ORIGINAL ARTICLE

Evaluation of antioxidant potential of *Amalakayas Rasayana*: A polyherbal *Ayurvedic* formulation

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ABSTRACT

Amalakayas Rasayana (AR) is a polyherbal formulation mentioned in Ayurveda to treat aging and age-associated diseases. Being an antiaging drug, AR may have antioxidants and free radical scavenging activity to minimize free radical-induced damage which is a key cause of aging. The methanolic extract of AR was evaluated *in vitro* for total phenolic and tannin content, free radical scavenging activity, superoxide radical scavenging activity, and reducing power. The total phenolic content was measured using Folin-ciocalteu reagent against gallic acid [relative standard deviation (R^2) = 0.998]. Total tannin was estimated using the Stephen method and was found to be 2.82% w/w. Free radical scavenging activity was measured by 2,2-diphenyl-1-picryl hydrazyl assay and R^2 was 1. Superoxide radical scavenging activity was done by ethylene diamine tetra acetate and Nitro Blue Tetrazolium Chloride assays against ascorbic acid and R^2 was 0.976 (EC_{50} = 77.5 µg/ml). Ferrous reducing power was evaluated by Oyaizu method where R^2 was 0.986. All studies showed that AR possesses antioxidant activity. The results of this study suggest that the antioxidant and free radical scavenging activity of AR may explain its rasayana effect and justify its use as a medicine for age associated diseases.

Key words: Antioxidant, anti-radical, *Amalakayas Rasayana,* DPPH, reducing power

INTRODUCTION

It is increasingly being realized that many of today's diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of free radicals.

Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids, and deoxyribonucleic acid in healthy human cells and cause protein and deoxyribonucleic acid damage along with lipid peroxidation. These changes contribute to formation of cancers, atherosclerosis, cardiovascular diseases, many other inflammatory diseases, and aging.[1,2]) All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol, and glutathione.[3] Sometimes, these protective mechanisms are disrupted by various pathological processes. Hence, antioxidant supplements are vital to combat oxidative damage.

Recently, much attention has been directed toward the development of "ethno medicines" that possess strong antioxidant properties and beneficially less toxicity. *Rasayana tantra* is a unique branch of Ayurveda and the drugs mentioned in this chapter have been described to both cure disease and also promote health.[4] In general, *Rasayana* drugs promote healthy longevity, memory and intellect, preserve youthfulness, luster of skin, and clarity of voice, and strengthen the all organs in the body.[5]

AR is a polyherbal compound in which *Phyllanthus emblica* Gaertn is the principal ingredient. The other ingredients are added in smaller proportions. This classical formulation is said to improve health and youthfulness (*Vayasthapana*). To maintain antioxidant pro-oxidant balance, human body is protected by a complex antioxidant system. Antioxidants

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Submission Date: 06-08-10 Accepted Date: 25-05-11

Access this article online	
Quick Response Code:	Website: www.ijaronline.com
	DOI: 10.4103/0974-7788.83186

delay or prevent free radical damage.[6] The antioxidant defense system consists of a network of enzymatic and nonenzymatic as well as endogenous and exogenous factors acting synergistically. Polyphenols, tannins, and flavonoids are plant-based dietary antioxidants that have beneficial effect on health and aging.[7] On this ground, because of being a combination of plant materials known to have health promoting effects, this study was designed to provide scientific basis to the efficacy of AR mentioned in the texts. The objective of this study was to evaluate the antioxidant potential and free radical scavenging activity of methanol extract of AR.

Materials and Methods

Drug preparation

Fine powder of dried fruits of *Phyllanthus emblica* (9 kg) was subjected to *Bhavana* (triturating of material with media)[8] with fresh juice of *P. emblica* —21 times as per textual reference.[9] Then, the ingredients of *vayasthapana kashaya* including *P. emblica (Dhatri)*, *Alpenia galanga Willd (Sweta)*, *Asparagus racemosus Willd (Satavari), Boerhavia diffusa Linn (Punarnava), Centella asciatica Linn and Urban (Mandukaparni), Desmodium gangiticum (Linn) DC (Salaparni), Leptadenia reticulata (Retz) Wt. and Arn. (Jivanti), Pluchea lanceolata C.B and Clarke (Mukta), Terminalia chebula Retz (Abhaya), Tinospora cordifolia (Willd) Miers ex. Hook. F. and Thoms (Amrita)*, and incinerated iron-Fe_2O_3 (*Lauha Bhasma*)[10,11] were taken in equal amounts (900 g each) and a decoction made by adding 36 l of water which was reduced to 9 l. After filtering the decoction, *P. emblica* powder was added, mixed well, and cooked until it became semi-solid. Then, 1.5 kg of *Lauha bhasma* was added and mixed well. Finally, the mixture was sun-dried and powdered. This formulation is known as AR. A sample of this drug was used for this study.

Chemicals

2,2-Diphenyl-1-picryl hydrazyl (DPPH, Lancaster, UK), riboflavin (Loba-India), Nitro Blue Tetrazolium Chloride (NBT, Loba, India), methanol GR (99.8%) (Loba-India), gallic acid (Loba, India), and ethylene diamine tetra acetate (EDTA, Loba, India) were purchased from Krishna Scientific Traders, Rajkot (Gujarat), India. *Folin Ciocalteu's reagent* and sodium carbonate solution were obtained from the pharmaceutical chemistry laboratory of IPGT and RA, Gujarat Ayurveda University, Jamnagar. Double-beam UV spectrophotometer-2201 (Systronics, India) was used for spectrophotometric analysis.

Preparation of methanol extract

As methanol is less polar in comparison to hydro alcohol, to generate selectivity preference to flavonoid type, more methanol extract is selected here; methanol is totally evaporated from the extracted material. Five grams of the powder was extracted with 100 ml of methanol (99.8%) in a conical flask by maceration followed by soaking for about 12 h. Then the extract was filtered and heated in a water bath until methanol is evaporated and dried. The solvent selection is based to give preference to flavonoid type of compound group.

Preliminary phytochemical testing

The extract was qualitatively tested for the presence of different chemical groups of compounds as per standard methods described in text of pharmaceutical chemistry.[12]

Estimation of total phenolic content[13]

The total phenolic content of the extract of AR was estimated according to the method described by Singleton and Rossi.[14] The extract was reacted with *Folin Ciocalteu's reagent* in the presence of 20% sodium carbonate and read at 765 nm after 30 min. Standard of 100 µg/ml was prepared and a standard curve of absorbance versus concentration of gallic acid (50–250 µg) was plotted, and total phenolics were expressed as % equivalent to gallic acid.

Free-radical scavenging activity (DPPH assay)[15]

Free radical scavenging activity was measured by a decrease in absorbance at 516 nm of a solution of colored DPPH in methanol brought about by the methanol extract of AR.[16-18)] A stock solution of DPPH (1.3 mg/ml in methanol) was prepared in such a way that 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of sample extract at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition (EC_{50}—50% inhibition observed at the minimum concentration).

A blank reading was obtained using methanol instead of the extract of AR. Ascorbic acid was used as positive control. Decrease in absorbance is the presence of sample extract, and standard at different concentrations was noted after 30 min. Absorbance was read out at 517 nm using double-beam U.V spectrophotometer-2201.

% Inhibition = $(A_{Blank} - A_{Test})/A_{Blank} \times 100$

Superoxide anion radical scavenging activity

Assay for superoxide radical scavenging activity was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system.[19,20)]

A 0.5 ml of each phosphate buffer, riboflavin, EDTA, NBT, and sodium cyanide solutions was taken in "Blank" (B), "Standard (S)," and "Test (T)" test tubes. A 0.5 ml of each of homogenate solution and distilled water was added to T and B test tubes, respectively, and mixed well.

After noticing initial readings, all the three test tubes (B, S,

and T) were kept under incandescent lamp for 15 min. Then, absorbance was taken at 530 nm in UV spectrophotometer, and percentage of inhibition was calculated by comparing the results of control and test samples (equation). Superoxide radical scavenging activity is expressed in terms of EC_{50}.

% Inhibition = $(A_{Blank} - A_{Test})/A_{Blank} \times 100$

Determination of reducing power

The Fe^{3+}-reducing power of the extract was determined by the Oyaizu of method[21] with a slight modification. A 0.0–0.4 mg/ml of the extract was mixed with 5 ml phosphate buffer (0.2 M, pH 6.6) and 1% potassium hexacyanoferrate, after incubation at 50°C in a water bath for 20 min. The 10% Trichloro acetic acid(TCA) was used to terminate the reaction, and supernatant portion was reacted with 1 ml of 0.1% $FeCl_3$ solution and measured at 700 nm against an appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power.

Quantitative estimation of tannin[22]

Accurately weighed sample of AR in aqueous media was boiled in water bath for 30 min, cooled, filtered, and made up to 250 ml. From this stock solution, 5 ml was diluted to 100 ml using distilled water, 12.5 ml of indigocarmine solution was added as indicator, then titrated against 0.1N $KMnO_4$ solution with constant stirring; color changes from blue, green and to bright yellow (reading A). In reading B, the stock solution, gelatin solution, and acidified sodium chloride solution were taken and kaolin powder was filtered through a filter paper. From this filtered solution 12.5 ml was titrated against 0.1N $KMnO_4$ solution.

$$\% \text{ of Tannin} = \frac{\text{A.B X O.OO42 X 250}}{\text{Wt. of Sample X Vol. taken}} \times 100$$

Results

The qualitative study reported the presence of tannins, tertiary-amines, flavonoids, phenols, cynogenic glycosides, and alkaloids in the extract of AR.[23]

In estimation of total phenolic content, when the concentration is increased, absorbance increased in both gallic acid and methanolic extract of AR. Relative standard deviation (RSD) of AR was superior (0.998) to that of gallic acid (0.966) indicating good linear correlation for AR [Table 1]. In probability theory and statistics, the RSD or (%RSD) is the absolute value of the coefficient of variation (CV). It is often expressed as a percentage. A similar term that is sometimes used is the relative variance, which is the square of the CV. In addition, the relative standard error is a measure of a statistical estimate's reliability obtained by dividing the standard error by the estimate, then multiplied by 100 to be expressed as a percentage. The RSD is widely used in analytical chemistry to express the precision and repeatability of an assay. RSD is a measure of precision (not accuracy). RSD is sometimes called CV and often is calculated as a percentage.

s = standard deviation, x = mean, RSD = s/x, as a percentage, $(s/x) \times 100$

The RSD allows standard deviations of different measurements to be compared more meaningfully. For example, if one is measuring the concentration of two compounds A and B and the result is 0.5 (+/−) 0.4 ng/ml for compound A and 10 (+/−) 2 ng/ml for compound B, one may look at the standard deviation for compound A and say because it is lower (0.4 vs. 2) than for B, the measurement for A was more precise. Actually this is not the case. When the %RSD is used, the new values for compounds A and B are 0.5 (+/−) 80% and 10 (+/−) 20%, respectively; therefore, the measurement for compound B is more precise.

Estimation of total tannin content was found to be 2.82% w/w, indicating AR is a good source of tannin which is a well-known natural antioxidant.

Absorbance (at 765 nm) of gallic acid and methanol extract of AR in different concentrations (µg/ml)was linear for both methanolic extract of AR and ascorbic acid [Table 1, Figure 1].

Free radical scavenging activity on DPPH assay revealed that when the concentration increased percentage inhibition was increased, but there was no good linearity of both methanolic extract of AR and ascorbic acid [Table 2 and Figure 2]. In third-degree polynomial plotting, RSD was 1 for both AR and ascorbic acid [Figures 3a and b]. EC_{50} for both test (AR) and standard were EC_{50} at 10–12 µg/ml with polynomial trend line extrapolated on graph [Table 2 and Figures 2, 3a and b].

EDTA and NBT assays revealed that when concentrations of methanol extract of AR and ascorbic acid increased, percentage inhibitions are also increased indicating good linear correlation. RSD of AR (0.976) found to be superior to that of ascorbic acid (0.925) [Table 3 and Figure 4].

The experiment on reducing power revealed that RSD of AR was 0.986, i.e., linear indicating good correlation between

Table 1: Absorbance comparisons of gallic acid and methanol extract of AR in different concentrations (µg/ml)

Solution	Concentration (µg/ml)	Absorbance at 765 nm
Standard (gallic acid)	25	0.196
	50	0.36
Test (methanol extract of AR)	25	0.14
	50	0.208
	100	0.298
	250	0.601

The range of concentration is 25, 50, 100, and 200 µg/ml as in table.

Table 2: Free radical scavenging activity (DPPH assay) of ascorbic acid and methanol extract of AR

Concentration (µg/ml)	% Inhibition	
	Ascorbic acid	Methanol extract of AR
10	65.54	91.12
20	88.37	—
40	95.98	96.83
60	98.30	97.57
80	—	99.26

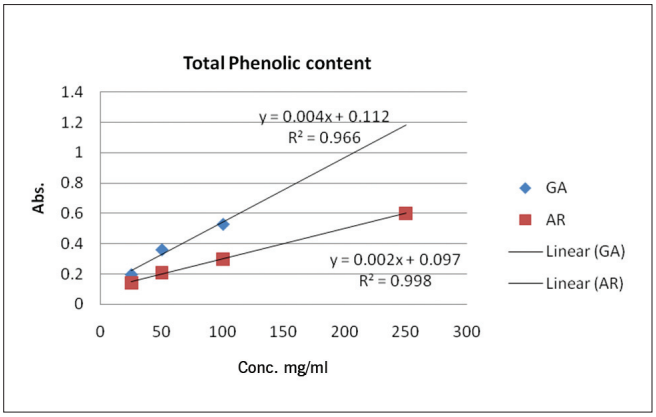

Figure 1: Absorbance (at 765 nm) of gallic acid and methanol extract of AR in different concentrations (µg/ml)

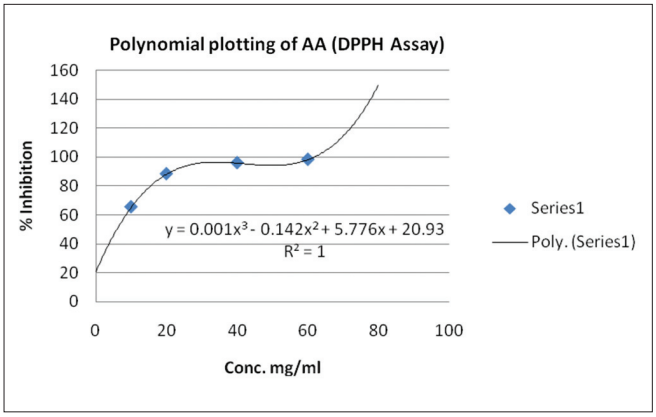

Figure 3a: DPPH assay—polynomial plotting of third degree of Ascorbic acid

concentration of AR and reducing capacity. The ferric reducing/antioxidant power assay was used to measure the total antioxidant power of AR [Figure 5]. Results showed that concentration tested antioxidant power and that the antioxidant capacity was strongly correlated ($r = 0.986$) with the total phenolics content of the AR.

Discussion

In living systems, free radicals are constantly generated and they cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and also accelerated aging. Many synthetic drugs are said to protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines. Recently, many natural antioxidants have been isolated from different plant materials. AR is one among classical drugs which is said to have properties to work against age-related deterioration in the body. AR is a polyherbal preparation composed of *P. emblica, A. galanga,*[24] *A. racemosus,*[25] *B. diffusa,*[26] *C. asciatica,*[27] *D. gangiticum,*[28] *L. reticulata,*[29]

Table 3: Superoxide anion scavenging activity of ascorbic acid and methanol extract of AR

Concentration (µg/ml)	% Inhibition	
	Ascorbic acid	Methanol extract of AR
40	13.25	34.80
60	38.95	45.02
80	52.49	49.45
100	59.11	60.50

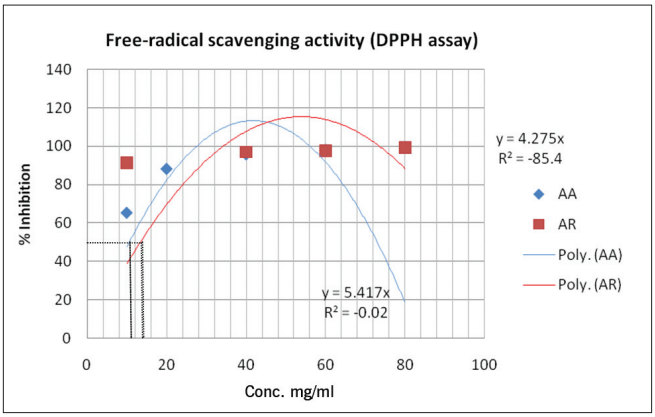

Figure 2: Free radical scavenging activity, % inhibition versus concentration for standard ascorbic acid (AA) and AR (polynomial plotting of second degree)

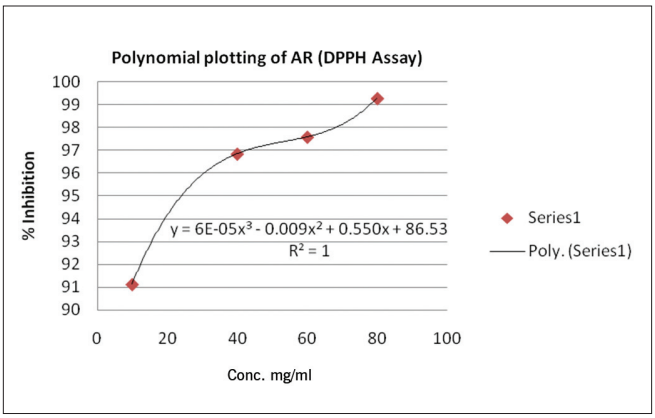

Figure 3b: DPPH assay—polynomial plotting of third degreeof AR

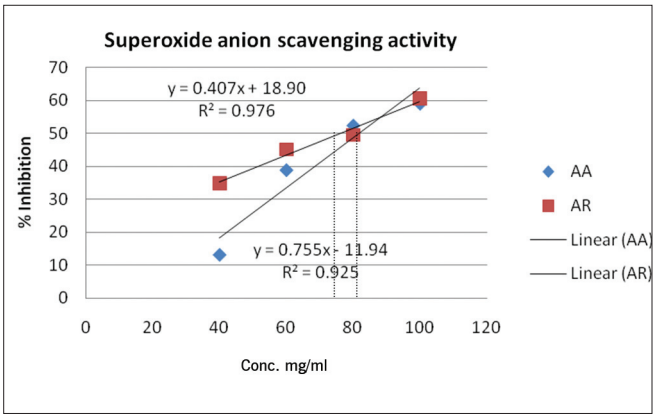

Figure 4: Superoxide radical scavenging activity, % inhibition versus concentration for standard ascorbic acid and test drug AR

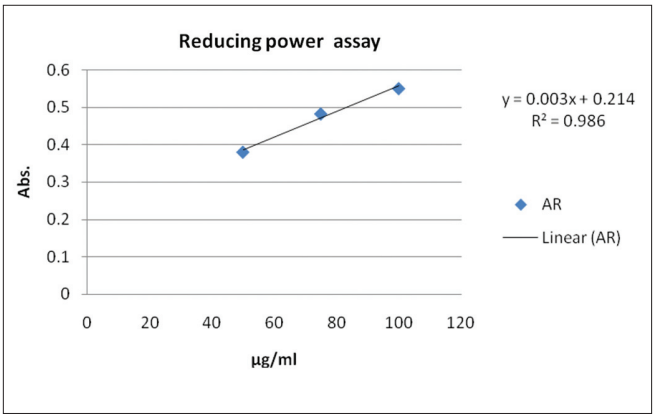

Figure 5: Reducing power of methanol extract of AR

T. chebula,[30] and *T. cordifolia*[31] which are all well known to exert immunomodulator, antioxidant, and free radical scavenging activities, as individually identified plants. When all the ingredients are made into a single formulation, their pharmacodynamic actions may differ from their individual effects. The hypothesis of this study was that all the ingredients may act synergistically to have potent phytochemical combination by which more antioxidant activity could be shown than the individual ingredients had.

Superoxide anion is very harmful to cellular components. The superoxide radical scavenging activity of methanolic extract of AR is increased markedly with increasing concentrations with RSD (R^2) 0.976, in comparison to that of ascorbic acid (RSD = 0.925). The EC_{50} = 77.5 µg/ml observed from graph for AR. The results suggest that methanolic extract of AR showed good superoxide anion scavenging activity in comparison to standard.

Variation in DPPH assay focus on reducing capacity of AR. The reducing capacity of AR shows an RSD 0.986, indicating good correlation between readings against ferrous ion; but when the drug is tested against DPPH molecule, a linear correlation was not possible. The polynomial equation of third degree shows RSD = 1 for both test and standard, and from polynomial equation of second-degree trend line, EC_{50} was extrapolated. Thus, the experiment requires further validation and robustness study for DPPH reduction assay. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity, and free radical scavenging. The reducing power of the extract of AR is found to be remarkable. The results indicate that AR extract contains significant amounts of tannins and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effect on human nutrition and health is considerable. The total phenolic content shows R^2 = 0.998 (RSD) hence, considered good correlation between readings; but the slope values is exactly half of the standard (gallic acid). It shows that the change in absorption is low with respect to concentration change that may be due to matrix effect and responsible for variation in DPPH assay. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.

Conclusions

On the basis of the results obtained in this study, it can be concluded that methanol extract of AR which contained large amounts of phenol compounds exhibits high antioxidant and free radical scavenging activities. It also has reducing power. These *in vitro* assays indicate that this combination of plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of numerous oxidative stresses which trigger at various age-related diseases and in retarding aging and preventing premature aging. However, the components responsible for the antioxidant activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the extract of AR.

Acknowledgments

This study was supported financially by National Centre for Advanced Studies (NCAS) of University Grants Commission of Sri Lanka. The authors acknowledge the NCAS and the Ph.D. scholars Nayan R. Bhalodia and Pankaj B. Nariya of Institute of the *Ayurvedic* Pharmaceutical Studies for extending technical assistance.

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How to cite this article: Samarakoon S, Chandola HM, Shukla VJ. Evaluation of antioxidant potential of Amalakayas Rasayana: A polyherbal Ayurvedic formulation. Int J Ayurveda Res 2011;2:23-8.
Source of Support: National Centre for Advanced Studies (NCAS) of University Grants Commission of Sri Lanka,
Conflict of Interest: None declared.