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Full Length Research Paper

Antioxidant and *a*-glucosidase inhibitory activity of *Adina rubella* Hance *in vitro*

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Antioxidant activity of extracts from *Adina rubella* Hance (AR) was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid diamonium salt (ABTS) radical scavenge and ferric reducing antioxidant power assay (FRAP) with BHT (2,6-Di-tert-butyl-4-methyiphenol) as positive control, and α-glucosidase inhibitory activity assay of AR extracts with acarbose as positive control *in vitro*. DPPH radical scavenging activity was observed in ethyl acetate extract (AREA) and *n*-butanol extract (ARBU). Their IC_{50} values were 21.63 and 23.16 µg/mL, respectively, slightly weaker than that of BHT (IC_{50} = 18.71 µg/mL). ABTS radical scavenging activity of AREA (IC_{50} = 17.25 µg/mL) was higher than that of petroleum ether extract (ARPE) and ARBU (IC_{50} = 25.73 and 20.64 µg/mL, respectively), but they were weaker than that of BHT (IC_{50} = 7.72 µg/mL). Ferric reducing antioxidant power for ARPE, ARBU and TEAC values were 1649.6 ± 16.09 and 1734.27 ± 68.53 µmol TE/g, respectively, and was higher than that of BHT with TEAC value of 1581.68 ± 97.41 µmol TE/g. AREA (IC_{50} = 862.2 µg/mL) had the best α-glycosidase inhibitory activity, followed by ARBU (IC_{50} = 924.9 µg/mL) and ARPE (IC_{50} = 994.0 µg/mL). Their inhibitory activity was higher than that of acarbose (IC_{50} = 1103.01 µg/mL) as positive control. Results indicated that antioxidant and α-glycosidase inhibitory activity of AREA were better than that ARPE and ARBU *in vitro*.

Key words: Antioxidant activity, α-glucosidase inhibitory activity, *Adina rubella* Hance.

INTRODUCTION

Adina rubella Hance (AR), belonging to Rubiaceae family, is known as Shuiyangmei in Chinese. It belongs to deciduous small shrubs distributed in Guangzhou, Guangxi, Fujian, Jiangsu, Zhejiang, Hunan, Jiangxi and Shanxi province in China and Korea. The whole plant can be used as emmenagogue, and ball-flower can be used for heat-clearing and detoxicating, treating bacillary dysentery and cough with lung heat. Decoction of AR roots can cure infantile convulsion (Editorial Commission of China Flora of Chinese Academy of Sciences, 2005).

Phytochemical research showed that triterpenoids (Fang et al., 1996; Lin et al., 1994), triterpenoid saponins (Fan and He, 1995; 1997), chromone glycosides (Fan and He, 1997), alkaloids (Yuan et al., 2009; Ye et al., 2007a; Lin et al., 1994), and catechins (Ye and Gao, 2007) were the main compounds. Pharmacological investigations showed that AR had the main effects of antibacterial (Bai et al., 2008), inhibiting the generation of nitric oxide (Liao et al., 2005), antiviral (Qiu, 2006), antitumor (Ye et al., 2007b).

At present, natural medicine are reported to have analgesic, anti-inflammatory, antifungal, antiviral, anti-oxidant, antidiabetic, antitumor, antileishmanial, insecticidal and cytotoxic activities (Alam et al., 2012; Asgarpanah and Ramezanloo, 2012; Nasri et al., 2012). Analgesic, antibacterial and anti-inflammatory that connected with the heart, lung and the eye are associated with the accumulation of reactive oxygen and nitrogen species. Antioxidants in blood, cells and tissue fluids play an important role in neutralizing the normal level of oxidative damage caused by free radicals. In an effort to minimize

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effort the impact of environmental pollution on humans, identification of natural product antioxidants has become a realistic and powerful tool in the dietary and natural products industry.

α-Glucosidase inhibitors are oral hypoglycemic drugs that can delay intestinal carbohydrate absorption to achieve the treatment of diabetes, the mechanism of action is through the reversible inhibition. α-Glucosidase was located in small intestinal brush border membrane of epithelial cells; the inhibitors could delay polysaccharide and disaccharides into glucose by inhibiting enzyme activity which could slow the rise of postprandial blood glucose. At present, synthetic α-glucosidase inhibitor such as acarbose and voglibose, had the shortcomings of high prices and side effects. Therefore, to screen α-glucosidase inhibitor, efficiency and safety has a broad market prospect from the natural product (Ali et al., 2006; Ashok et al., 2008). No research has so far been conducted concerning the antioxidant and a-glucosidase inhibitory activity of AR *in vitro*. Therefore in this paper, antioxidant activity of AR using DPPH, ABTS and FRAP methods and α-glucosidase inhibitory activity were investtigated for the first time *in vitro*.

MATERIALS AND METHODS

Plant material

AR were collected from Banping Town, Nanzhao County of Henan Province in China, in July 2011 and identified by Associate Professor Changqin Li (Institute of Chinese Materia Medica, Henan University), and a voucher specimen was deposited in the Institute of Chinese Materia Medica, Henan University (No. 20110704).

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Japan Chemical Industries, Limited, 2,6-Di-tert-butyl-4-methyiphenol (BHT) and 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) from Acros organics, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid diamonium salt (ABTS) from Fluka, α-glucosidase, 4-N-trophenyl-α-D-glucopyranoside (PNPG), acarbose and dimethyl sulfoxide (DMSO) were purchased from the United States Sigma Company.

Preparation of extracts

AR branches and flowers powders (12.1 kg) were extracted three times with methanol at room temperature, every time in 3 days. Filterring and combining extracting solution was done, and then evaporation of solvent *in vacuo* to get total extract. The total extract was suspended in water and extracted with petroleumether, EtOAC (ethyl acetate) and *n*-BuOH (*n*-butanol), respectively. The solution was concentrated under reduced pressure to yield petroleumether extract (ARPE, 1010 g), EtOAC extract (AREA, 1050 g) and *n*-BuOH extract (ARBU, 830 g).

Antioxidant activity using DPPH assay

DPPH radical scavenging activity was carried out as described by Kang et al., (2010) and Çetinkaya et al. (2012). Extracts (ARPE, AREA and ARBU) were made into 2.0 mg/mL as preliminary screening concentration with methanol, and then diluted to 1.0, 0.5, 0.25 and 0.125 mg/mL. 0.1 mL sample solutions were mixed with 3.5 mL DPPH methanol solution (0.06 mmol/L). After 30 min stand at room temperature in dark, the absorbance of the resulting solution and blank (with same chemicals, except for the sample) were recorded against BHT as positive control. The absorbance of DPPH was measured at 515 nm using UV-2000 type Ultraviolet-visible Spectrophotometer. DPPH radical scavenging rate was calculated in the following way:

$$\text{DPPH radical scavenging rate (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$$

Where, A_{blank} is the absorbance of DPPH itself and A_{sample} is the absorbance of the samples on DPPH.

According to the above scavenging rates, IC_{50} value was calculated by origin 6.0 software, which represented the concentration of the extracts (tested samples) that caused 50% reduction of DPPH radical.

Antioxidant activity using ABTS assay

ABTS assay was carried as described by Wei et al., (2012), Kang and Wang (2010) and Bursal and Gülçin (2011). Extracts were made into 2.0 mg/mL as preliminary screening concentration with methanol, and diluted to 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/mL. Sample (0.15 mL) was mixed with 2.85 mL ABTS radical working liquid. After 10 min at room temperature in dark, the absorbance of the resulting solution and blank (with same chemicals, except for the sample) were recorded against BHT as positive control. Absorbance of ABTS was measured at 734 nm using UV-2000 type Ultraviolet-visible Spectrophotometer. ABTS radical scavenging rate was calculated in the following way:

$$\text{ABTS radical scavenging rate (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$$

Where A_{blank} is the absorbance of ABTS itself and A_{sample} is the absorbance of the samples on ABTS.

According to the above scavenging rates, IC_{50} value was calculated by origin 6.0 software which represented the concentration of the extracts (tested samples) that caused 50% reduction of ABTS radial.

FRAP reducing activity assay

The FRAP of the extracts was determined according to the method of Kang et al., (2011) and Gülçin et al. (2011). Extracts were made into a series of concentrations using methanol 2.0, 1.0, 0.5, 0.25 and 0.125 mg/mL. 0.2 mL Samples were mixed with 3.8 mL TPTZ working liquid freshly prepared. After 30 min response period at 37°C, the resulting solution absorbance was recorded against BHT as the positive control. Absorbance was measured at 734 nm using UV-2000 type Ultraviolet-visible Spectrophotometer. At the same time, with Trolox as reference, results were expressed in μmol Trolox equivalents (TEAC) (TE)/g sample.

α-Glucosidase inhibition assay

Making standard curve

According to the reaction system, 1000 μmol/L PNP was prepared in phosphoric acid buffer with pH value of 6.8, and then made into seven different concentrations 400, 300, 200, 150, 100, 50, 25 and

Table 1. Antioxidant activity of different extracts from AR.

Extract	DPPH assay	ABTS assay	FRAP assay
	IC_{50} (μg/mL)		TEAC (μmolTE/g)
ARPE	32.83	25.73	1649.6±16.09
AREA	21.63	17.25	846.8±5.622
ARBU	23.16	20.64	1734.27±68.53
BHT	18.71	7.72	1581.68±97.41

BHT is positive control.

5, 0 μmol/L. Seven different concentrations were taken in 160 μL, respectively, mixed with 80 μL of 0.2 mol/L Na_2CO_3 solution. Resulting solution absorbance was measured at 405 nm using Multiskan MK3 type microplate reader from United States Thermo Electron Company. In the end, a group set of three parallels were used, taking the means of absorbances with absorbance values as the vertical axis and PNP concentrations as abscissa to make the standard curve.

Determination of α-glucosidase activity

According to the reaction system 112 μL of potassium phosphate buffer (pH 6.8), 20 μL of 0.2 U/mL α-glucosidase and 8 μL of DMSO were mixed. After 15 min response period at 37°C in LCH-150 Biochemical Incubator from Shanghai-Heng Technology Company Limited, 20 μL of 2.5 mmol/L PNPG was added to the above reaction system, mixed and continued to react for 15 min at 37°C. Finally after the 80 μL of 0.2 mol/L Na_2CO_3 solution was added, absorbance values were measured at 405 nm. The definition of enzyme activity unit was determined under the conditions of 37°C and pH value of 6.8 hydrolysis of the substrate to produce 1 μmol of nitrophenol per minute that required the amount of enzyme.

Test method

The extracts of AR were dissolved in DMSO and stored at 4°C in a refrigerator. The α-glucosidase inhibitory activity of the extracts was assessed according to the 96 microplate screening method reported by Kang et al., (2011) and Kang and Wang (2010). Absorbance (A) was detected at 405 nm. Enzymatic inhibition data were expressed as IC_{50} values (concentration of inhibitor required for 50% inhibition against α-glucosidase). The inhibitory rates (%) were calculated according to the formula: [1 - (OD_{test} - OD_{blank}) / (control OD_{test} - control OD_{blank})] × 100%. All reactions were carried out with three replications. Acarbose was used as positive control.

Statistical analysis

All data were evaluated with origin 6.0 software. To draw relationship diagram using at least five concentrations and the corresponding inhibition rates, all results were expressed as IC_{50} values for extracts.

RESULTS

Antioxidant activity

Table 1 showed that the DPPH radical scavenging activity of AREA and ARBU (IC_{50} = 21.63 and 23.16 μg/mL, respectively) were similar to BHT (IC_{50} = 18.71 μg/mL) as positive control. In Figure 1, when the concentrations of extracts were lower than 23.77 μg/mL, three extracts showed weaker DPPH radical scavenging activity than that of BHT as positive control. When the concentrations were higher than 23.77 μg/mL, the activity of AREA was higher than that of BHT. With the extracts concentration increasing, ARBU concentration at 34.57 μg/mL showed higher activity than that of BHT. In the experiment concentration range, DPPH radical scavenging activity of the three extracts and BHT had positive dose-effect relationship with concentration.

In Figure 2, the same concentration, ABTS radical scavenging activity of three extracts (ARPE, AREA and ARBU) were lower than that of BHT as positive control, and IC_{50} values were also greater than that of BHT in Table 1. In the experiment for concentration range, when the concentrations of three extracts were 3.125 μg/mL, ABTS radical scavenging rates were low (8.98, 12.05 and 12.3%, respectively). With the increase in the concentration, scavenging rates were increased. When the concentration increased to 50 μg/mL, radical scavenging rates of three extracts increased to 84.26, 99.82 and 99.25%, respectively. It illustrated that ABTS radical scavenging activity of three extracts had positive dose-effect relationship with concentration. When the concentration was higher than 50 μg/mL, there was almost no change in the scavenging rates of AREA and ARBU.

In Table 1, results indicated that Ferric reducing activity of ARPE and ARBU (TEAC values = 1649.6 ± 16.09 and 1734.27 ± 68.53 μmol TE/g, respectively) were stronger than that of BHT (TEAC value = 1581.68 ± 97.41 μmolTE/g), and AREA (TEAC value = 846.8 ± 5.622 μmol TE/g) was weaker than that of BHT; half of BHT.

α-Glucosidase inhibition activity

In Figure 3, α-glucosidase inhibition rates of three extracts of AR were greater than that of acarbose as positive control in the preliminary screening with concentration at 1500 μg/mL. Table 2 showed that AREA (IC_{50} = 862.2 μg/mL) had the best α-glycosidase inhibitory activity, followed by ARBU (IC_{50} = 924.9 μg/mL) and ARPE (IC_{50} = 994.0 μg/mL). Their inhibitory activities were higher than that of acarbose (IC_{50} = 1103.01 μg/mL). Figure 4 showed that α-glucosidase inhibition activity of the three extracts of AR had dose-dependent. When the concentration was lower than 1.5 mg/mL, the inhibition rate of AREA was higher than that of ARPE and ARBU, when the concentration increased to 1.5 mg/mL, AREA was slightly lower than ARPE.

DISCUSSION

It is well known that free radicals are one of the causes of several diseases, such as Parkinson's disease and Alzheimer type dementia (Sacan and Yanardag, 2010).

Table 2. The α-glucosidase inhibitory activity of extracts.

Extract	Preliminary screening		IC_{50} (μg/mL)
	Concentration (μg/mL)	Inhibition rate (%)	
ARPE	1500	80.61	994.0
AREA	1500	80.44	862.2
ARBU	1500	76.07	924.9
Acarbose	1500	57.26	1103.01

Acarbose as positive control. Acarbose is a very strong glucosidase inhibitor; it is strange that its IC_{50} value is so large.

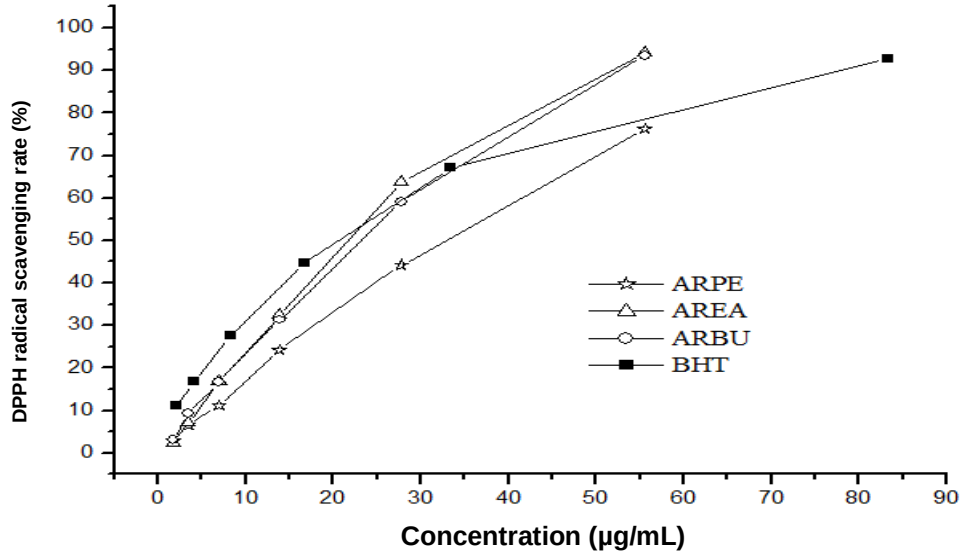

Figure 1. Effect of concentration of extracts on DPPH free radical.

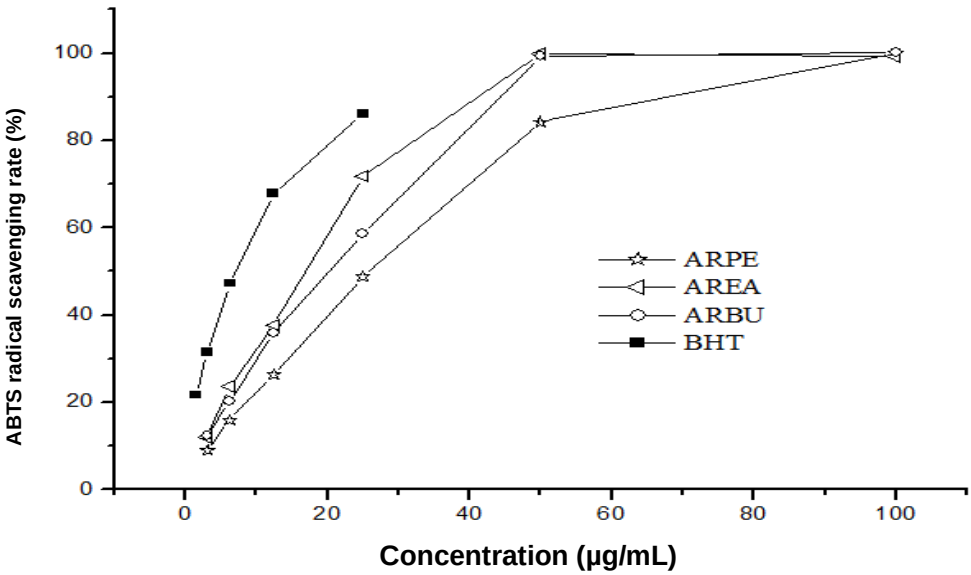

Figure 2. Effect of concentration of extracts on ABTS free radical.

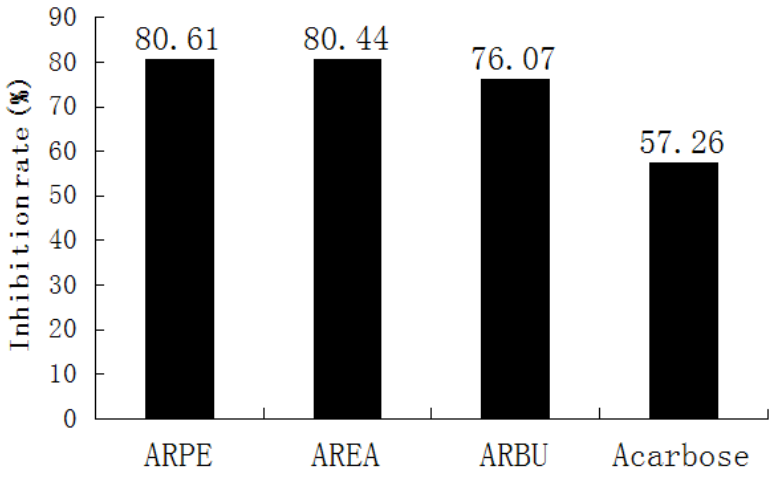

Figure 3. The comparison of α-glucosidase inhibitory activity of extracts.

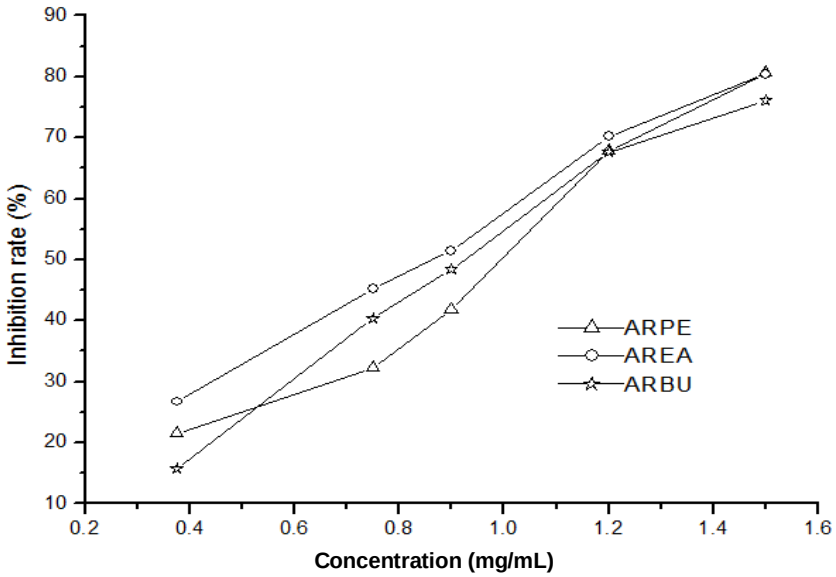

Figure 4. The concentration of extracts effect on inhibitory activity of α-glucosidase.

Therefore, antioxidant activities indicated the potential medicinal value. To assess the antioxidant activities of extracts from plants, a variety of methods based on different mecha-nistic principles which must be used in parallel, because different methods often got different results was used. In this experiment, the antioxidant activity of *A. rubella* was determined by three methods for the first time.

DPPH is a very stable nitrogen-centered radical, if the sample can clear it. This indicates that the tested substance can reduce the effective concentration of hydroxyl radical, alkyl radical and peroxyl radical, and also can interrupt the lipid peroxidation chain reaction (Shi and Yang, 2007). ABTS is a water soluble free radical initiator, can be isoxidized by reactive oxygen to form a stable blue-green radical $ABTS^{\cdot+}$. When analytes contains anti-oxidants, the substances will react with the $ABTS^{\cdot+}$, the reaction system color fades, the maximum absorption in 734 nm can reflect substance antioxidant activity (Zhu and Jiao, 2005). FRAP assay reflects no sample for a certain kind of free radical scavenging activity but samples of the total reducing capacity. Therefore, some scholars deter-mine that this method can be used to reflect total anti-oxidant activity of the sample (Guo et al., 2003). At the same time, there are many antioxidant functional factors that can eliminate reactive oxygen species in the plant. For example, flavonoids and tannins have the effect of antioxidant and scavenging oxygen free radical (Wang et al., 2011). Alkaloid can enhance the antioxidant ability and reduce the antioxidant stress

injury of liver and kidney in rats without toxic effects (Guo et al., 2009). Phytochemical research showed that chromone glycosides, alkaloids and catechins are main compounds in AR. So, these compounds may be the active compounds in AR and the antioxidant activity of ARPE, AREA and ARBU is relative to these chemical compositions.

As a result of α-glucosidase inhibitor having similar chemical structure with monosaccharides or oligosaccharides, it can competitively inhibit the activity of carbohydrate hydrolytic enzyme. Therefore, it can effectively control postprandial blood glucose levels of diabetes and delay the use of insulin, which plays an important role in the process of the treatment of patients with diabetes and health care. In addition, α-glucosidase inhibitor can inhibit the process of protein glycolsylation and lipid glycosylation; it also has anti-cancer, anti-viral and immune stimulating activity (Xu et al., 2005).

α-Glucosidase inhibitors have been listed as the third kind of oral hypoglyceimic agents in medical profession (Du et al., 2005). Voglibose that developed by the Japanese Takeda Pharmaceutical Company and acarbose that developed by Bayer AG, Germany have been widely used for the prevention and treatment of diabetes and its complications. By establishing α-glucosidase inhibitory model *in vitro*, α-glucosidase inhibition activity of the three extracts of AR were slightly higher than that of acarbose as positive control.

Currently, the structure types α-glucosidase inhibitor from plants are diversity, flavonoids and their glycosides, polyphenols, triterpenoid glycosides, alkaloids, peptides, lipids, and acids which had inhibition activity. Triterpenoids, triterpenoid saponins, chromone glycosides, alkaloids and catechins as main compounds in AR may be the active compounds, and this work is carried out in our research group.

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