

Research Article

Anti-Aging Activity and Non-Toxic Dose of Phytooxyresveratrol from *Artocarpus lakoocha* Roxb

Prasit Suwannalert^{1*}, Nasapon Povichit^{2,3}, Pranom
Puchadapirom¹ and Mutita Junking⁴

¹Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, ²Detox (Thailand) Co. Ltd., Chiang Mai 50200, ³Youcando R&D Institute, Youcando Co Ltd., Bangkok 10510, ⁴Division of Medical Molecular Biology, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Abstract

Purpose: To determine the anti-aging activity and toxicity doses of phytooxyresveratrol extracted from *Artocarpus lakoocha* Roxb.

Methods: *Artocarpus lakoocha* 100 g was extracted with 2 ml of 95 % ethanol to obtain phytooxyresveratrol (POV). Total phenolic content, as well as free radical scavenging and anti-glycation activities of POV were characterized in order to assess its anti-aging properties. The models of DNA nicking and bacterial reverse mutation (Ames) were applied to the extract in order to determine its effective and toxic doses, respectively.

Results: Phytooxyresveratrol (POV) exhibited antiaging activity. It also showed high levels of phenolic content, radical scavenging activity and anti-glycation. A POV concentration of 25 µg/ml promoted strong anti-DNA nicking. Furthermore, it was non-toxic at concentrations ranging from 5 - 100 µg/ml.

Conclusion: In vitro, phytooxyresveratrol (POV) extracted from *Artocarpus lakoocha* exerted effective anti-aging activity at a concentration of 25 µg/ml. Thus, the substance should be further studied for possible formulation into pharmaceutical products.

Keywords: Aging, *Artocarpus lakoocha*, Free radicals, Glycation, Phytooxyresveratrol, DNA nicking.

Received: 26 June 2011

Revised accepted: January 17 2012

*Corresponding author: **E-mail:** parasit109@yahoo.com, scpsw@mahidol.ac.th; **Tel:** +66-2-201-5578; **Fax:** +66-2-354-7158

INTRODUCTION

Oxidative damage to nucleic acid, lipid membrane and protein are associated with progressive loss of cellular structure and function [1]. An imbalance of free radical species: reactive oxygen species and reactive nitrogen species are associated with aging and some chronic diseases such as neurodegenerative disorder and cancers [2,3]. Glycation is the reaction that occurs when the reducing portion of monosaccharides, such as glucose or fructose, binds to macromolecules of protein or lipid structure to form advanced glycation end-products (AGEs) [4,5]. AGEs are related to the pathogenesis of skin aging and diabetic complications [4,6]. Phytocompounds play a crucial role in the process of anti-glycation and anti-oxidation, leading to the prevention of cellular aging [7].

Oxyresveratrol (OV) is a hydroxylated analog of resveratrol. It can be used to promote anti-cellular aging [7,8]. *Artocarpus lakoocha* is a plant used in traditional Thai medicine for anti-inflammatory therapy and as well as an anti-skin aging agent [8].

In this study, we investigated the effective anti-aging dose of phytooxyresveratrol (POV) isolated from *Artocarpus lakoocha*. To this end, total phenolic content, free radical scavenging and antiglycation were defined for anti-aging activity.

EXPERIMENTAL

Experimental samples

Oxyresvenox (Sabinsa Co Ltd, Germany) was used as the oxyresveratrol standard. Phytooxyresveratrol was extracted from *A. lakoocha* which was obtained from Detox (Thailand) Co Ltd.

Extraction of POV

The heartwood of *A. lakoocha* was dried at 50 °C and then ground into powder, 100 g of

which was mixed with 2 ml of 95 % ethanol in the dark. The mixture was left for 4 h and then centrifuged at 1500 rpm for 15 min. The dried extract was obtained by drying the supernatant in a rotary evaporator at 50 °C and 50 mmHg. Phytooxyresveratrol was isolated by a high performance liquid chromatography (HPLC) and its structure confirmed with the ¹H and ¹³C-NMR spectroscopic methods.

Total phenolic content

Total phenolic content was assayed using Folin-Denis reagent [9]. The extract, POV (20 µl), was diluted with 780 µl of distilled water and then mixed with 50 µl of fresh Folin-Denis reagent. Thereafter, 150 µl of 7.5 % w/v of sodium carbonate was added and the mixture incubated at room temperature in the dark for 30 min. The spectrophotometric absorbance of the mixture was measured at 765 nm. Gallic acid equivalent (GAE) was used as a standard. Phenolic content of the sample was expressed as mg gallic acid/g sample equivalent based on the calibration standard curve of gallic acid standard.

Evaluation of total scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) method

A working solution of DPPH was freshly prepared with 95 % ethanol with absorbance of 0.95 ± 0.01 at 540 nm, 180 µl of which was mixed with 20 µl of POV. The absorbance of the mixture was immediately measured spectrophotometrically (Shimadzu UV-2550, Japan) at a wavelength of 540 nm. Total antioxidant activity of the sample was expressed as mg Vit C/g sample equivalent based on the calibration curve of standard vitamin C [9].

Ferric reducing antioxidant power (FRAP) method

FRAP reagent was freshly set by mixing a solution of 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution, 2.5 ml of 20 mM ferric chloride hexahydrate (FeCl₃.6H₂O) and

25 ml of acetate buffer (pH 3.6). This method is based on the reduction of ferric complex (Fe³⁺-TPTZ) to the ferrous complex (Fe²⁺-TPTZ) at low pH [10]. The extract was mixed with FRAP reagent in 96 well plates of a multimode detector model of Beckman, DTX 880 and then incubated in dark conditions at room temperature for 15 min. Ferrous sulfate (Fe₂SO₄) was used as reference standard at 595 nm. The FRAP activity was expressed as μmol Fe²⁺/g sample equivalent.

Protein antiglycation

The antiglycation activity of POV extract was investigated as described in a previous study [8]. The extract (50 μl) was added to a solution consisting of 20 mg/ml bovine serum albumin, D-glucose (235 mM) and D-fructose (235 mM) in 200 mM of potassium phosphate buffer (pH 7.4). The mixture was incubated at 60 °C for 30 h. The fluorescence intensity was obtained in a multimode detector and excitation and emission at wavelengths of 370 nm and 440 nm, respectively, were used. Anti-glycation of standard oxyresveratrol (OV) and phytooxyresveratrol (POV) were assessed as the 50 % inhibition concentration (IC₅₀). A solution of aminoguanidine (AG) was used as a reference standard.

DNA nicking assay

DNA nicking was evaluated using supercoiled pUC 18 DNA model [11]. Supercoiled pUC 18 DNA was isolated from an *E. coli* strain, DH 5-alpha. Plasmid DNA 0.5 μg was added to Fenton reagent (consisting of a mixture of 30 mM H₂O₂, 50 μM ascorbic acid and 80 μM FeCl₃) and contained phytooxyresveratrol (either 5, 10, 25 or 50 μg/ml). The mixture was incubated for 30 min at 37 °C and then stained with ethidium bromide on a 1 % agarose gel to obtain the intensity of DNA nicked.

Bacterial reverse mutation (Ames) test

This test was performed using plate incorporation method [12]. The toxicity of oxyresveratrol standard and

phytooxyresveratrol were investigated for their capacity to induce reverse mutation by *Salmonella typhimurium* strains, TA98 and TA100. Doses ranging from 5, 25 - 100 μg/ml of oxyresveratrol and phytooxyresveratrol were determined for their mutagenic toxicity. 4-Nitroquinoline-1-oxide (4NQO, 0.2 μg/ml) was used as positive mutagen control while 100 % DMSO was used as negative control.

Statistical analysis

All results were expressed as mean ± standard error (SE). The difference among groups was obtained by one-way ANOVA using SPSS program, version 13.0. Statistic significance was set at *p* < 0.05.

RESULTS

Total phenolic content and radical scavenging activity of phytooxyresveratrol and OxyResvenox (reference standard) are shown in Table 1. The phenolic contents of phytooxyresveratrol (isolate) and OxyResvenox (reference) were not statistically significant (*p* = 0.671). There was also no significant difference between the scavenging activities of phytooxyresveratrol isolate and OxyResvenox, based on DPPH and FRAP data (*p* = 0.227 and *p* = 0.373, respectively).

Table 1: Total phenolic content and radical scavenging of oxyresveratrol (mean ± SEM)

Test substance	Total phenolic content (mg gallic acid/g sample)	Radical scavenging activity	
		DPPH (mg Vit C/g sample)	FRAP (μmol Fe ²⁺ /g sample)
Phytooxy-resveratrol	12.671 ± 0.552	0.578 ± 0.047	4.003 ± 0.152
OxyResvenox	11.639 ± 0.716	0.569 ± 0.025	4.193 ± 0.093

Phytooxyresveratrol exhibited a high level of antiglycation with IC₅₀ value of 3.343 ± 0.414 μg/ml. Interestingly, phytooxyresveratrol had

stronger antiglycation activity than aminoguanidine, a hydrazine-like compound that blocks the formation of advanced glycation end-products (AGEs) ($p = 0.001$), as shown in Fig 1, but is not statistically different from that of the standard OxyResvenox ($p = 1.000$).

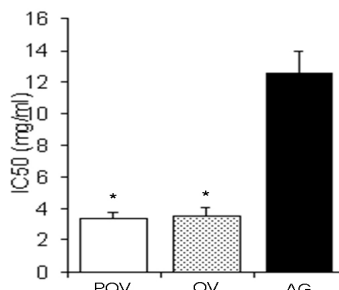


Fig 1: Anti-glycation activity of oxyresveratrol (*statistically significant at $p < 0.001$)

The effect of phytooxyresveratrol in protecting against DNA damage (using the DNA nicking model) is illustrated in Fig 2. Isolated supercoiled pUC 18 DNA of *E.coli* strain DH 5-alpha is shown in lane 1. Nicked DNA treated Fenton's reagent is presented in lane 2 while mixed plasmid DNA, Fenton's reagent (H_2O_2 , ascorbic acid, and $FeCl_3$) and phytooxyresveratrol at concentration of 1, 5, 25 or 50 $\mu\text{g/ml}$ are displayed in lanes 3, 4, 5 and 6, respectively. Lane 7 shows 50 $\mu\text{g/ml}$ of standard gallic acid to supercoiled pUC 18 treated with Fenton's reagent.

The standard gallic acid at 50 $\mu\text{g/ml}$ (lane 7) served as positive control to prevent DNA

damage in the mixed reaction. In this study it was found that the phytooxyresveratrol dose of 25 $\mu\text{g/ml}$ (lane 5) had a beneficial anti-DNA damage activity but at a dose of 50 $\mu\text{g/ml}$ (lane 6), the activity was lower.

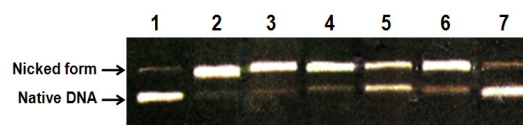


Fig 2: Anti-DNA nicking profile of phytooxyresveratrol (Lane 1 = Supercoiled pUC 18 DNA of *E.coli* strain DH 5-alpha; Lane 2 = The nicked DNA treated with fenton's reagent ; Lanes 3-6 = Supercoiled pUC 18 DNA treated fenton's reagent and phytooxyresveratrol at 1, 5, 25 and 50 $\mu\text{g/ml}$, respectively; Lane 7 = Supercoiled pUC 18 DNA treated fenton's reagent and 50 $\mu\text{g/ml}$ of standard gallic acid)

In the toxicity test, phytooxyresveratrol did not show any mutagenic activity against the test bacterial strains of T98 and T100 at concentrations 5, 25, 50 and 100 $\mu\text{g/ml}$ of the isolate. Table 2 shows that the toxicity profiles of the test isolate and the standard, OxyResvenox, were not significantly different at concentrations of 5, 25 and 50 $\mu\text{g/ml}$. Treatment with 0.2 $\mu\text{g/ml}$ 4NQO, a positive mutagen to T98(S9-) and T100(S9-), had high reverting colony per plate activity compared with control (DMSO), phytooxyresveratrol and reference (OxyResvenox). Thus, phytooxyresveratrol dose of 100 $\mu\text{g/ml}$, like OxyResvenox

Table 2: Toxicity of oxyresveratrol using Ames' test

Treatment	Number of reverting colony/plate (Mean \pm SE)	
	TA98(S9-)	TA100(S9-)
Phytooxyresveratrol (5 $\mu\text{g/ml}$)	21.166 \pm 1.990	114.000 \pm 6.608
OxyResvenox (5 $\mu\text{g/ml}$)	20.333 \pm 1.201	117.000 \pm 6.191
Phytooxyresveratrol (25 $\mu\text{g/ml}$)	20.833 \pm 2.749	106.500 \pm 4.780
OxyResvenox (25 $\mu\text{g/ml}$)	21.000 \pm 0.730	115.333 \pm 3.955
Phytooxyresveratrol (50 $\mu\text{g/ml}$)	22.833 \pm 2.072	119.500 \pm 4.169
OxyResvenox (50 $\mu\text{g/ml}$)	20.166 \pm 1.222	113.333 \pm 4.104
Phytooxyresveratrol (100 $\mu\text{g/ml}$)	21.833 \pm 1.249	115.116 \pm 8.658
OxyResvenox (100 $\mu\text{g/ml}$)	17.333 \pm 0.557*	109.000 \pm 6.233
100 % DMSO	21.333 \pm 1.542	112.833 \pm 4.784
4NQO (0.2 $\mu\text{g/ml}$)	247.500 \pm 18.957	990.833 \pm 18.430

standard, did not show mutagenic activity. OxyResvenox (100 µg/ml) was less toxic than 100 % DMSO to T98(S9-) and T100(S9-) ($p = 0.040$ and $p = 0.564$, respectively).

DISCUSSION

Free radicals and glycation products have important roles in cellular aging development [6,13]. External radicals, especially ultra violet radiation-generating singlet oxygen, are linked to cellular aging [14].

In the present study, phytooxyresveratrol isolated from *A. lakoocha* showed both antioxidant and anti-glycation activities. This supports previous findings on the phytocompound [15,16]. As the results indicate, the high level of radical scavenging and antiglycation activities of the phytooxyresveratrol isolate suggests that it is bioequivalent to the reference standard, OxyResvenox.

The similarity of the activities of both phytooxyresveratrol isolate and the oxyresveratrol standard indicate that the latter is a useful anti-aging agent. An imbalance of antioxidant molecules often leads to the formation of oxidant species [17,18]. The anti-aging activity can thus be attributed to the radical scavenging and anti-glycation properties of the compound.

Although the phytooxyresveratrol isolate demonstrated the capacity to prevent cellular aging and also exhibited good safety profile at the effective dose, there is a need to undertake studies to develop and clinically evaluate pharmaceutical formulations of phytooxyresveratrol derived from *A. lakoocha* in order to determine their suitability for anti-cellular aging applications.

CONCLUSION

Phytooxyresveratrol derived from *A. lakoocha* has the capacity to prevent cellular aging due to its antioxidant and anti-glycation activities.

At a dose of 25 µg/ml, the compound prevented DNA damage and was cellularly non-toxic. However, further studies are required to develop it into suitable pharmaceutical formulations.

ACKNOWLEDGMENT

Phytooxyresveratrol compound was obtained free of charge from the Detox (Thailand) Co Ltd, Chiang Mai, Thailand. This research project was supported by Faculty of Science, Mahidol University, Bangkok, Thailand.

REFERENCES

1. Toyokuni S, Akatsuka S. Pathological investigation of oxidative stress in the post-genomic era. *Pathol Int* 2007; 57(8): 461-473.
2. Bauer ME, Jeckel CM, Luz C. The role of stress factors during aging of the immune system. *Ann NY Acad Sci* 2009; 1153: 139-152.
3. Favier A. Oxidative stress in human diseases. *Ann Pharm Fr* 2006; 64(6):390-396.
4. Gillery P. Oxidative stress and protein glycation in diabetes mellitus. *Ann Biol Clin* 2006; 64(4): 309-314.
5. Kim HY and Kim K. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *J. Agric. Food Chem* 2003; 51(6): 1586-1591.
6. Ashok BT AR. The aging paradox: free radical theory of aging. *Exp Gerontol* 1999; 34(3): 293-303.
7. Pietta PG. Flavonoids as antioxidants. *J Nat Prod* 2000; 63(7): 1035-1042.
8. Povichit N, Phrutivorapongkul A, Suttajit M, Chaiyasut C, Leelapornpisid P. Phenolic content and in vitro inhibitory effects on oxidation and protein glycation of some Thai medicinal plants. *Pak J Pharm Sci* 2010; 23(4): 403-408.
9. Rattanachitthawat S, Suwannalert P, Riangrojpitak S, Chaiyasut C, Pantuwatana S. Phenolic content and antioxidant activities in red unpolished Thai rice prevents oxidative stress in rats. *J Med Plant Res* 2010; 4(9): 796-801.
10. Suwannalert P, Boonsiri P, Khampitak T, Khampitak K, Sriboonlue P, Yongvanit P. The levels of lycopene, alpha-tocopherol and a marker of oxidative stress in healthy northeast Thai elderly. *Asia Pac J Clin Nutr* 2007; 16 Suppl 1: 27-30.
11. Cowan R, Collis CM, Grigg GW. Breakage of double-stranded DNA due to single-stranded nicking. *J Theor Biol* 1987; 127(2): 229-245.
12. Mortelman K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res* 2000; 455: 29-60.

13. Jenkins G. Molecular mechanisms of skin ageing. *Mech Agein Dev* 2002; 123(7): 801-810.
14. Rabe JH, Mamelak AJ, McElgunn PJ, Morison WL and Sauder DN. Photoaging: mechanisms and repair. *J Am Acad Dermatol* 2006; 55(1): 1-19.
15. Conforti F, Menichini F. Phenolic compounds from plants as nitric oxide production inhibitors. *Curr Med Chem* 2011; 18(8): 1137-1145.
16. Suwannalert P, Rattanachitthawat S, Chaiyasut C, Riengrojpitak S. High levels of 25-hydroxyvitamin D₃ [25(OH)D₃] and α-tocopherol prevent oxidative stress in rats that consume Thai brown rice. *J Med Plants Res* 2010; 4(2):120-124.
17. Halliwell B. The wanderings of a free radical. *Free Radic Biol Med* 2009; 46(5): 531-542.
18. Sies H. Total antioxidant capacity: appraisal of a concept. *J Nutr* 2007;137(6): 1493-1495.