known for its antimicrobial and anti oxidant activities [14, 15]. In the present study the fractions collected from the root of *Walsura trifoliata* methanol extract was screened for the inhibitory activity towards NO production by measuring its production in LPS-stimulated RAW 264.7 cells.

2. Materials and Methods

2.1. Preparation of Extract

Sample collection, identification and extraction of *Walsura trifoliata* root was reported earlier [15]. The herbarium specimen was submitted to Presidency College Herbarium (Voucher number - 6742).

2.2. Isolation of active fractions

The methanol extract (30 g) was chromatographed over silica gel column(100–200 mesh, Sissco, Mumbai) in hexane. The column was eluted with hexane and hexane: chloroform with increasing polarity of chloroform. A total of 78 fractions were obtained. Each fraction was spotted on a TLC plate over silica gel 60F254 (precoated aluminium plate, layer thickness 0.2 mm) (Merck). All the fractions with similar retention factor (Rf) inTLC pattern were combined together. Finally eight fractions were obtained.

2.3. In-vitro anti-inflammatory activity of fractions

Cell culture

Murine macrophage RAW 264.7 cells were purchasedfrom National Centre for Cell Science Pune. They were cultured in Dulbecco's modified Eagle's medium (DMEM)containing 2 mM glutamine, 10 mM 4-[2-hydroxyethyl]-1-piperazine ethane sulfonic acid (HEPES), penicillin (100units/mL), streptomycin (100 µg/mL) and 10% foetal bovineserum. Cells were cultured at 37 °C in a humidified incubator with an atmosphere of 5% CO2.

2.4. MTT assay for cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and only freshly dead cells do not reduce significant amounts of MTT. RAW264.7 cells were cultured in 96-well plates for 18 hr, followed by treatment with LPS (1 µg/mL) in the presence of plant extracts at concentrations of 100 µg/mL. After a 24h incubation, MTT was added to the medium for 4h. Finally,the supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 540 nm. The percentage of dead cells was determined relative to the control group.

2.5. Nitric oxide assay

The nitric oxide assay was performed with slight modification [6]. After pre-incubation of RAW 264.7 cells (1.5 × 105 cells/mL)with LPS (1 µg/mL) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amounts of nitrite, a stable metabolite of NO,were measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5%phosphoric acid). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.6. Infrared spectroscopy (IR)

Sample was ground along with Potassium bromide (KBr) and pellets were prepared. IR spectrum was recorded in Shidmazu IR spectrophotometer by KBr pellet method. IR gives a strong absorption pattern at a particular frequency for a particular functional group.

2.7. Mass spectrometry

By mass spectroscopy the molecular mass of a compound and its elemental composition can be easily determined. Further this method involves very little amount of the test sample, which will give molecular weights accurately using Gas chromatography Mass spectroscopy (GC-MS). Mass spectrometric studies have been performed in Shimadzu.

3. Results and Discussions

Inflammation is a basic way in which the body reacts toinfection, irritation or other injury which causes redness, warmth, swelling and pain. An inflammatory reaction directs immune system components to the site of injury or infection, and is manifested by increased blood supply and vascular permeability, which allows chemotactic peptides, neutrophils and mononuclear cells to leave the intravascular compartment [16,17]. Inflammatory reaction is uncontrolled and prolonged, it leads to the pathogenesis of chronic diseases such as cancer, arthritis, autoimmune disorder and vascular disease [18,19].The Griess reaction, a spectrophotometric determination for nitrite, was carried out to quantify the nitrite levels in the conditioned medium of RAW 264.7 cells treated with LPS. In murine macrophage RAW 264.7 cells, LPS stimulation alone has been demonstrated to induce iNOS transcriptionand its protein synthesis, with a corresponding increase in NO production [20]. Therefore, this cell system is an excellent model for drug screening and the subsequent evaluation of potential inhibitors against iNOS and NO production [11].The goal is to inhibit excessive formation of NO without interfering with the production of small quantities of NO generated by the endothelial and neuronal NOS isoforms [21, 22].

In plants phenols and flavonoids have been referred to as nature's biological response modifiers because of their inherent ability to modify the bodys reaction to allergies and virus and they showed their anti-allergic, anti-inflammatory, antimicrobial and anti-cancer activities [23]. In our study, column was packed using methanol root extract of *W. trifoliata*. Totally 79 fractions were obtained. Based on TLC same fractions were combined together to form 8 major fractions. Among the 8 major fractions, fractions 4 and 7 showed significant anti-inflammatory activity in RAW 264.7 cells. Similar results were shown in plants extracts. Aqueous extract of leaves of *Pentatropis capensis* showed anti- inflammatory effect against paw oedema after 3h of carrageenan induction [4]. In *Salvia bicolor* the methanol extract exerted a stronger effect than the standard anti-inflammatory drug indomethacin after 1h and 4h of administration [24]. Andrographolide inhibits NO synthesis in RAW 264.7 cells by reducing the expression of iNOS protein [25]. *Raphidophora pertusa* and *Epipernum pinnatum* at the doses 125 and 250 mg/kg significantly inhibited the carrageenan-induced paw oedema in rats [26]. Nitric oxide inhibition and cytotoxicity of the fractions isolated from *Walsura trifoliata* root extract was given in table 1.

In NO inhibition assay the fractions 4 and 7 of *W. trifoliata* showed significant activity (78.08% and 80.16% respectively) at a concentration of 100µg/ml. Significant cytotoxic effect was also found in fractions 4 and 7. In column chromatography, fraction-4 was eluted with ethyl acetate: methanol (20:80) which yielded 80 mg. It showed a single spot on TLC plate with Rf value 0.48. Fraction-7 was eluted with Chloroform: ethyl acetate (40:60) and 35 mg was obtained which showed single spot on TLC plate with Rf value 0.62. The purified compound was subjected to spectroscopic analysis.

IR spectrum of fraction 4 was IRKBr/max cm^{-1}: 3496.94, 3375.43 and 3280.92 cm^{-1} (O-H stretch, H-bonded; hydroxyl group); 3062.96 cm^{-1} (C-H stretch; aromatics); 2999.31 and 2839.22 cm^{-1} (C-H stretch; alkanes); 2779.42 cm^{-1}(H-C = O:C-H stretch; aldehydes); 1703.14 cm^{-1} (C=O; alpha, beta- unsaturated aldehydes, ketones) and 1616..35 cm^{-1} (C-C; aromatics).The Gas chromatography mass spectrometry of fraction 4 showed a single peak in 11.75 minutes corresponding to 3,4,-5 trihydroxy benzoic acid.

IR spectrum 7 was IRKBr/max cm^{-1}: 3439.08 cm^{-1} (O-H stretch, H-bonded; hydroxyl group); 2856.58 cm^{-1} (C-H stretch; alkanes); 1735.93 cm^{-1} (C=O stretch; aldehydes, saturated aliphatic); 1631.78 cm^{-1} (N-bend; primary amines); 1458.18 cm^{-1} (C-H bend; alkanes) and 1242.16, 1165, 1055.06 and 1024.2 cm^{-1} (C-N stretch; aliphatic amines). Metabolites of phytosterols were identified using GC-MS for fraction 7. Two peaks were eluted at 29.01 and 29.65 minutes and had characteristic molecular ions m/z 414.38 g/mol corresponding to β-sitosterol.

Table 1. Nitric oxide inhibition and cytotoxicity of the fractions isolated from *Walsura trifoliata* root extract

Fractions	Percentage of NO inhibition (100µg/ml)	Percentage of cell viabilities (100µg/ml)
F1	23.45	22.76
F2	40.59	35.29
F3	48.12	50.95
F4	78.08	70.12
F5	61.25	55.37
F6	50.79	40.51
F7	80.16	90.75
F8	38.13	27.83

4. Conclusion

The fractions were collected from the root methanol extract of *Walsura trifoliata* (fractions 4 and 7) inhibited the production of nitric oxide which leads to inflammation. The fractions were identified as 3, 4,-trihydroxy benzoic acid and β-sitosterol as per IR and GC-MS analysis. Further the study was focussed on NMR analysis to predict the structure of the compound.This work may leads to the identification of green medicine in anti-inflammatory treatment in health-care industry.

5. References

1. N. Praveen, S. Nayak, D.M. Kar, P. Das. Pharmacological evaluation of ethanolic extracts of the plant *Alternanthera sessilis* against temperature regulation. *Journalof Pharmacy Research*. 2010; 3(6), 1381-1383.
2. B. Latha, Y. Rumaisa, C.K. Soumya, S. Shahul, N. Sadhiya. Phytochemical studies on *Leucas aspera*. *Journal of Chemical and Pharmaceutical Research*. 2013; 5(3), 222-228.
3. A.L. Tariq, Reyaz. Significances and importance of Phytochemical Present in *Terminalia chebula*. *International Journal of Drug Development and Research*. 2013; 5(3), 256-22.
4. S. Chowdhury, K. Nishteswar, M.K. Nariya. Analgesic and Anti-inflammatory effects of aqueous extract of leaves of *Pentatropis capensis* Linn. f. (Bullock). *Ancient Science of Life*. 2014; 34(2), 64-67.
5. D. Hersh, J. Weiss, A. Zychlinsky. How bacteria initiate inflammation: aspects of the emerging story. *Current Opinion in Microbiology*. 1998; 1, 43-48.
6. W.J. Yoon, Y.M. Ham, K.N. Kim, S.Y. Park, N.H. Lee, C.G. Hyun, W.J. Lee. Anti-inflammatory activity of brown algae *Dictyota dichotoma* in murine macrophage RAW 264.7 cells. *Journal of Medicinal Plants Research*. 2009; 3(1), 001-008.
7. C.A. Dinarello. Pro-inflammatory cytokines. *Chest*. 2000; 118, 503-508.
8. V. Turcanu, N.A. Williams. Cell identification and isolation on thebasis of cytokine secretion: a novel tool for investigating immune response. *Natural Medicines*. 2001; 7, 373-376.
9. G. Cirino, E. Distritti, J.L. Wallace. Nitric oxide and Inflammation. *Inflammation and Allergy-Drug Targets*. 2006; 5, 115-119.
10. J.H. Oh, T.J. Lee, J.W. Park, T.K. Kwon. Withaferin A inhibits iNOS expressionand nitric oxide production by Akt inactivation and down-regulating LPS-induced activity of NF-kappaB in RAW 264.7 cells. *European Journal of Pharmacology*. 2008; 599, 11-17.

11. E.J. Yang, E.Y. Yim, G. Song, G.O. Kim, C.G. Hyun. Inhibition of nitric oxide production in lipopolysaccharide activated RAW 264.7 macrophages by Jeju plants extracts. *Toxicology*. 2009; 2(4), 245-249.
12. J. MacMicking, Q.W. Xie, C. Nathan. Nitric oxide and macrophage function. *Annual Review of Immunology*. 1997; 15, 323-350.
13. K.S.R. Murthy, N. Kandimalla. Antimicrobial spectrum and phytochemical study of *Walsura trifoliata*(A.Juss.) Harms. (Meliaceae) Bark extracts. *Journal of Pharmacology and Toxicology*. 2008; 3(4), 267-271.
14. J. Josphin Mini, Natarajan Gajendran. Phytoconstituents and Antimicrobial activity of *Walsura trifoliata* (A. JUSS.) Harms . *Indian Journal of Drugs and Diseases*. 2014; 3(2), 317-323.
15. J. Josphin Mini, Natarajan Gajendran. In vitro α -glucosidase inhibition and antioxidant activity of *Walsura trifoliata* (A. Juss.) Harms. *Indian Journal of Science and Technology*. 2015; 8(S7), 16-23.
16. G.Y. Chen, G.Nunez. Sterile inflammation: sensing and reacting to damage.*Natural Reviews Immunology*. 2010; 10, 826-837.
17. G.M. Park, J.G. Jun, J.K. Kim. XH-14, a novel danshen methoxybenxo [b]furan derivative, exhibits anti-inflammatory properties in lipopolysaccharide treated RAW 254.7 cells. *Journal of Inflammation.* 2013; 10, 1-7.
18. F. Montecucco, F. Mach. Common inflammatory mediators orchestrate pathophysiological processes in rheumatoid arthritis and atherosclerosis.*Rheumatology*. 2009; 248, 11-22.
19. P.J. Murray, T.A. Wynn. Protective and pathogenic functions of macrophage subsets. *Natural Reviews Immunology*. 2011; 11, 723-737.
20. T. Henkel, T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, P.A. Baeurle. Rapid proteolysi of I Kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*. 1993; 365, 182-185.
21. S.Moncada, E.A. Higgs. Molecular mechanisms and therapeutic strategies related to nitric oxide. *The Journal of the Federation of American Societies for Experimental Biology*. 1995; 9, 1319-1330.
22. J. Pfeilschifte, W. Eberhardt, R. Hummel D. Kunz, H. Muhl, D. Nitsch, C. Pluss, G. Ealker. Therapeutic strategies for the inhibition of inducible nitric oxide Synthase-potential for a novel class of anti-infammatory agents. *Cell Biology Internationl*. 1996; 20(1),
5. 51-58.
23. S.B. Swami, N.S.K. Thakor, M.M. Patil, P.M. Haldankar. Jamun (*Syzygium cumini* (L.)): A Review of Its Food and Medicinal Uses. *Food and Nutrition Sciences*. 2012; 3(8), 1100-1117.
24. T. A. Ibrahim. Chemical composition and biological activity of extracts of *Salvia bicolor* Desf. Growing in Egypt. *Molecules*. 2012; 17, 11315-11334.
25. W.H. Chiou, C.F. Chen, J.J. Lin. Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells by andrographolide. *British Journal of Pharmacology*. 2000; 129, 1553-1560.
26. A. Linnet, P.G. Latha, M.M. Gincy, G.I. Anuja, S.R. Suja, S. Shyamal, V.J. Shine, S. Sini, P. Shikha, Mathew Dan, S. Rajasekharan. Anti-inflammatory, analgesic and anti-lipid peroxidative effects of *Raphidophora pertusa* (Roxb.) Schott and *Epipernum pinnatum* (Linn.) Engl. aerial parts. *Indian Journal of Natural Products*. 2010; 1(1), 5-10.

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