

In-vitro Assays to Show the Antioxidant Potential of β -sitosterol from *Lawsonia inermis* Leaves

Sanjukta Badhai¹, Durga P. Barik², Bairagi C. Mallick^{2,*}

¹School of Biotechnology, KIIT University, Bhubaneswar-751024, Odisha, India

²Department of Botany, Ravenshaw University, Cuttack-753003, Odisha, India

²Department of Chemistry, Ravenshaw University, Cuttack-753003, Odisha, India

Corresponding Author

*Dr. Bairagi C. Mallick

Department of Chemistry, Ravenshaw University, Cuttack-3, Odisha, India

E-mail: bcmallick@ravenshawuniversity.ac.in

Contact No.: +91-9178890581

Abstract: *Apprehensions about the therapeutic efficacy and safety of synthetic drugs fuel the research to discover holistic remedies. The antioxidant effect of methanolic extract of Lawsonia inermis leaves (MELIL) β -sitosterol have been investigated against the in-vitro oxidant systems by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical, superoxide anion, FRAP (ferric reducing antioxidant power) and nitric oxide assays. The results obtained clearly shows that β -sitosterol exerts significant antioxidant effect across all the studied oxidant systems.*

Keywords: *Lawsonia inermis* leaves, β -sitosterol, antioxidant property, DPPH, FRAP assays

1. Introduction

The human body encompasses an variety of different enzymatic and non-enzymatic antioxidant defense system, which prevents the detrimental effects of free radicals and other oxidative stressors. Oxidants may be free-radicals (e.g: superoxide anion, hydroxyl radical, lipid peroxide, peroxy nitrite, nitric oxide, etc.) or non-free radicals (e.g: hydrogen peroxide) derived from oxygen or nitrogen species which cause serious injury to the cells when the oxidant levels overwhelm the levels of antioxidants. A defying factor in the assay of antioxidant potential is that within biological systems, there are various forms of antioxidants including enzymes (e.g. thioreductase, catalase, etc.), large molecules (e.g. ceruloplasmin, ferritin, etc.), small molecules (e.g. ascorbate, glutathione, thioredoxin, etc) and some hormones (e.g. melatonin) which follow diverse antioxidant mechanisms. Thus, devising a universal method to accurately assess the antioxidant potential of any compound is difficult [1]. This robust threat to the oxidant resistance mechanism may result in cancer, cardiovascular, neuropsychological, gastro-intestinal or other diseases [2]. Recent reports have shown that many natural compounds exhibit potential medicinal properties against these diseases. Thus, "herbal renaissance" is now ensuing the progressive innovation and hunt for novel therapeutics remedial effects [3]. The World Health Organization (WHO) has proposed that 80% of the population from developing countries are still dependent on the holistic folklore medicines [4]. Intriguingly, many clinicians have started to prescribe complementary and alternative medicine (CAM) to their patients for the successful treatment of various diseases [5, 6].

Lawsonia inermis (popularly known as "Henna") is a well-known traditional herb used in India for over 9,000 years for various cosmetic and medicinal purposes. A meticulous review by Chaudhary et al., revealed that the leaf, flower, seed, bark and root of *Lawsonia inermis* shows diverse medicinal useses in treating hepatobiliary, dermatological, neurological, venerea and other diseases [7]. The herb has been reported to exhibit anti-oxidant, anti-microbial, antiviral, anti-cancer, anti-coagulant, wound-healing, abortifacient, analgesic and anti-inflammatory activities. There have been research evidences to ascertain the antioxidant effects of *Lawsonia inermis* due to the presence of various phytonutrients including flavonoids, tannins, terpenoids and coumarins [8]. On top of its antioxidants potential, *Lawsonia inermis* has been reported to exhibit anti-inflammatory, antibacterial, antifertility, wound healing and anticancer activities [7, 9]. In this study, we have investigated the antioxidant effects of methanolic extract of *Lawsonia inermis* leaves (MELIL) β -sitosterol against the oxidant systems, using 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical, superoxide anion, FRAP (ferric reducing antioxidant power) and nitric oxide assays.

2. Materials and Methods

1,1-Diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside, nitro-blue tetrazolium, xanthine oxidase, 2, 4, 6-tripyrindyl-s-triazine (TPTZ) and 2-deoxyribose were obtained from Sigmaaldrich, USA. All other reagents used were of superior analytic grade. Fresh *Lawsonia inermis* leaves were collected from the local garden in School of Biotechnology, KIIT University, Bhubaneswar, Odisha. The fresh leaves of *Lawsonia inermis* were washed through running tap water, air dried and pulverized to obtained coarse powder. Then, the powder material was

sieved, weighed and stored in an air tight container. The powder was extracted successively with 250 ml of methanol in a soxhlet extractor for 8 hrs. The solvent was allowed to evaporate at about 30- 35 °C and the dried extract was stored in a dessicator for further use.

2.1. HPLC analysis

Chromatographic separation was performed with Merck Hitachi high performance liquid chromatograph equipped with C18 column (150 x 4.6, 5 µm) and L-7100 pump fitted with L- 7455 auto sampler, and HSM-LACHROM Multi HSM manager chromatographic software was used for data acquisition. The mobile phase comprising of 100 % acetonitrile was filtered through a 0.45 µm membrane filter (Millipore) and degassed by sonication. Throughout the run time, a flow rate of 1.0 mL min⁻¹ was maintained in the analysis. The column effluent was monitored at a wavelength 198 nm with L-2400 series multi-wavelength UV Detector.

2.2. Antioxidant assays

i) DPPH assay: We employed the method of Mensor et al. [10] to resolve the free radical scavenging effect of MELIL, determined by the discoloration of methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The MELIL extracts were assayed at various concentrations ranging from 10–500 µg/mL. The free radical quenching effect of MELIL was evaluated spectrophotometrically at 518 nm against the absorbance of the DPPH radical. A freshly prepared DPPH solution was used for the DPPH assays; MELIL solution was used as a blank and methanolic solution of DPPH was used in our study as a negative control. Trolox was used as positive control to assess the efficacy of MELIL. The degree of discoloration denotes the free radical scavenging potential (expressed as EC₅₀ in µg/ml) of MELIL.

ii) Nitric oxide assay: The interaction of the MELIL with nitric oxide was evaluated by the method proposed by Green et al. [11]. NO was engendered from spontaneous decomposition of 200 nM sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Nitric oxide reacts with oxygen to initiate formation of stable products, thereby forming nitrites, which were calculated by the Griess reaction. The reaction mixture comprising 10 mM sodium nitroprusside in phosphate buffer and the MELIL were incubated at 37 °C for 1h. An aliquot was taken and homogenized with Griess reagent (0.1% α-naphthylethylenediamine in water and 1% sulfanilic acid in 5% H₃PO₄). The concentration of nitrite was measured at 540 nm wavelength and calculated with reference to the absorbance of the standard nitrite solutions. Trolox was used as positive control. The results with respect to nitric oxide inhibition were expressed as EC₅₀ in µg/ml.

iii) Superoxide scavenging activity: A modified method of Chang et al. [12] has been employed to assess the superoxide scavenging activity of MELIL. Nitro-blue tetrazolium (NBT) solution (100 ml of 4.1 mM/l) was made ready in situ by adding 3.15 g Tris-HCl, 0.1 g MgCl₂, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate

and 34.0 mg 4-nitro-blue tetrazolium chloride to double distilled water. The reaction mixture (100 ml) was prepared by mixing 0.53 g sodium carbonate (pH 10.2), 4.0 mg ethylene diamine tetraacetic acid (EDTA) and 500 mg xanthine in 0.025 mM NBT solution. The mixture was refrigerated at 4 °C, transferred into a microcuvette and placed in a 25 °C cell holder of spectrophotometer. Superoxide generation was kicked-off by adding 1.0 ml of xanthine oxidase (XOD) (20 U/ml). The optical density (OD) readings were observed at 560 nm for 120 s by using Lambda 2S spectrophotometer. MELIL was dissolved in the reaction mixture at the concentration of 200 mg/ml. The stock solution measuring about 200 ml was added to 799 ml of the reaction mixture and positioned in a cell holder to autozero. One microliter of XOD (20 U/ml) was then added, mixed thoroughly and also calculated for the XOD curves. Trolox (antioxidant) was used as positive control. The superoxide radical scavenging efficiency of MELIL was expressed as EC₅₀ in µg/ml.

iv) Ferric reducing/antioxidant power (FRAP) assay:

The ferric reducing power of MELIL was assessed by the modified method of Benzie and Strain [13]. The stock solution comprised 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM FeCl₃ 6H₂O solution. The fresh working solution was made by the addition of 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃ 6H₂O at 37 °C. In a dark atmosphere, samples (10 µL) were allowed to react with 300 µl of the FRAP solution after 4 min. Readings of the final colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. Trolox was used as positive control. Results were expressed as EC₅₀ in µg/ml.

v) Hydroxyl radical scavenging activity: The effect of MELIL on hydroxyl radical was evaluated by using the deoxyribose method [14]. The reaction mixture contained 450 µl of 0.2 M sodium phosphate buffer (pH 7.0), 150 µl of 10 mM 2-deoxyribose, 150 µl of FeSO₄-EDTA, 150 µl of H₂O₂, 500 µl of H₂O, and about 100 µl of sample solution (0.02–5.00 mg/ml) and the mixture was set to react by the addition of H₂O₂. After incubation at 37 °C for 4 h, the reaction was halted by the addition of 750 µl of 2.8 % trichloroacetic acid and 750 µl of 1% thiobarbituric acid in 50 mM NaOH. Then, the solution was boiled for about 10 min, and then cooled in ice-cold water. The absorbance of the solution was measured at 520 nm. Results were expressed as EC₅₀ in µg/ml. Trolox was used as reference standard.

3. Results and Discussion

To explore the presence of β-sitosterol, a well reported antioxidant [15], we performed HPLC analysis of MELIL (Fig. 1) and compared that with the commercially available β-sitosterol at 198 nm (Fig.2). It was observed from the HPLC analysis of the methanolic extract of *Lawsonia inermis* leaves extracts that MELIL showed the presence of β-sitosterol. Retention time, measured - from the time at which the sample (MELIL or commercially obtained β-sitosterol) is injected - to the point at which the chromatogram shows a maximum peak height for β-sitosterol was taken as the key criterion to ascertain the

presence of β -sitosterol in the extract (Fig. 1 and 2). The chromatograms portrayed that the retention time of β -sitosterol in extract and commercial sample were almost similar and stood at 2.604 and 2.645 min respectively. Thus, we conjecture that along with the presence of

flavonoids and other phenolic compounds, the presence of β -sitosterol may at least in part account for the in vitro antioxidant activities exhibited by the plant extract.

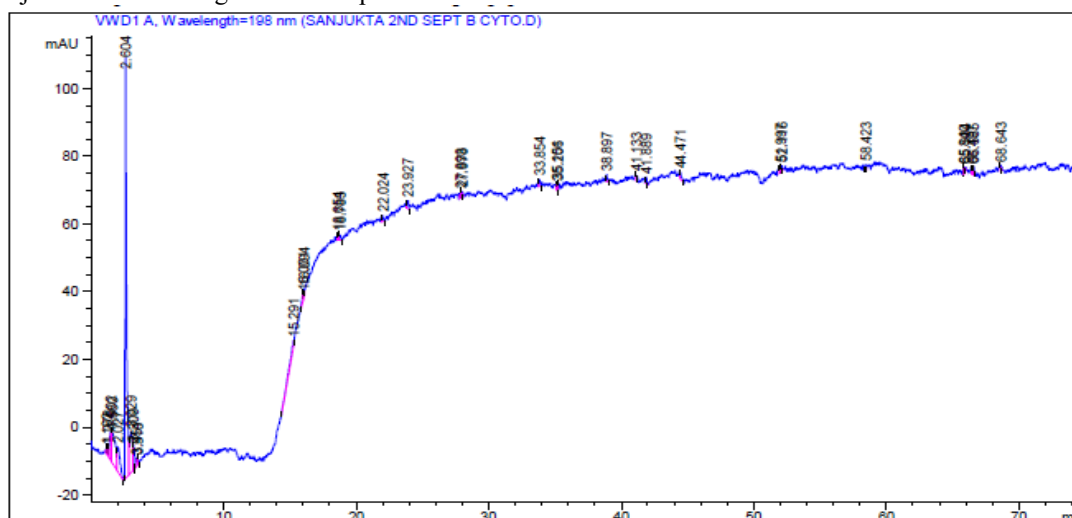


Figure 1: HPLC chromatogram analysis profile of *Lawsonia inermis* leaves extract (MELIL) showing the presence of β -sitosterol (Wavelength = 198 nm).

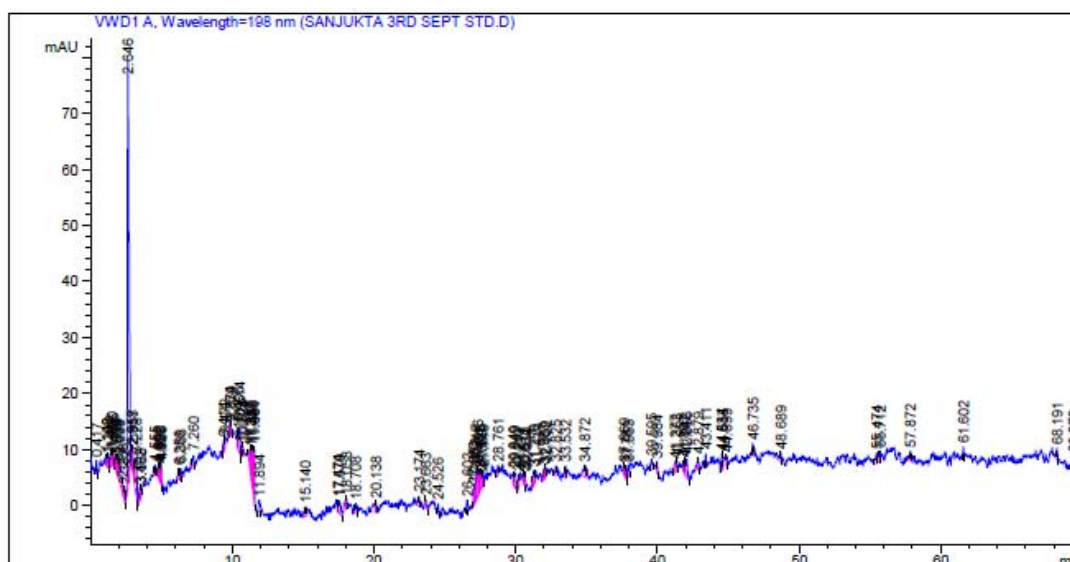


Figure 2: HPLC chromatogram analysis profile of commercially obtained β -sitosterol standard (Wavelength = 198 nm).

Five different *in-vitro* assay methods such as DPPH, hydroxyl radical, superoxide anion, FRAP and nitric oxide assays were employed to investigate the antioxidant properties of the leaf extract and the result obtained are listed in Table 1. DPPH assay is one of the widely used and rapid methods to evaluate the in vitro antioxidant capability of compounds. In this study, MELIL depicted significantly better antioxidant effect when compared with the standard. It was observed that MELIL exerted the antioxidant effect in the DPPH assay with about 0.22-fold decrement in the concentration as compared to the standard; thus ascertaining its better antioxidant capacity. Similarly, the results of nitric oxide, superoxide anion, hydroxyl radical and FRAP assays showed substantially better antioxidant effect with 22.9, 79.1, 18.0 and 11.3 fold lesser concentration respectively than that of the standard (Table 1).

Table 1: EC₅₀ values of methanolic extract of *Lawsonia inermis* leaves (MELIL) and trolox, as standard. * MELIL significance ($p < 0.05$) in comparison with the standard.

Assay	Samples	EC ₅₀ (μ g/ml)
DPPH	MEIL	189.09 \pm 1.27*
	Standard	243.11 \pm 2.18
NO	MEIL	54.22 \pm 0.91*
	Standard	77.06 \pm 0.99
Superoxide anion	MEIL	104.08 \pm 1.09*
	Standard	183.20 \pm 1.77
Hydroxyl radical	MEIL	47.59 \pm 0.35
	Standard	65.46 \pm 0.41*
FRAP	MEIL	19.72 \pm 0.23*
	Standard	31.03 \pm 0.33

These results are in correlation to the earlier studies on the antioxidant effect of *Lawsonia inermis* leaves methanolic

extracts exhibited significant antioxidant effect in the ABTS [2,2'-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid)] assay [16, 17].

4. Conclusions

From previous studies and from this study it is underscored that the leaf extracts of *Lawsonia inermis* have many phytoconstituents including gallic acid, mannitol, lawsone (2-hydroxy-1,4-naphthoquinone), apigenin, p-coumaric acid, 2-methoxy-3-methyl-1,4-naphthoquinone, apigenin-7-apiosylglucoside (apiin), eugenol, hexadecanoic acid, phytol, α -terpineol, luteolin and cosmosiin [18]. Although other phytoconstituents were reported, phytosterol like β -sitosterol is not reported for its presence in the leaves. This study needs further critical investigations to show the *in-vivo* effects of β -sitosterol as a potential antioxidant.

References

- [1] Prior RL, Wu X, Schaich K. J Agric Food Chem. 2005;53:4290.
- [2] Alam MN, Bristi NJ, Rafiquzzaman M. Saudi Pharm J. 2013; 2: 143.
- [3] Pan SY, Litscher G, Gao SH, Zhou SF, Yu ZL, Chen HQ et al. Evid Based Complement Alternat Med. 2014; 52: 340.
- [4] Ibrahim MA, Na M, Oh J, Schinazi RF, McBrayer TR, Whitaker T et al. Proc Natl Acad Sci U S A. 2013; 110: 16832.
- [5] Manek NJ, Crowson CS, Ottenberg AL, Curlin FA, Kaptchuk TJ, Tilburt JC. BMC Complement Altern Med. 2010;10:5.
- [6] N Suzuki. Evid Based Complement Alternat Med, 2004, 1, 113.
- [7] Gagandeep C, Sandeep G, Priyanka P. Int J Pharm Sci Drug Res. 2010; 2: 91.
- [8] Hsouna AB, Trigui M, Culioli G, Blache Y, Jaoua S. Food Chem. 2011; 125:193.
- [9] Pradhan R, Dandawate P, Vyas A, Padhye S, Biersack B et al. Curr Drug Targets. 2012;13:1777.
- [10] Mensor LL, Menezes FS, Leitão GG, Reis AS, dos Santos TC et al. Phytother Res. 2001;15:127.
- [11] Green LC, Wagne DA, Glogowski J, Skipper PL, Wishnok JK, Tannenbaum SR. Anal Biochem. 1982; 126: 131..
- [12] Chang WS, Lin CC, Chiang HC. Am J Chin Med. 1996; 24:11.
- [13] Benzie IF, Strain J. Anal Biochem. 1996; 239:70.
- [14] Halliwell B, Gutteridge JM, Aruoma OI. Anal Biochem. 1987; 165:215.
- [15] Gupta R, Sharma AK, Dobhal MP, Sharma MC, Gupta RS., J Diabetes. 2011;3(1):29-3
- [16] Oyesola O. Ojewunmi, Tope Oshodi, Omobolanle I. Ogundele, Chijioke Micah and Sunday Adenekan. In vitro Antioxidant, Antihyperglycaemic and Antihyperlipidaemic Activities of Ethanol Extract of *Lawsonia inermis* Leaves. British Journal of Pharmaceutical Research. 2014;4(3):301-314.
- [17] Mikhaeil BR1, Badria FA, Maatooq GT, Amer MM., Z Naturforsch C. 2004;59(7-8):468-76.
- [18] Tekle kebede kidanemariam, tesfahun kebede tesema, kesatebrhan haile asressuand aman dekebo boru. Chemical Investigation of *Lawsonia inermis* L. Leaves from Afar Region, Ethiopia. Orient J Chem. 2013;29;(3):1129-1134