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Antioxidant activities and exhibit to stimulate immune cell production in the *in vitro* of *Hibiscus sabdariffa* Linn. and *Phyllanthus emblica* L. extracts

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Abstract

Hibiscus sabdariffa Linn. and *Phyllanthus emblica* L. are widely used in traditional Thai medicine as natural remedies. The aim of this study was to investigate the total phenolics and flavonoids contents, antioxidant activities, NO inhibition and cytotoxicities of methanolic extracts. The result showed that *H. sabdariffa* Linn. and *P. emblica* L. extracts contain high amount of total phenolics and flavonoids contents which were related to the antioxidant activities. *P. emblica* L. extract showed to inhibit NO production in dose dependent manner. In addition, both extracts showed to stimulate the number of mouse macrophage cell line in the *in vitro*. These data suggest that *H. sabdariffa* Linn. and *P. emblica* L. extracts may be potentially beneficial in the treatment of inflammatory diseases through the inhibition of NO production as well as stimulation of immune system via increasing number of immunize cells.

Keywords: *Hibiscus sabdariffa* Linn.; *Phyllanthus emblica* L.; Antioxidant; Anti-inflammation; Immune cell stimulation; Methanolic extract

1. Introduction

Inflammation is the first response of the immune system to infection, irritation, as well as to various other diseases such as cancer, cardiovascular diseases, obesity, hypertension, diabetes, autoimmune, and neurodegenerative disorders [1]. It involves directed migration and activation of leukocytes (neutrophils, monocytes and eosinophils) to the site of damage. Inflammation is linked to oxidant/antioxidant imbalance (an elevation in the reactive oxygen species in parallel to low levels of antioxidants). High levels of inflammatory markers and low level of antioxidants are observed in many inflammatory diseases [2, 3], as well as in many pathological conditions such as malignancy, cardiovascular disease, diabetes type 2, kidney malfunction, gastrointestinal disorders, microbial infection, fibrogenesis, and neurological diseases. The imbalance between production of antioxidants versus getting rid of free radical species leads to oxidative stress [4 – 6].

Medicinal plants have attracted huge attention due to their diverse range of biological and therapeutic properties. Evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary, and alternative systems [7 – 9]. The traditional medicinal plants, *H. sabdariffa* Linn. and *P. emblica* L. are widely used in traditional Thai medicine as natural remedies. *H. sabdariffa* Linn. is used in traditional Thai medicine to treat urinary tract diseases as well as prevention of heart disease. *P. emblica* L. is used as longevity medicine and treatment of many diseases including diabetes mellitus, liver disease, peptic ulcer and others.

In this study, the anti-inflammatory, cytotoxicity and the antioxidant activity in the *in vitro* of *H. sabdariffa* Linn. and *P. emblica* L. extracts were assessed in mouse macrophage cultures.

2. Materials and Methods

Chemicals and reagents

Butylated hydroxytoluene (BHT), ammonium molybdate, sodium phosphate, sulphuric acid, gallic acid, ferric chloride, potassium ferrocyanide, Folin-ciocalteu reagent (FCR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate was purchased from Prolabo (Paris, France). All the other chemicals and solvents used were of analytical grade.

Preparation of plant extracts

Samples were collected from local market located in Ubon Ratchathani and were dried in hot air oven at 50 ºC for 48 h and ground into fine powder. Fifty grams of powder were extracted in methanol and concentrated at 55 ºC in a rotary vacuum evaporator. The obtained extracts were stored at –20 ºC until use.

Total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex in acid pH [10]. A total volume of 0.30 mL extract dissolved in methanol was added to 3 mL of reagent solution (0.60 mol L^{-1} sulphuric acid, 28 mmol L^{-1} sodium phosphate and 4 mmol L^{-1} ammonium molybdate). The mixtures were incubated at 95 °C for 90 min then cooled to room temperature. The absorbance was measured at 695 nm. The total antioxidant activity was expressed as the number of equivalence of ascorbic acid and BHT.

$ABTS^{+}$ free radical scavenging activity

An ABTS assay was determined using the method reported by N. Tlili *et al.* (2015). To produce an ABTS radical cation, 7 mM of ABTS solution was mixed with 2.45 mM of potassium persulphate and after that, the mixture was allowed to stand in the dark at room temperature. Then, the ethanol was used to dilute the $ABTS^{+}$ solution to an absorbance of 0.70 ± 0.02 at 734 nm. The aliquot (25 µL) of each diluted sample or Trolox standard was added to 2 mL of $ABTS^{\bullet+}$; after that the absorbance was read at 734 nm [11]. $ABTS^{\bullet+}$ scavenging activity is presented as an IC_{50} value. Trolox was used as a reference compound.

FRAP assay

The ferric reducing antioxidant power (FRAP) of methanolic extract was estimated according to the procedure described by M. Zheng *et al.* (2017). FRAP reagent (900 µL), prepared freshly and incubated at 37 ºC for 30 min, was mixed with 90 µL of distilled water and 30 µL of test sample or methanol (for the reagent blank) or BHT (1 mg mL^{-1} as positive control). Then reaction mixture was incubated at 37 ºC for 30 min in a water bath. The FRAP reagent contained 2.50 mL of 20 mmol L^{-1} TPTZ solution (2,4,6-Tris 2-pyridyl)-s-triazine) in 40 mmol L^{-1} HCI and 2.50 mL of 20 mmol L^{-1} $FeCl_3.6H_2O$ and 25 mL of 0.30 mol L^{-1} acetate buffer (pH 3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm using the Spectrophotometer [12]. Methanolic solutions of known Fe(II) concentrations ranging from 100 to 2000 µmol L^{-1} $FeSO_4.7H_2O$ were used for the preparation of calibration curve. The antioxidant activity of methanolic extracts was expressed in mmol L^{-1} Fe(II) per gram DW extract.

*Total phenolics content*s

The total phenolics contents were determined using the FCR [10]. The reaction mixture contained 100 µL of methanolic solution (1 mg mL^{-1}) of the extract, 0.50 mL of FCR, 1.5 mL of 20% (w v^{-1}) sodium carbonate and 10 mL of distilled water. After 2 h of reaction at ambient temperature, the absorbance was measured at 765 nm and used to calculate the phenolics contents, using gallic acid as

a standard. Then the total phenolics contents were expressed in term of gallic acid equivalents (mg GAE g^{-1} dry extract).

Total flavonoids contents

The flavonoids contents of the ethanolic and ethyl acetate extracts of *I. iscosa* were assessed based on the formation of a complex flavonoidaluminium [10]. Briefly, 1 mL of diluted sample (20 μg mL^{-1}) was mixed with 1 mL of 2% aluminium chloride methanolic solution, after incubation for 10 min at room temperature. The absorbance was read at 430 nm and the flavonoids contents were expressed in mg quercetin equivalent (QE) per gram of dry extract.

Anti-inflammatory activity

Macrophage cells were cultured in monolayer using RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Co. Ltd., USA). The cells were grown in logarithmic phase at 37 °C in a moisturized atmosphere of 5% CO_2 and 95% air. Experiments were performed with cells not surpassing 30 passages. For extract treatment, cells were grown until 70% – 80% confluence followed by 2 h pretreatment with either *H. sabdariffa* Linn. or *P. emblica* L. extracts. Then, the cells were stimulated with lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS) (10 μg/mL; Sigma-Aldrich Co Ltd., USA) for 24 h by adding LPS directly into CBD-, moringin-, and CBD-moringin-treated cell culture medium. Untreated cells and treated cells without LPS activation were also included as controls. After LPS stimulation, the cells were harvested for further analyses as anti-inflammatory and cytotoxic effects. All the experiments were made in triplicates and repeated for three independent times [13, 14].

3. Results and Discussion

Total phenolics and flavonoids contents of extracts

As show in Table 1, the extract of *H. sabdariffa* Linn. showed the total phenolics and flavonoids contents at 63.26 ± 18.05 and 5.56 ± 0.30, respectively, whereas *P. emblica* L. extract showed 106.17 ± 8.17 and 2.03 ± 0.08, respectively.

Table 1 Total phenolics and flavonoids contents of *H. sabdariffa* Linn. and *P. emblica* L. extracts.

Extracts	Total phenolics contents (mg GAE g^{-1} extract ; n = 7)	Total flavonoids contents (mg QE g^{-1} extract ; n = 7)
H. sabdariffa Linn	63.26 ± 18.05	5.56 ± 0.30
P. emblica L.	106.17 ± 8.17	2.03 ± 0.08

Total antioxidant capacity of extracts

The extracts of *P. emblica* L. showed the higher total antioxidant capacities (123.80 ± 0.55 equivalent to ascorbic acid and 185.72 ± 0.82 equivalent to BHT) when compared to *H. sabdariffa* Linn. extracts (37.84 ± 0.49 equivalent to ascorbic acid and 56.88 ± 1.11 equivalent to BHT). The data was expressed as mg g^{-1} of dry extract as show in Table 2.

Table 2 Total antioxidant capacity of *H. sabdariffa* Linn. and *P. emblica* L. extracts

Extracts	Equivalent to ascorbic acid (mg g^{-1} dry extract ; n = 5)	Equivalent to BHT (mg g^{-1} dry extract ; n = 5)
H. sabdariffa Linn.	37.84 ± 0.49	56.88 ± 1.11
P. emblica L.	123.80 ± 0.55	185.72 ± 0.82

Ferric Reducing Antioxidant Power of extracts

As show in Table 3, *P. emblica* L. showed the power on reducing antioxidant in similar to positive control BHT (13.93 ± 1.78 versus 14.97 ± 0.95 equivalent to mmol Fe^{+2} g^{-1} dry extract, respectively). Methanolic extract of *H. sabdariffa* Linn. showed the lower antioxidant power when compared to *P. emblica* L. and positive control BHT.

Table 3 Ferric Reducing Antioxidant Power of *H. sabdariffa* Linn. and *P. emblica* L. extracts

Extracts	**FRAP (Equivalent to mmol Fe^{+2} g^{-1} dry extract)**
H. sabdariffa Linn.	2.64 ± 0.18
P. emblica L.	13.93 ± 1.78
BHT	14.97 ± 0.95

$ABTS^{+}$ radical scavenging activity of extracts

As similar to FRAP result, *P. emblica* L. extracts showed to inhibit $ABTS^{+}$ radical as similar as positive control trolox at an EC_{50} at 0.44 mg mL^{-1}, which was higher than in *H. sabdariffa* Linn. extract (EC_{50} 2.38). The data is shown in Table 4.

Table 4 $ABTS^{+}$ radical scavenging activity of *H. sabdariffa* Linn. and *P. emblica* L. extracts

Extracts	**EC_{50} (mg mL^{-1})**
H. sabdariffa Linn.	2.38
P. emblica L.	0.44
Trolox	0.51

Anti-inflammatory activity of extracts

P. emblica L. extract showed to inhibit NO production in dose dependent manner at the concentration of 62.50 – 1000 μg mL^{-1}, whereas *H. sabdariffa* Linn. extract showed no effect on inhibition of NO production in the *in vitro* model using mouse macrophage cell line. The data is shown in Fig. 1.

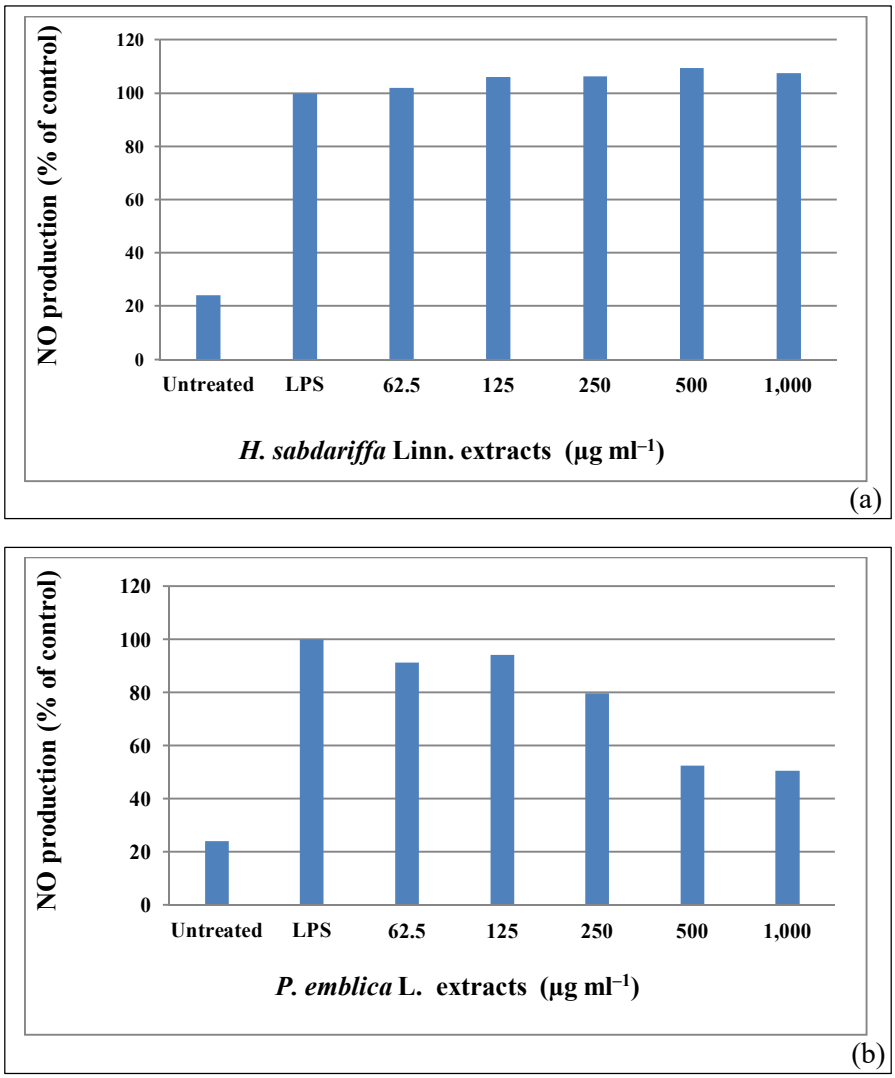
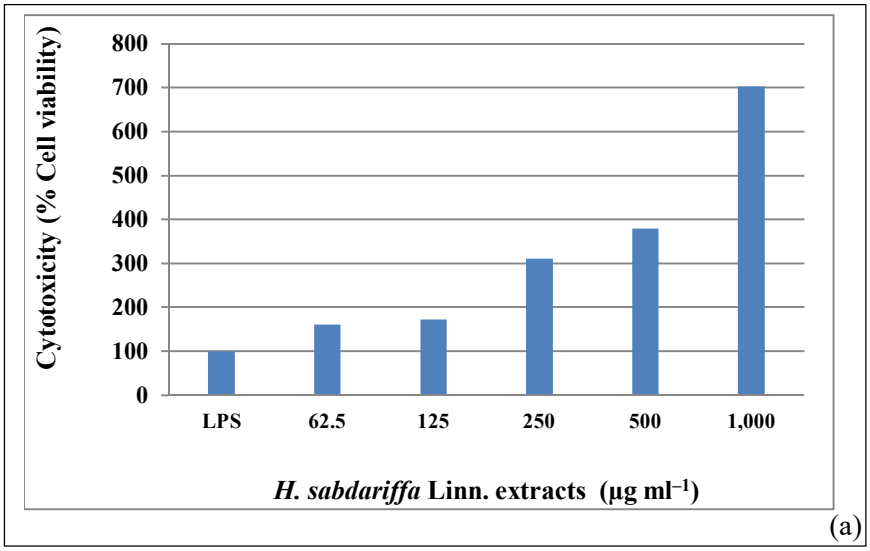


Fig. 1 Anti-inflammatory activity of (a) *H. sabdariffa* Linn. and (b) *P. emblica* L. extracts

Cytotoxicity of extracts on mouse macrophage cell line

The results in Fig. 2 demonstrated that both of *H. sabdariffa* Linn. and *P. emblica* L. extracts showed the number of cell line increasing after incubation with the extracts in the dose dependent manner at the concentration of 62.50 – 1000 µg ml^{-1}. *P. emblica* L. extract show the higher activity of stimulating on cell production than those found in *H. sabdariffa* Linn. extract.



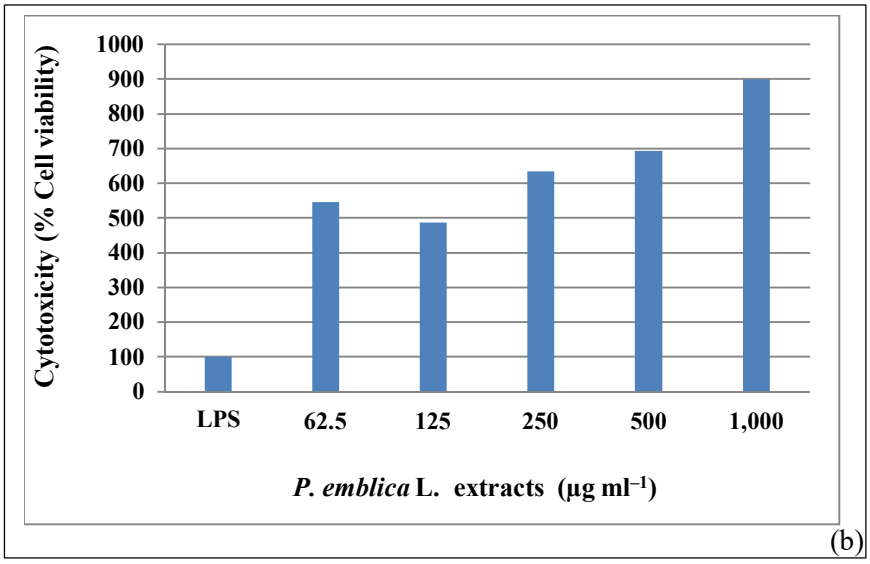


Fig. 2 Cytotoxicity of (a) *H. sabdariffa* Linn. and (b) *P. emblica* L. extracts on mouse macrophage cell line

4. Conclusion

In this study, we investigated the total phenolics and flavonoids contents of *H. sabdariffa* Linn. and *P. emblica* L. extracts as well as antioxidant activities, inhibition of NO production and cytotoxicity effects. In addition to a pivotal role in many body functions, NO has also been implicated in the pathology of many inflammatory diseases, including arthritis, myocarditis, colitis, and nephritis. Therefore, NO inhibitors are essential for prevention of inflammatory diseases. Recent evidence suggests that some phytochemical agents are involved in inflammatory processes, and that COX-2, an inducible isoform of COX, is mainly responsible for the production of large amounts of these mediators [13 – 15]. Our results indicated that *H. sabdariffa* Linn. and *P. emblica* L. extracts showed the higher antioxidant activity. *P. emblica* L. extracts also show in inhibit NO production in mouse macrophage cell line. This can be implied that *P. emblica* L. extract can inhibit inflammation. The results also indicated that both of *H. sabdariffa* Linn. and *P. emblica* L. extracts showed the number of macrophage cell line increasing after incubation with the extracts in the dose dependent

manner. This can be implied that studied extracts may be used as potential agents for increasing immune system. The previous data have demonstrated that one of the increasing of immune system is to simulate the ability of the macrophage both the phagocytosis process and also to the macrophage number of cell [16].

The results firstly showed, at the first time, that *H. sabdariffa* Linn. and *P. emblica* L. extracts showed to increase the number of immune cell. These data suggest that *H. sabdariffa* Linn. and *P. emblica* L. extracts may be potentially beneficial in the treatment of inflammatory diseases through the inhibition of NO production as well as stimulation of immune system via increasing number of immunize cells [17].

5. Acknowledgement

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6. References

[1] S.M. Cardona, J.A. Garcia, A.E. Cardona, The fine balance of chemokines during disease: trafficking, inflammation, and homeostasis, Methods Mol Biol. (1013) (2013) 1 – 16.

[2] C. Murr, B.M. Winklhofer-Roob, K. Schroecksnadel, M. Maritschnegg, H. Mangge, B.O. Böhm, Inverse association between serum concentrations of neopterin and antioxidants in patients with and without angiographic coronary artery disease, Atherosclerosis. 202(2) (2009) 543 – 549.

[3] D. Fuchs, P. Avanzas, R. Arroyo-Espliguero, M. Jenny, L. Consuegra-Sanchez, J.C. Kaski, The role of neopterin in atherogenesis and cardiovascular risk assessment, Curr Med Chem. 16(35) (2009) 46 – 53.

[4] E. Ginter, V. Simko, V. Panakova, Antioxidants in health and disease, Bratisl Lek Listy. (115) (2004) 603 – 606.

[5] K. Apel, H. Hirt, Reactive oxygen species: Metabolism, oxidative stress, and signal transduction, Annu Rev Plant Biol. (55) (2004) 373 – 399.

[6] J.M. McCord, The evolution of free radicals and oxidative stress, Am J Med. 108(8) (2000) 652 –659.

[7] B. Saad, O. Said, Greco-Arab and Islamic Herbal Medicine: Traditional System, Ethics, Safety, Efficacy and Regulatory Issues, Wiley-Blackwell John Wiley & Sons Inc, New Jersey, 2011.

[8] B. Saad, Integrating traditional Greco-Arab and Islamic diet and herbal medicines in research and clinical practice, in: I. Ramzan (Eds.), Phytotherapies: Efficacy, safety and regulation, Wiley-Blackwell John Wiley & Sons, Inc., New Jersey, 2015, pp. 142 – 148.

[9] H. Zaid, B. Saad, State of the Art of Diabetes Treatment in Greco-Arab and Islamic Medicine. In: R.R. Watson, V.R. Preedy (Eds.), Bioactive Food as Dietary Interventions for Diabetes, Academic Press, San Diego, 2013, pp. 327 – 337.

[10] N. Chahmi, J. Anissi, S. Jennan, A. Farah, K. Sendide, M.E. Hassouni, Antioxidant activities and total phenol content of *Inula viscosa* extracts selected from three regions of Morocco, Asian Pac J Trop Biomed. 5(3) (2015) 228 – 233**.**

[11] N. Tlili, H. Mejri, F. Anouer, E. Saadaoui, A. Khaldi, N. Nasri, Phenolic profile and antioxidant activity of *Capparis spinosa* seeds harvested from different wild habitats, Ind Crops Prod. 76 (2015) 930 – 935.

[12] M. Zheng, Q. Xia, S. Lu, Study on drying methods and their influences on effective components of loquat flower tea, LWT – Food Sci. Technol. 63 (2015) 14 – 20.

[13] D. Jang, G.A. Murrell, Nitric oxide in arthritis, Free Radic Biol Med. 24(9) (1998) 1511 – 1519.

[14] N.W. Kooy, S.J. Lewis, J.A. Royall, Y.Z. Ye, D.R. Kelly, J.S. Beckman, Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite, Crit Care Med. 25(5) (1997) 812 – 819.

[15] E. Iwashita, T. Miyahara, K. Hino, T. Tokunaga, H. Wakisaka, Y. Sawazaki, High nitric oxide synthase activity in endothelial cells in ulcerative colitis, J Gastroenterol. 30(4) (1995) 551 – 554.
[16] C.M. Hogaboam, K. Jacobson, S.M. Collins, M.G. Blennerhassett, The selective beneficial effects of nitric oxide inhibition in experimental colitis, Am J Physiol Gastrointest Liver Physiol. 268(4) (1995) 673 – 684.
[17] C.J. Kelly, D.P. Gold, Nitric oxide in interstitial nephritis and other autoimmune diseases, Semin Nephrol. 19(3) (1999) 288 – 295.