*Medical Sciences*

# Diabetic Wound Healing and Activation of Nrf2 by Herbal Medicine

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**Nrf2 defense is a very important cellular mechanism to control oxidative stress, which is implicated in wound healing. Nrf2 can induce many cytoprotective genes, including HO-1, NQO1 and G6PD. Among many natural products that have been reported as Nrf2 activators, sulforaphane and curcumin have been studied more widely than any others, and both are in clinical trials for non-cancerous disorders. Recently, we reported 4-ethyl catechol and 4-vinyl catechol as Nrf2 co-factors that can induce Nrf2 as potently as sulforaphane and curcumin. These new Nrf2 co-factors were identified in hot aqueous extract of an herbal medicine *Barleria lupulina*, and fermented Noni (*Morinda citrifolia*) juice, which are used traditionally for diabetic wound healing.**

Nrf2 | diabetic wound healing | herbal medicine | *Barleria lupulina* | *Morinda citrifolia* | alkyl catechols

## The Nrf2 defense pathway

In mammals, the master regulator of antioxidant defense is the Nrf2 pathway[1,2]. The Nrf2 transcription factor controls expression of anti-oxidant and detoxifying enzymes that maintain a healthy cellular redox state and protect against toxic foreign chemical substances through phase II modification[3], which neutralizes dangerous species, generating less reactive and more soluble substances that are readily eliminated[4]. The network of cyto-protective genes regulated by Nrf2 is more than two hundred[5], accounting for more than 1% of the genome[6]. In the absence of stress, Nrf2 with a half-life of about 20 minutes[7] is sequestered within the cytosol by the actin-binding protein Kelch-like ECH-associated protein 1 (KEAP1) and Cullin 3, which degrade Nrf2 through ubiquitination[8]. Under conditions of oxidative stress, Nrf2 is released from Keap1 and rapidly moves to the nucleus to induce transcription of anti-oxidant and detoxifying enzymes. The "redox sensor" mechanism that releases Nrf2 from Keap1, resulting in Nrf2 transport to the nucleus, involves oxidation-sensitive sulfhydryl groups in cysteine residues of Keap1[9].

## Nrf2 induction of cytoprotective genes and diabetic wound healing

Compelling evidence for the importance of the Nrf2 pathway comes from numerous studies with mice lacking the Nrf2 gene. These Nrf2 null mice exhibit increased sensitivity to a multitude of chemical toxins, resulting in increased inflammation and damage to brain, lung, and kidney[2,10]. Similarly, Nrf2 null mice are considerably more sensitive to chemical carcinogens, with increased incidence of cancers demonstrated in skin, stomach, colon, and bladder[2,11,12]. Also, mice engineered with a dominant negative Nrf2 mutant transgene develop skin cancer at three times the frequency of control mice in a classical two-stage model of chemical carcinogenesis[13]. Additional problems for Nrf2 null mice include impaired liver regeneration[14], accelerated UVB-induced photo-ageing of skin[15], increased rheumatoid arthritis[16], development of lupus-like autoimmune nephritis[17], and development of age-related retinopathy[18]. Thus, the protective importance of the Nrf2 pathway is well established.

Diabetes often causes slow-healing wounds that can worsen rapidly. The Keap1-Nrf2 system is a critical target for preventing the onset of diabetes mellitus[19]. Nrf2 transcription factor, a novel target of keratinocyte growth factor action, regulates gene expression and inflammation in the healing skin wound[20]. In a streptozotocin-induced diabetes mouse model, $Nrf2^{-/-}$ mice have delayed wound closure rates compared with $Nrf2^{+/+}$ mice[21]. Hence, Nrf2 can be activated to potentially heal diabetic wounds and improve overall health.

## Nrf2 small molecule co-factors were first discovered as "cancer-protective" compounds, which could be utilized for diabetic wound healing

Long before the discoveries of Nrf2 and the antioxidant response element (ARE) to which Nrf2 binds, a variety of small chemical compounds were observed to protect rodents from chemically induced carcinogenesis[22]. Remarkably, these "cancer-protective" compounds were from distinctly different chemical classes, but they all shared the critical property of high susceptibility to oxidation-reduction reactions[23]. The simplest of these active cancer-protective compounds were identified as 1,4-diphenol (hydroquinone) and 1,2-diphenol (catechol), and more complex examples include the isothiocyanate sulforaphane isolated from broccoli[24] and curcumin from the turmeric plant[25] (see Figure 1). With the discovery of Nrf2, it became clear that these previously identified redox-sensitive, cancer-protective compounds worked as co-factors for Nrf2 activation[26]. Collectively, these findings suggested that support of Nrf2 activation by redox-sensitive co-factors, particularly dietary factors such as sulforaphane and curcumin, could be employed as an effective anti-cancer strategy[27]. This provided impetus for clinical trials[28,29], which are continuing. Sulforaphane and curcumin are also currently in clinical trials for non-cancer disorders in which the Nrf2 pathway has been implicated. Current challenges with the application of sulforaphane and curcumin for clinical benefit appear to involve bioavailability of these compounds[28-32]. Acrolein (CHO-CH=CH) induces Nrf2 translocation and ARE-luciferase reporter activity[33]. Cinnamaldehyde (Ph-CH=CH-CHO, *trans double bond*) inhibits thioredoxin reductase and induce Nrf2[34].

Recent studies have demonstrated the protective role of Nrf2 and the potential therapeutic effect of Nrf2 activators, sulforaphane and cinnamaldehyde in a diabetic nephropathy animal model[21].

Conflict of Interest: no conflicts declared.

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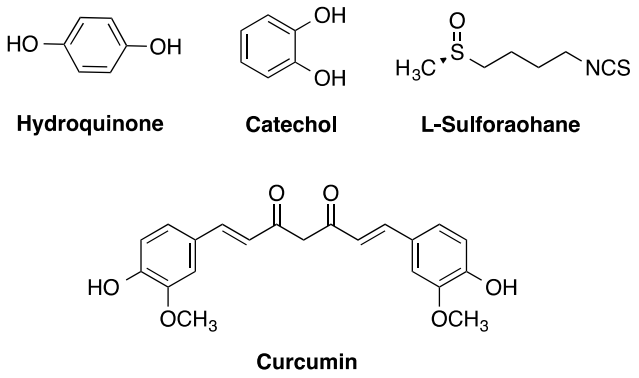


**Figure 1.** Known natural Nrf2 co-factors

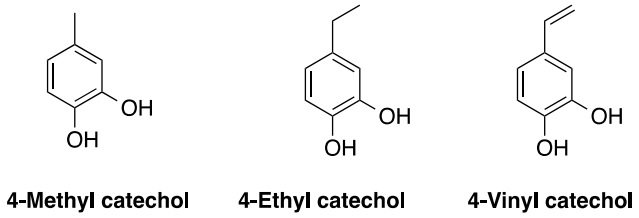


**Figure 2.** New Nrf2 co-factors identified in *Barleria lupulina* and *Morinda citrifolia.*

### The alkyl catechols, 4-ethyl catechol and 4-vinyl catechol, potent Nrf2 co-factors, from *Barleria lupulina* and *Morinda citrifolia*, both of which are used traditionally for diabetic wound healing

Barleria lupulina (BL): Recently, our work with a traditional Vietnamese medicine (*Barleria lupulina*, BL) has identified 4-ethyl catechol and 4-vinyl catechol as potently active, natural Nrf2 co-factors[35] (Figure 2) in the hot water extract of *Barleria lupulina*. Although these compounds had been reported, they had not been recognized as important Nrf2 co-factors. Nonetheless, these compounds each satisfy the well-defined structural criteria for "oxidation-reduction lability" that is required for a compound to induce protective enzymes[23,36]. Interestingly, catechol (Figure 2) was among the first compounds recognized as an Nrf2 co-factor[23], but 4-ethyl catechol and 4-vinyl catechol had not received attention. Importantly, we found that 4-ethyl catechol and 4-vinyl catechol are much more potent Nrf2 co-factors than catechol and that they are comparably potent to sulforaphane and curcumin[37]. Thus, apart from sulforaphane and curcumin, we believe that 4-ethyl catechol and 4-vinyl catechol may be the most potent of the naturally occurring Nrf2 co-factors. Moreover, the relatively small size and simple structure of these compounds, suggests the likelihood of better bioavailability than sulforaphane and curcumin. We have reported the activities of these alkyl catechols recently[37]. Besides inducing Nrf2, BL, 4-EC and 4-VC significantly improved the organization of the endothelial cell actin cyto-skeleton, reduced actin stress fibers, organized cell-cell junctions, and induced expression of mRNA encoding claudin-5 that is important for formation of endothelial tight junctions and reducing vascular leak[35].

Morinda citrifolia (Noni): Encouraged by the discovery of new Nrf2 co-factors, 4-MC, 4-EC, and 4-VC from the hot water extract of *Barleria lupulina*, we screened many herbs for their activity of Nrf2 activation. Only fermented Noni juice (Order Number 809979, Virgin Noni juice, http://www.virginnonijuice.com) showed Nrf2 activation, which was as strong as BL.

The traditional medicinal plant *Morinda citrifolia L.* (Rubiaceace) is believed to have the origin in Southeast Asia and later on distributed to Polynesia. It is called Indian mulberry in India, ba ji tian (巴戟天) in China, nono in Tahiti, and noni in Hawaii[38]. Noni is now widely cultivated in tropical areas of the South Pacific, including Hawaii[39]. Traditionally, noni bark and roots were used as dye or clothing, while medicinal usage of all plant parts, including leaves and fruits, were mostly restricted to treat wounds, infections, menstrual cramps, bowel irregularities, diabetes, high blood pressure or as a purgative[40]. Pacific Islanders and Native Hawaiians consume fresh fruits or noni juice prepared by fermenting the fruits[39,41]. Claims of its 'healing powers' have fuelled much of the commercial interest in noni and promoting a worldwide market for noni-based dietary supplements including fruit juice, in North America, Mexico, Australia, and Asia.

**Figure 3.** A Noni tree on campus of University of Hawaii at Hilo and fruits (Young: bottom left, green & hard; Ripe: bottom right, whitish & soft).

Young Noni fruits are green, and will turn yellow before ripe, but harvested Noni fruits are mainly whitish (Figure 3), and will turn dark after fermentation. We tested FNJ from ripe Noni fruits, and juices from both green and whitish Noni fruits for their activity of Nrf2 activation. Results showed that juice from young green Noni fruits was inactive, and both juice from ripe whitish Noni fruits and FNJ exhibited Nrf2 activation (Figure 4, Figure 5).

Using the same assay-guided separation method as described in our previous publication[35], we have identified 4-MC, 4-EC, and 4-VC (Figure 2, Figure 6) in FNJ, which partially accounted for the Nrf2 activation. We are in progress of identifying other Nrf2 activators. At the same time, questions remain why juice from ripe whitish Noni fruits with a fouling smell was active while juice from young green Noni fruits was not, and why people prefer FNJ to juice from ripe whitish Noni fruits.

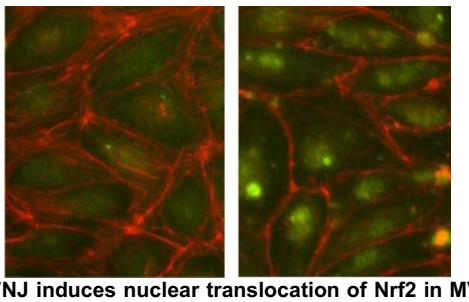

**Figure 4. FNJ induces nuclear translocation of Nrf2 in MVECs.** MVECs were incubated with FNJ at 1:25 dilution. Staining of Nrf2 in human MVECs incubated with FNJ (right), in comparison with control (left). Green = Nrf2, red = F-actin. Note increased nuclear staining for Nrf2 in cells incubated with FNJ. Overall intensity of Nrf2 staining is also increased, because Nrf2 activation typically involves Nrf2 stabilization in combination with nuclear translocation.

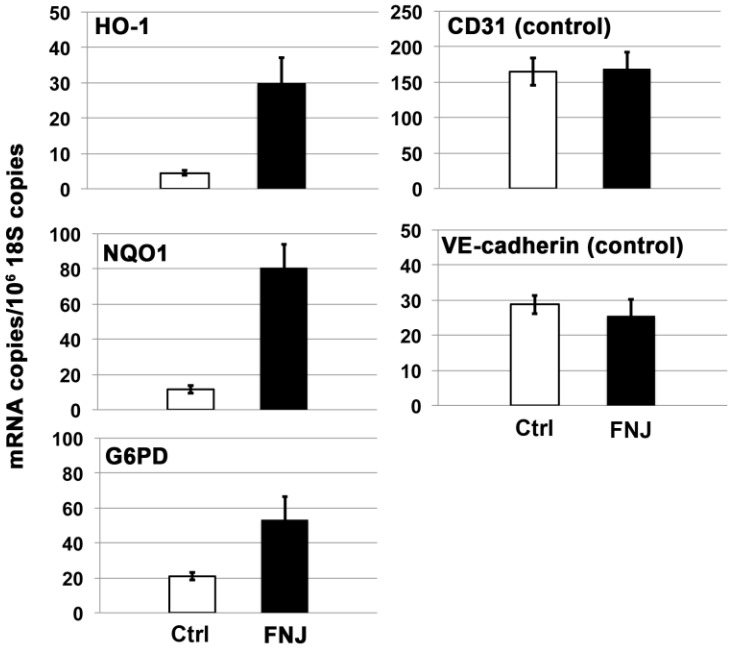


**Figure 5. Induction of Nrf2 target gene RNAs by FNJ in MVECs, as measured with RT-PCR.** Y-axis = (mRNA copies)/($10^6$ 18S rRNA copies). Nrf2 target genes = heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glucose 6-phosphate dehydrogenase (G6PD). Control, non-NRF2 target mRNAs = CD31 (PECAM-1) and VE-cadherin (cadherin-5). FNJ was added to a final dilution of 1:25, and cells were harvested at 24 hours. For all panels, error bars = $\pm$ standard deviation (S.D.); n $\geq$ 4 for each data point. Statistical significance: For HO-1, NQO1, and G6PD panels: FNJ vs. vehicle Ctrl = all extremely significant ($p<0.001$); for CD31 and VE-cadherin panels: no significant differences.

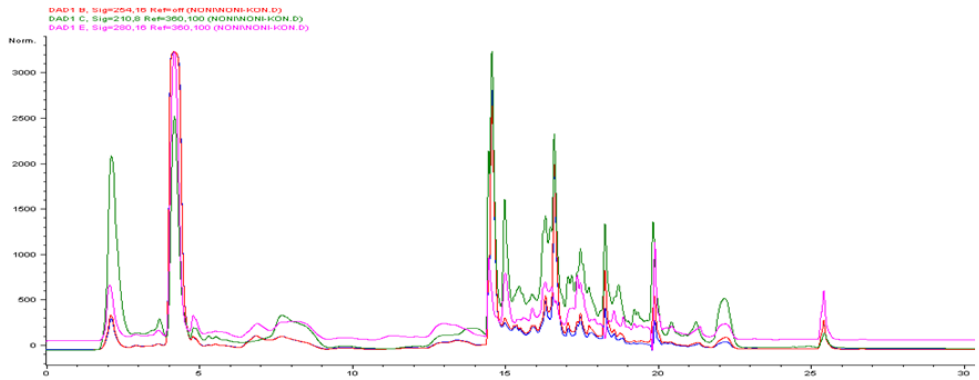


**Figure 6. Agilent prep-HPLC HPLC chromatogram of the aqueous acetonitrile eluent of FNJ**. Sample: FNJ (2 mL/injection, about 150 mg); Flow-rate: 10 mL/min; Solvents: 100% water (0.1% formic acid) for 10 min (0-10 min), then to 100% acetonitrile (0.1% formic acid) in 20 min (10-30 min), finally 100% acetonitrile for 10 min (30-40 min). Y-axis = absorbance at 254nm (mAU), X-axis = minutes. Fraction 11 ($t_R$ = 29-31 min) was active and Nrf2 activators 4-MC, 4-EC and 4-VC (minor compounds) were identified.

## Discussion

Nrf2 is a master regulator that can modulate many cytoprotective genes. Many small molecules, including natural products, were reported as Nrf2 activators, but only a few showed strong Nrf2 induction. Sulforaphane and curcumin are in clinical trials for non-cancer disorders, and dimethyl fumarate ($CH_3O$-CO-CH=CH-OC-$OCH_3$, *trans*), an Nrf2 activator, is also in phase III clinical trials for multiple sclerosis[42]. Many traditional herbal medicines have been used for diabetic wound healing, but most of the active compounds and mechanisms of actions are unknown. We have studied BL and more recently Noni, and we have identified 4-MC, 4-EC, and 4-VC as Nrf2 co-factors. 4-EC and 4-VC demonstrated Nrf2 induction, which were as potent as sulforaphane and curcumin. Since Nrf2 activation by Noni has not been fully characterized, it is worthy of further investigation.

## Abbreviation

BL, *Barleria lupulina*; Noni, *Morinda citrifolia*; FNJ: fermented Noni juice; 4-MC, 4-methyl catechol; 4-EC, 4-ethyl catechol; 4-VC, 4-vinyl catechol.

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1. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends in molecular medicine. 2004;10(11):549-557.
2. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annual review of pharmacology and toxicology. 2007;47:89-116.
3. Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends in biochemical sciences. 2014;39(4):199-218.
4. Kwak MK, Wakabayashi N, Kensler TW. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. Mutat Res. 2004;555(1-2):133-148.
5. Carmona-Aparicio L, Perez-Cruz C, Zavala-Tecuapetla C, et al. Overview of Nrf2 as Therapeutic Target in Epilepsy. International journal of molecular sciences. 2015;16(8):18348-18367.
6. Holmstrom KM, Baird L, Zhang Y, et al. Nrf2 impacts cellular bioenergetics by controlling substrate availability for mitochondrial respiration. Biology open. 2013;2(8):761-770.
7. Kobayashi A, Kang MI, Okawa H, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Molecular and cellular biology. 2004;24(16):7130-7139.
8. Itoh K, Mimura J, Yamamoto M. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxidants & redox signaling. 2010;13(11):1665-1678.
9. Holland R, Fishbein JC. Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. Antioxidants & redox signaling. 2010;13(11):1749-1761.
10. Liu M, Grigoryev DN, Crow MT, et al. Transcription factor Nrf2 is protective during ischemic and nephrotoxic acute kidney injury in mice. Kidney international. 2009;76(3):277-285.
11. Khor TO, Huang MT, Prawan A, et al. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer prevention research. 2008;1(3):187-191.
12. Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(6):3410-3415.
13. auf dem Keller U, Huber M, Beyer TA, et al. Nrf transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing. Molecular and cellular biology. 2006;26(10):3773-3784.
14. Beyer TA, Xu W, Teupser D, et al. Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated insulin/IGF-1 resistance. The EMBO journal. 2008;27(1):212-223.
15. Hirota A, Kawachi Y, Yamamoto M, Koga T, Hamada K, Otsuka F. Acceleration of UVB-induced photoageing in nrf2 gene-deficient mice. Experimental dermatology. 2011;20(8):664-668.
16. Wruck CJ, Fragoulis A, Gurzynski A, et al. Role of oxidative stress in rheumatoid arthritis: insights from the Nrf2-knockout mice. Annals of the rheumatic diseases. 2011;70(5):844-850.
17. Yoh K, Itoh K, Enomoto A, et al. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. Kidney international. 2001;60(4):1343-1353.
18. Zhao Z, Chen Y, Wang J, et al. Age-related retinopathy in NRF2-deficient mice. PloS one. 2011;6(4):e19456.
19. Uruno A, Furusawa Y, Yagishita Y, et al. The Keap1-Nrf2 system prevents onset of diabetes mellitus. Molecular and cellular biology. 2013;33(15):2996-3010.
20. Braun S, Hanselmann C, Gassmann MG, et al. Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. Molecular and cellular biology. 2002;22(15):5492-5505.
21. Long M, Rojo de la Vega M, Wen Q, et al. An Essential Role of NRF2 in Diabetic Wound Healing. Diabetes. 2016;65(3):780-793.
22. Wattenberg LW, Coccia JB, Lam LK. Inhibitory effects of phenolic compounds on benzo(a)pyrene-induced neoplasia. Cancer research. 1980;40(8 Pt 1):2820-2823.
23. Prochaska HJ, De Long MJ, Talalay P. On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. Proceedings of the National Academy of Sciences of the United States of America. 1985;82(23):8232-8236.
24. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(6):2399-2403.
25. Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer research. 1995;55(2):259-266.
26. Xu C, Huang MT, Shen G, et al. Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer research. 2006;66(16):8293-8296.
27. Cornblatt BS, Ye L, Dinkova-Kostova AT, et al. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. Carcinogenesis. 2007;28(7):1485-1490.
28. Egner PA, Chen JG, Wang JB, et al. Bioavailability of Sulforaphane from two broccoli sprout beverages: results of a short-term, cross-over clinical trial in Qidong, China. Cancer prevention research. 2011;4(3):384-395.
29. Kensler TW, Egner PA, Agyeman AS, et al. Keap1-nrf2 signaling: a target for cancer prevention by sulforaphane. Topics in current chemistry. 2013;329:163-177.
30. Shureiqi I, Baron JA. Curcumin chemoprevention: the long road to clinical translation. Cancer prevention research. 2011;4(3):296-298.
31. Carroll RE, Benya RV, Turgeon DK, et al. Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. Cancer prevention research. 2011;4(3):354-364.
32. Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer research and treatment : official journal of Korean Cancer Association. 2014;46(1):2-18.
33. Wu CC, Hsieh CW, Lai PH, Lin JB, Liu YC, Wung BS. Upregulation of endothelial heme oxygenase-1 expression through the activation of

the JNK pathway by sublethal concentrations of acrolein. Toxicology and applied pharmacology. 2006;214(3):244-252.
34. Wondrak GT, Cabello CM, Villeneuve NF, et al. Cinnamoyl-based Nrf2-activators targeting human skin cell photo-oxidative stress. Free radical biology & medicine. 2008;45(4):385-395.
35. Senger DR, Hoang MV, Kim KH, Li C, Cao S. Anti-inflammatory activity of Barleria lupulina: Identification of active compounds that activate the Nrf2 cell defense pathway, organize cortical actin, reduce stress fibers, and improve cell junctions in microvascular endothelial cells. J Ethnopharmacol. 2016.
36. Bensasson RV, Zoete V, Dinkova-Kostova AT, Talalay P. Two-step mechanism of induction of the gene expression of a prototypic cancer-protective enzyme by diphenols. Chemical research in toxicology. 2008;21(4):805-812.
37. Senger DR, Li, D., Jaminet, S-C., Cao, S. . Activation of the Nrf2 Cell Defense Pathway by Ancient Foods: Disease Prevention by Important Molecules and Microbes Lost from the Modern Western Diet. PloS one. 2016;11(2):e0148042. .
38. Abbott IA, Shimazu C. The geographic origin of the plants most commonly used for medicine by Hawaiians. J Ethnopharmacol. 1985;14(2-3):213-222.
39. Wang MY, West BJ, Jensen CJ, et al. Morinda citrifolia (Noni): a literature review and recent advances in Noni research. Acta pharmacologica Sinica. 2002;23(12):1127-1141.
40. Brown AC. Anticancer activity of Morinda citrifolia (Noni) fruit: a review. Phytotherapy research : PTR. 2012;26(10):1427-1440.
41. McClatchey W. From Polynesian healers to health food stores: changing perspectives of Morinda citrifolia (Rubiaceae). Integrative cancer therapies. 2002;1(2):110-120.
42. Fox RJ, Miller DH, Phillips JT, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. N Engl J Med 2012;367:1087-97.