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Investigation of *Morinda citrifolia* Activity Related Collagen Type II Synthesis Through Gene Expression

Unchaleeporn Ameamsri[1], Runglawan Sudmoon[2], Warin Wonok[1], Sanit Kaewdaungdee[1], Tawatchai Tanee[3], Arunrat Chaveerach[1*]

[1]*Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[2]*Faculty of Law, Khon Kaen University, Khon Kaen 40002, Thailand*
[3]*Faculty of Environment and Resource Studies, Maha Sarakham University, Maha Sarakham 44150, Thailand*

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ABSTRACT

To extend plant benefits, three different concentrations of five *Morinda citrifolia* part was investigated on the collagen type II which is the primary collagen in human cartilage through the expression of the genes, COL2A1, COL-II and COLL2 regions in normal human dermal fibroblasts by qRT-PCR method. The results showed that 1) fibroblasts cultured in the presence of *M. citrifolia* extracts produced many times more collagen type II gene expression than control cells depending on the plant parts and concentrations, 2) the expression levels of the collagen type II gene stimulated by all fruit parts yields the higher percentages than leaves. Next, *Morus alba* leaf extract at a concentration expected to be a precursor protein source for collagen synthesis, working together with *M. citrifolia* stimulation, was added to the selected concentration indicating high expression in the *M. citrifolia* fruit experiments. The result showed various gene expression levels depended on the kind of gene and fruit part. Therefore, *M. citrifolia* fruits can benefit the creation of collagen type II with or without *M. alba*. The *M. citrifolia* fruit can be further benefited in product production for both the elderly and young for maintaining the typical structure and function of the skin, tendon, and bone.

1. Introduction

Collagen is an extracellular matrix protein synthesized mainly by fibroblasts in the body, the main structural protein in the human body that has several functions in the skin, tendon, and bone, playing a connective role in biological structures. There are about 28 types of collagens, and the collagen type I, II, and III are used as supplements with disease treatment, such as with osteoarthritis, wound healing, cosmetics, and establishment in the skin, bones, teeth, cartilage, tendons, ligaments, fascia, cornea and sclera (Wang 2021). Collagen type I in the dermis forms thick bundles of irregular fibers, and is the most important type due to its wide prevalence in almost all connective tissues of the dermis providing strength and elasticity to the skin, while type III forms a fibril network (Drąg-Zalesińska *et al.* 2019). So, collagen degradation and creation are very crucial mechanisms which can cause skin aging and diseases. The major components of the skin are 85-90% collagen type I and 10-15% collagen type III (Wang 2021). However, Rodriguez *et al.* 2018 reported that an excellent option target in the aging process is mainly happens in the dermis where collagens type I and III compose 90% of this skin layer in a distribution of 60-80% type I and 15-20% type II. So, the collagen type I is the most used in cosmetics for skin regeneration, anti-wrinkle, anti-aging and wound healing. Collagen is damaged over time, decreasing collagen density and dermal thickness, as well as decreasing synthesis and replacement of important structural proteins (Wang 2021). These mechanisms combined with other factors such as genetic conditions, environmental factors as smoke, uv radiation, etc. lead to skin aging (Drąg-Zalesińska *et al.* 2019). In addition to collagen type II being a component of the human skin, it is important subject inside the human body where it is the main component of cartilage tissue and has the potential to be used as a treatment for osteoarthritis. The sources of collagens are animals, plants, and recombinant protein production from chicken breast cartilage and feet (Wang

* Corresponding Author
E-mail Address: raccha@kku.ac.th

2021). Collagen type I is mainly found in marine collagen, collagen type II is original from chicken collagen and bovine collagen, a mixture of collagen type I and III can be obtained from porcine collagen and bovine collagen. With various uses, there are many collagen products on the market, such as supplements, healthy food, and cosmetics for the face and body are the most popular forms. However, when taken into the body, collagen can be hydrolyzed/digested to be a group of peptides that is returned to be a unit for a protein synthesis (Drąg-Zalesińska *et al.* 2019; Aguirre-Cruz *et al.* 2020; Wang 2021). When used as face and body topical cosmetics as its important role of collagen in the skin, there is a scientific data to consider as collagens' large molecular weights make it unable to penetrate through the top layers of skin. Typically, molecules of size 500 Daltons or less can effectively cross the skin barrier (Bos and Meinardi 2000). Therefore, adding collagen to a topical product such as face serum, cream, or body lotion is worthless in terms of anti-aging benefits, as the products have never been shown to stimulate collagen synthesis or growth, but only moisturize the skin. Collagen hydrolysates for skin protection have been used instead, is a hydrolyzed collagen, a group of peptides with low molecular weight (3-6 KDa) that can be obtained by enzymatic action in acid or alkaline media at a specific incubation temperature. For cosmetics, it functions as an improvement for controlling of cell proliferation, water-holding capacity, moisture absorption, retention, and anti-aging in skin (León-López *et al.* 2019). According to Aguirre-Cruz *et al.* 2020, they studied hydrolyzed collagen which is widely utilized as an antioxidant due to its excellent biocompatibility, easy biodegradability, and weak antigenicity, and is therefore a safe cosmetic biomaterial with good moisturizing properties on the skin.

Even though collagen and collagen hydrolysates are used orally and dermally, consumers will not be getting the anti-aging benefits of the collagen molecule. Another choice which can be effective and thought to be better as it will not create a collagen shortage in the body: to stimulate the body to create its own collagen. Phytochemicals have long been sources for stimulating collagen synthesis and reducing skin aging. In the past, triterpenes, madecassoside, madecassic and asiatic acid from *Centella asiatica*; ascorbic acid as a co-factor of proline hydrolyase; and the triterpene betulinic acid, a natural derivative of betulin (Drąg-Zalesińska *et al.* 2019), have been used as a collagen synthesis stimulant. Of all of these three materials, it was found that betulinic acid is the strongest stimulant for collagen synthesis (Drąg-Zalesińska *et al.* 2019). Later, in 2013, Matsuda *et al.* reported on the anti-photoaging effects of noni (*M. citrifolia*) revealing a melanogenesis inhibitory effect, and collagen degradation inhibition by its phytochemical containing, 3'-bisdemethylpinoresinol (pinoresinol), α-melanocyte stimulating hormone (α-MSH) inhibitors, and human leucocyte elastase (HLE). Its effect are skin whitening stimulation and wrinkle inhibition. Recently, in 2022, Sudmoon *et al.* reported their research results of *M. citrifolia* activities through collagen type I and type III gene expression in normal human dermal fibroblast. The species contained the main substances α-EG and pinoresinol stimulating the gene expression of collagen type I and type III. Topliss *et al.* 2002 reported on the similarities between natural and synthetic substances in the overall range of properties with regard to efficacy and safety, in terms of their impact on human health. The actions of individual substances are determined by their molecular structures and dose, not whether they are of natural or synthetic origin. However, the users should consider in their differences which may affect human health such as: synthetics are a higher risk of overdose deaths when manufactured and mixed with drugs like heroin or cocaine; they are designed in labs to mimic the effects of natural, slightly altered molecular structures; and the production process on various transformed forms of ingredients and batches. There are various phytochemicals present in plants that may interfere with human metabolic processes which are known and used for a long time, common daily-used substances, like caffeine and vitamins. Conversely, there are many plants and beneficial substances that have not been studied and researched for use in human health, both traditional and modified forms such as for medicine, dietary supplements, cosmetics, medicinal foods, nutraceuticals, etc.

Therefore, the aim of this research is to find the efficacy of collagen type II stimulating fibroblast from *M. citrifolia* components, supported by plant protein sources as the precursor for the collagen type II synthesis.

2. Materials and Methods

Plant materials and extract preparation; Gene expression analysis by qRT-PCR included cell activation, collagen type II gene quantification, and collagen type II gene quantification protocols were followed Sudmoon *et al.* 2022. There is a difference in this study that was conducted on collagen type II with the gene-specific primers below.

Conducted on collagen type II with the gene-specific primers, included GAPDH (Bogaki *et al.* 2017) Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′; GAPDH Reverse: 5′-ACCACCCTGTTGTGTAGCCAA-3′; COL2A1 (Lian *et al.* 2017) Forward: 5′-GGCAATAGCAGGTTCACGTACA-3′; COL2A1 Reverse: 5′-CGATAACAGTCTTGCCCCACTT-3′, COLL2 (Pugliano *et al.* 2017) Forward: 5′-CGTCCAGATGACCTTCCTACG-3′; COLL2 Reverse: 5′-TGAGCAGGGCCTTCTTGAGT-3′; COL-II (Grande *et al.* 2021) Forward: 5′-AGAGCGGAGACTACTGGA-3′; COL-II Reverse: 5′-TCTGGACGTTAGCGGTGT-3′.

3. Results

Investigation of the influence of *M. citrifolia* extract parts on the collagen type II gene expression research quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was succeeded following reports. The three concentration levels started from the first working concentration and its two time of 10-fold dilution of the studied samples were tested (Table 1). The results indicated high various percentages compared to the control (without the plant extracts) in plants effective at stimulation, COL2A1, COLL2 and COL-II collagen type II genes. Graphs of various relative gene expression and the studied plant extract parts with various concentrations are shown in Figure 1 according to the values in Table 1. Two main results were: fibroblasts cultured in the presence of *M. citrifolia* extracts produced many times of more collagen type II gene expression percentages than control cells depending on the plant parts and concentrations, and the expression levels of collagen type II genes stimulated by all fruit parts are higher percentages than leaves. The percentages are following: concentrations of leaves at 0.04 mg/ml and 3.90 mg/ml stimulated 6.15% and 13.74% COL2A1, and 21.86% COL-II gene expression; of unripe fruit pulp at 0.065 and 6.05 mg/ml posted 28.84% in COLL2 gene and 43.00% in COL-II gene, of ripe fruit pulp at 0.10 mg/ml expressed 27.10% in COLL2 and 25.90% in COL-II gene, of raw fruits with seeds at 5.06 and 0.05 mg/ml showed 31.12% in COL2A1 gene and 18.70% in COL-II, and of seeds at 1.04 mg/ml showed 24.25% in COLL2 gene.

The three *M. citrifolia* extract parts which were shown for higher percentage collagen type II gene expression including unripe fruit pulp at concentration of 6.50 mg/ml, raw fruits with seeds at 5.06 mg/ml and seeds at 1.04 mg/ml were selected for experiments adding with *M. alba* leaf extract at a concentration, 3.11 mg/ml expected to be precursor protein source for collagen synthesis, work together with *M. citrifolia* extract stimulant. The result showed various gene expression levels depended on the kind of gene and fruit parts, namely: the unripe fruit pulp showed a high percentage expression at 25.02% with *M. alba* in COL2A1, and the highest percentage expression without *M. alba* at 43.00% in COL-II genes, while there is little difference in the COLL2 gene expression with (4.86%) and without (3.85%) *M. alba.* The raw fruits with seeds showed the highest percentage without *M. alba* at 31.12% in the COL2A1 gene, but the other two studied genes showed higher percentages with *M. alba* at 15.08% and 13.50% than the much lower percentage of 0.08% and 0.74% without *M. alba.*

The seeds showed both higher and lower expression with and without *M. alba*, depending on the gene, including the highest percentage of 24.25% without *M. alba* in COLL2, but higher percentages with *M. alba* at 19.77% and 10.89% in COL2A1 and COL-II genes (Table 2 and Figure 2).

4. Discussion

The study of collagen type II is very essential, even though it seems like a short topic, since its result can potentially profoundly affect human health and be used for both cosmetics and foods in the form of supplements, functional foods and nutraceuticals. As mentioned, collagen serves an important function in body, specifically type II which is a minor component of the skin, and the main component of cartilage tissue, so it can potentially be used to treat osteoarthritis (Wang *et al.* 2021). More when taken into the body, collagen can be hydrolyzed/digested to be a group of peptides that is returned to be a unit for a protein synthesis (Drąg-Zalesińska *et al.* 2019; Aguirre-Cruz *et al.* 2020; Wang 2021). When used as face and body topical cosmetics due to the important role of collagen in the skin, it is important to consider the large molecular weights for both collagen and hydrolysate collagen (a group of peptides that has a molecular size over 500 Daltons), which are unable to penetrate through the top layers of skin, and it only functions to increase cell proliferation, water-holding capacity, moisture absorption, retention, and

Table 1. The summary of the calculation of collagen type II gene expression value by *Morinda citrifolia* extracts, which is derived from Ct number of reference gene from that of the targeted genes study

Samples	Conc. (mg/ml)	Ct value of GAPDH	Human dermal fibroblast cells		
			Gene expression value (%)		
			COL2A1	COLL2	COL-II
Control	-	18.36	2.27	1.07	1.25
Leaves	0.039	27.27	6.15	13.74	11.84
	0.39	25.74	0.80	8.00	0.58
	3.90	26.30	1.27	0.01	21.86
Unripe fruit pulp	0.065	26.10	2.00	28.84	6.34
	0.65	23.35	6.73	2.43	6.70
	6.50	25.10	7.46	4.86	43.00
Ripe fruit pulp	0.103	24.47	5.17	27.10	25.90
	1.03	25.00	1.18	0.01	2.37
	10.3	26.50	5.21	0.26	3.38
Raw fruits with seeds	0.0506	25.80	1.23	13.55	18.70
	0.506	23.52	1.21	1.61	3.93
	5.06	28.50	31.12	0.08	0.74
Seeds	0.0104	23.20	5.86	4.59	3.08
	0.104	21.84	0.69	2.06	0.90
	1.04	26.10	6.77	24.25	1.50

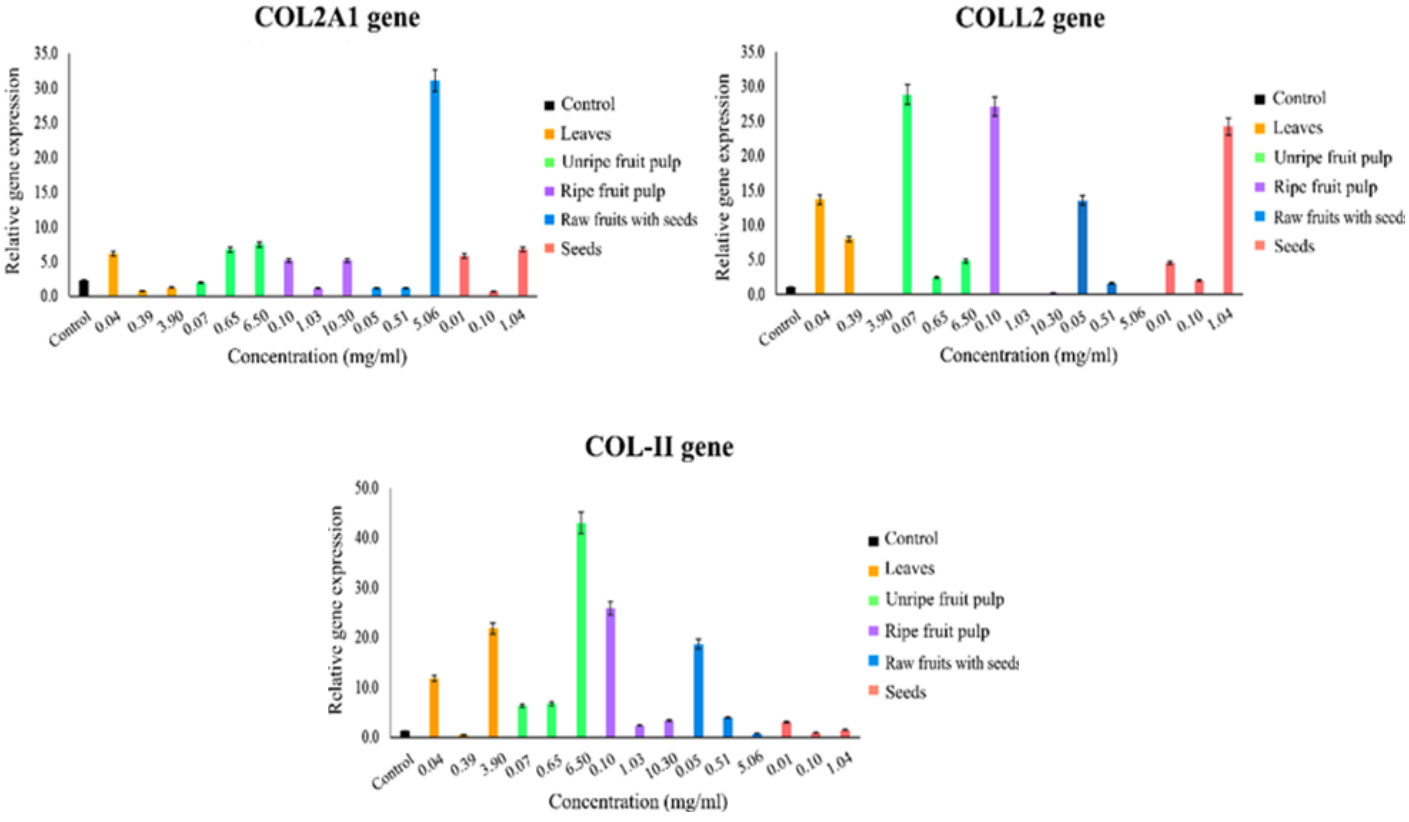

Figure 1. Graph showing the stimulatory effects of *Morinda citrifolia* extracts on collagen type II gene expression using qRT-PCR analysis

Table 2. The comparative value of stimulation gene expression values of *Morinda citrifolia* (Mc) with and without *Morus alba* (Ma) leaf extracts, which is derived from Ct number of reference gene and target gene

Treatment	Conc. (mg/ml)	Ct value of GAPDH	Human dermal fibroblast cells					
			Gene expression value (%)					
			COL2A1		COLL2		COL-II	
			-	+ *Ma*	-	+ *Ma*	-	+ *Ma*
Control	-	18.75	2.27	0.59	1.07	0.72	1.25	0.37
Mc unripe fruit pulp	6.50, 3.11	34.45	7.46	25.02	4.86	3.85	43.00	19.90
Mc raw fruits with seeds	5.06, 3.11	37.19	31.12	28.15	0.08	15.08	0.74	13.50
Mc seeds	1.04, 3.11	34.88	6.77	19.77	24.25	11.43	1.50	10.89

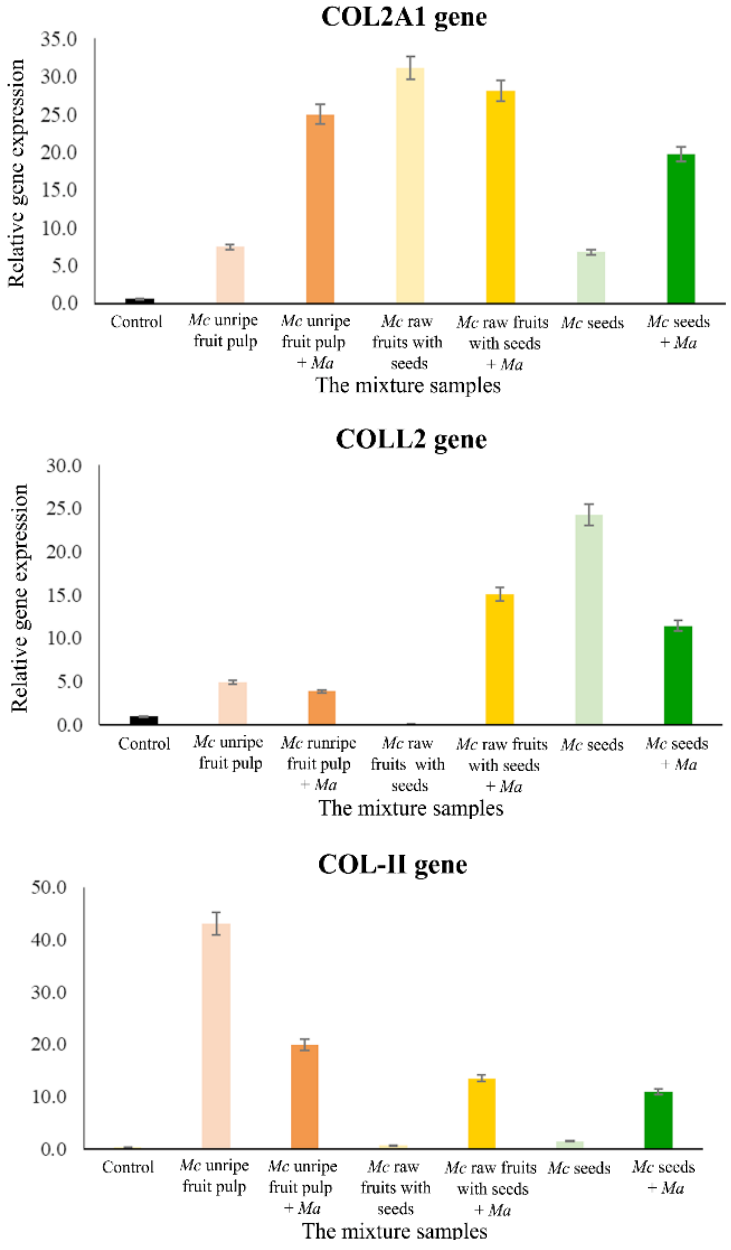

Figure 2. Stimulation gene expression values and the mixture of plant parts with various concentrations of *Morinda citrifolia* (Mc) extracts and *Morus alba* (Ma) leaf extract at 3.11 mg/ml

anti-aging in skin (Bos and Meinardi 2000; León-López *et al.* 2019). These effects lead to the hypothesis that, collagen creation in the body by itself should be the best way to get the collagen to its most effective target sites. Although there are many raw materials from foods to use in collagen synthesis, if there is an additional process stimulant, it should make the process happen efficiently and rapidly. Additionally, a natural antioxidant stimulant should replace the use of synthetic ingredients, due to the natural substances' safety, nutritional and therapeutic values (Aguirre-Cruz *et al.* 2020).

Previously, published research showed that *M. citrifolia* components can stimulate collagen type I and type III synthesis from the fruit more than the leaves stimulated by α-EG and pinorasinol contained in the fruit. These two substances function on collagen stimulation, decrease collagen degradation, inhibit melanogenesis (Masuda *et al.* 2009; Matsuda *et al.* 2013; Sudmoon *et al.* 2022). These research results agreed with the mentioned results, including stimulation of collagen type II, showing a higher percentage of expression from fruit parts than leaves, including unripe fruit pulp: 28.84% of COLL2 gene at 0.065 mg/ml and 43% of COL-II at 6.50 mg/ml, ripe fruit pulp: 27.10% of COLL2 and 25.90% of COL-II at 0.103 mg/ml, raw fruit with seeds: 18.70% of COL-II at 0.0506 mg/ml and 31.12% of COL2A1 at 5.06 mg/ml, and seeds: 24.25% of COLL2 at 1.04 mg/ml. More importantly, the percentages of all collagen type II gene expression are many times higher than the control (Table 1). When *M. alba* at concentration of 3.11 mg/ml, contained 20.48 g total protein/100 g, which is expected to be protein precursor was added in the selected fruit parts, including unripe fruit pulp, raw fruits with seeds, and seeds which were shown to induce a high percentage of gene expression, the result revealed higher and lower expression distributed in all three collagen type II genes studied, COL2A1, COLL2 and COL-II, compared between with and without *M. alba* depending on the kind of gene and fruit part (Table 2). For example, *M. citrifolia* raw fruits with seeds showed the highest percentage of COL2A1 gene without *M. alba* at 32.12%, but without *M. alba* in COLL2 and COL-II genes, the *M. citrifolia* raw fruits with seeds showed lower percentages at 15.08% and 13.50% (Table 2). Additionally, collagen type I and III had higher and lower expression percentages both with or without *M. alba* fruit extracts, and in this regard, *M. citrifolia* parts do not have toxicity on both the cell and DNA level, so it can be used safely (Sudmoon *et al.* 2022). Therefore, *M. citrifolia* fruit extracts are the most effective parts of the plant, which can benefit all type I, II, and III collagen gene expression that are essential for maintaining the normal structure and function of cartilage and skin while having whitening, anti-aging, anti-wrinkle, delaying collagen degradation, collagen creation stimulation effects, both with and without the support of *M. alba* on genes according to these research results and the results reported by Sudmoon *et al.* 2022.

The summary of this research is that the *M. citrifolia* raw fruit and pulp with seeds can be used to benefit collagen type II stimulation which is a key component of the connective tissue of both skin and joints. This research report can be used for the great benefit of mankind. There is an even greater advantage that this positive effect is from a natural substance found in *M. citrifolia* which is a plant that grows all over the world.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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