Free Radical Scavenging and Antibacterial Activities of Amrycard Powder (A Ayurvedic Formulation)

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Citation

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Abstract

Free radicals are implicated for more than eighty diseases including diabetes mellitus, arthritis, cancer, ageing, etc. in treatment of theses diseases; antioxidant therapy has gained an utmost importance. Current research is now directed towards finding naturally occurring antioxidant of herbal drugs. Antioxidant activity of methanol extract of Amrycard powder was evaluated by using Phosphomolybdenum assay, DPPH radical scavenging assay, superoxide radical scavenging assay and ABTS assay. The total phenolic, total tannins, and total flavonoids content were determined. Antibacterial activity was also studied against Bacillus subtilis, Escherichia coli, Streptococcus aureus and staphylococcus by using cup-plate method. Erythromycin was used as standard antibacterial agent. The methanol extract was diluted into different concentration (1,2, 4, 6, 8, 10 mg/l00 µl) with DMSO. The results of the study revealed that, the Amrycard powder exhibited significant antibacterial activity.

INTRODUCTION

Reactive oxygen species (ROS), sometimes called active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O^-), and reactive hydroxyl radicals (OH^-), as well as non-free radical species such as hydrogen peroxide (H_2O_2) [12]. In living organisms various ROSs can form in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco, smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides [345], Free to their deterioration [67]. In addition, reactive oxygen species have been implicated immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer [891011]. When produced in excess, ROSs can cause tissue injury. However, tissue injury can itself cause ROS generation [12]. Nevertheless, all aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damages, and numerous damage removal and repair enzymes to remove or repair damaged molecules [41314 15]. However, this natural antioxidant mechanism can be inefficient, and hence dietary intake of antioxidant compounds is important [111617]. There are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), commonly used in processed foods. However it has been suggested that these compounds have some side effects [1819]. In addition, it has been suggested that there is an inverse relationship between dietary intake of antioxidant rich food and the incidence of human disease [20].

Plant products are being used as source of medicine since long. The medicinal properties of plants have been investigated in recent scientific developments through the world, due to their potent antioxidant activities, no side effects and economic viability [21]. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic, etc. they were also suggested to be a potential iron chelator [2223]. Among natural antioxidants, phenolic antioxidants are in the forefront since all the phenolic classes (simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids) have the structural requirements of free radical scavengers and antioxidants.

Amrycard powder traditional used for the treatment of diabetics, it consists of Tej patra (Cinnamomum Iners), Bilv patra (Aegle marmelos), Gular patra (Ficus racemosa),

Jamun patra (Psidium guyava), Methi beej (Foeniculum vulgare), Giloe (Tinospora cordifolia), and Neem Patra (Azardirachta indica). The ethnomedical claims of Tinospora cordifolia, Cinnamomum iners, Ficus racemosa and Azardirachta indica are antiseptic in urinary tract diseases, eczema, fever, cough, jaundice, laxative, diabetics, and wounds. Foeniculum vulgare, and Aegle marmelos as laxative, astringent, antipyretic and also contain tannins [2425].

MATERIALS AND METHODS

PREPARATION OF AMRYCARD POWDER

Tej patra, Bilv patra, Gular patra, Jamun patra, Methi beej, Giloe, and Neem Patra made were reduced to fine powder and passed through the Sieve no.100 and mixed in geometric proportion and packed in well-closed container.

CHEMICALS AND DRUGS

Folin Ciocalteu's reagent (SD Fine Chemicals, India, glacial acetic acid and sodium dodecy sulphate (SDS). 1.1 diphenyl-2-picryl hydrazyl (DPPH), foline denix reagent, ammonium molybdate, 2.2'-azubibis (3-ethylbenzothiazoline-6-sulfonate) ABTS HIMEDIA, India. trichloroacetic acid, thiobarbituric acid E.Merck India, India. Erythromycin (Ranbaxy lab. India)

TEST MICROORGANISMS

Bacterial strains were obtained from Microbial type culture collection (MTCC) Staphylococcus aureus MTCC 3160, Escherichia coli MTCC 40, Streptococcus MTCC 389 and Bacillus Subtilis MTCC 121, procured from Department of Biotechnology, Nagarjuna College of Engineering and Technology, Bangalore.

PREPARATION OF EXTRACT

Amarycard powder was extracted with methanol by maceration process. The different concentrations (1, 2, 4, 6, 8 and 10 mg/100 µl) were prepared with DMSO for antimicrobial activity.

ESTIMATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of the extract was estimated according to the method described by Singleton and Rossi. From the stock solution (1 mg/ml) of the Amrycard powder extract, suitable quantity was taken into a 25 ml volumetric flask and mixed with 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent. After 5 min, 4 ml of 20% (W/v) sodium carbonate solution was added and volume was made up to 25 ml with double distilled water .The absorbance was recorded at 765 nm, after 30 min. Percentage of total phenolics was calculated from calibration curve of Gallic acid (50-250 µg) plotted by using same procedure and total phenolics were expressed as % Gallic acid [26].

ESTIMATION TOTAL TANNINS

To the 5 ml of Folin Denis reagent (100g of sodium sulphate+20g of phosphomalybdic acid +50 ml of phosphoric acid and 750ml of distilled water was refluxed or boiled for 2 hrs and make up the volume 1000 ml with distilled water) mixed 10 ml of 35% sodium carbonate and add different concentrations of methanolic extract of Amrycard powder. Then make up the volume to 100 ml in volumetric flask with distilled water. Incubate reacting mixture for 30 min at room temperature and absorbance was recorded at 760 nm. Percentage of total tannins was calculated from calibration curve of tannic acid (100-1000 µg) plotted by using same procedure and total tannins were expressed as % tannic acid [26].

ESTIMATION OF TOTAL FLAVONOIDS

In a 10 ml volumetric flask add 4 ml of water 1 ml of methanolic extract of Amrycard powder keep aside for 5 min. then add 3 ml of 5% sodium nitrite and 0.3 ml of 10% aluminum chloride allow the reaction for 6 min. again add 2 ml of 1M sodium hydroxide. Know make up the volume to 10 ml with distilled water and measure the absorbance of pink chromogen at 510 nm. Percentage of total flavonoids was calculated from calibration curve of Quercetin (100-1000 µg) plotted by using same procedure and total flavonoids were expressed as % Quercetin [26].

PHOSPHOMOLYBDENUM ASSAY

The assay is based on the reduction of Mo^{VI} to Mo^{V} by the extracts and subsequent formation of a green phosphate/Mo^{V} complex in acidic pH. Separately extracts and formulations were mixed with 3ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate), incubated at 95°C for 90 min, cooled to room temperature, and absorbance measured at 695nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid (ASC) using standard plot [2627].

ASSAY FOR ANTIRADICAL ACTIVITY WITH DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm in spectrophotometer (Shimadzu model 1601) of methonalic solution of coloured DPPH brought about the sample. A stock solution of DPPH (1.3

ml/ml in methanol) was prepared such that 75 ml of it in 3 ml of methanol gave an initial absorbance of 0.9. This stock solution was used to measure the antiradical activity. For steady state measurements, 100 mm DPPH in methanol was added to extracts and formulation (2-1000mg/ml) in methanol, mixed well and kept in dark for 20 min. decrease in the absorbance in the presence of different concentration of methanolic extract of Amrycard powder was noted. IC_{50} was calculated from % inhibition. Ascorbic acid was used as positive control [28].

ABTS RADICAL SCAVENGING ASSAY

For steady state measurements, 100 mM $ABTS^{\bullet-}$ (prepared by the reaction of 2 mM [$ABTS^{2-}$] was mixed with 0.17 mM potassium persulphate in 20 mM phosphate buffer pH 7.4, kept overnight before use) and mixed with methanolic extract (2-1000 mg/ml) and decrease in absorbance was measured at 734 nm [29].

SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The assay was based on the capacity of the methanol extract of Amrycard powder to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [14]. The reaction mixture contained 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM EDTA, and NBT 0.1 mg/3 ml was added in the sequence. Reaction mixture was initiated by illuminating the reaction mixture with different concentrations (2-1000 µg) of methanol extract of Amrycard powder for 90 sec. immediately after illumination, the absorbance was measured at 590 nm, IC_{50} was calculated. Methanol was used for blank reading. Ascorbic acid was used as positive control.

ANTIBACTERIAL ACTIVITY

The antibacterial activity was evaluated by employing 24 hrs cultures of B. subtilis, E. coli, S. aureus and Staphylococcus, using nutrient agar medium. The bacterial strains were transferred to sterile plates aseptically. The plates were left at room temperature and allowed for solidification. In each plate one well of 6 mm diameter were made using a sterile borer. Accurately 100 µl different dilutions of methanol extract of Amarycard powder (1, 2, 4, 6, 8, 10 mg) and single concentration of erythromycin (5 mg/ml) solutions were transferred to wells aseptically and labeled accordingly. The plates were incubated at 37 ± 1°C for 24 hrs. The diameter of zone of inhibition surrounding each of wells was recorded [3031].

RESULTS

Preliminary phytochemical screening of the Amrycard powder showed the presence of phenolics, flavonoids, tannins, and steroids. Subsequent quantification showed shoed the presence of amount of total phenolics, tannins and total flavonoids were showed in Table 1.

The Phosphomolybdenum assay is based on the reduction of Mo^{VI} to Mo^{V} by antioxidant compounds and a formation of green phosphate/Mo^{V} complex with a maximal absorption at 695 nm and it was efficient to extend its application to plants polyphenols [32]. Total antioxidant capacity of Chitrakadi vati was found to be 3.25 µg.

The methanolic extract was able to reduce the stable radical DPPH to the coloured diphenylpricrylhydrazine. Both extracts and formulation exhibited a concentration-dependent DPPH radical scavenging activity but the formulation showed more potent activity when compared to extracts (Figure 1).Methanolic extract of Amrycard powder was found to have comparable activity to standard ASC (IC_{50}–3.6 µg/ml) in scavenging 100µM ABTS (Figure 2). For kinetic studies, the concentration of ABTS was kept at 100 µM. In the absence of the formulation, the ABTS signal did not show any decay and remained stable. However, in the presence of the methanolic extract of formulation the absorption due to the ABTS decayed completely in 20 seconds. This absorption time plot was fitted to a single exponential function to get observed decay rate constant, which was found to increase with increasing concentration of extract. The superoxide radical activity of the ayurvedic formulation increased with the increase in concentration (Figure 3). The IC_{50} value for formulation was found to be 482µg.

ANTIBACTERIAL ACTIVITY

Antibacterial activity of different concentration of methanol extract of Amrycard powder was measured in terms of Zone of Inhibition. It revealed that significant antibacterial activity was showed against bacterial strains like Escherichia coli, staphylococcus, Bacillus subtilis, and Streptococcus in comparison with standard erythromycin. Amrycard powder showed maximum effect against E.Coli and Streptococcus at small concentrations (Table 2).

DISCUSSION

Polyphenols and flavonoids used for the prevention and cure of various diseases, which is mainly associated with free radicals [33]. Free radical scavenging of phenolic compounds

is an important property underlying their various biological and pharmacological activities. Flavonoids and phenolic compounds possess antioxidant activity. Phenolic compounds are known to be powerful antioxidant agents. Since Amrycard powder contains good amount of total phenolics (1.35µg) it was thought of interest to screen it for its possible antioxidant activity. The antioxidant activity may result from the neutralization of free radical initiating oxidation processes, or from the termination of radical chain reactions. For this reason, the above mentioned methods of antioxidant activity estimation were used. Proton-radical scavenging action is an important attribute of antioxidants, which is measured by DPPH radical scavenging assay. The method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction resulting in a color change from purple to yellow. As seen from the results extract Amrycard powder exhibited a more potent activity. Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself as weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress [$_{1617}$] the capacity of different concentration of extract to scavenge superoxide radical reveals that the extract of formulation possess superoxide dismutase like activity. Although the activity was found to be lower than scavenging activity of ascorbic acid in entire dosage ranges.

The antibacterial activity of Amrycard powder showed significant activity against E. coli and S. aureus at 1 mg/ml concentration. This activity is due to the constituents like Tinospora cordifolia, Cinnamomum iners, Ficus racemosa and Azardirachta indica are antiseptic in urinary tract diseases, eczema, fever, cough, jaundice, laxative, diabetics, and wounds. Foeniculum vulgare, and Aegle marmelos as laxative, astringent, antipyretic and also contain tannins [$_{2425}$].

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Figure 1

Table 1: Preliminary phytochemical studies of Ayurvedic formulation

Sl no	Chemical group	Amrycard powders
01	Phenols	1.35µg
02	Tannins	2.28µg
03	Flavonoids	0.85µg

1000µg of formulation µg of gallic acid, tannic acid and quercetin

Figure 2

Table 2: Antibacterial activity of methanol extract of Amrycard Powder

Microorganisms	Zone of Inhibition of methanol extract in mm						
	1 mg	2 mg	4 mg	6 mg	8 mg	10 mg	Erythromycin 5 µg/100 µl
E. coli	15	18	22	20	20	23	18
Staphylococcus	4	10	15	20	20	21	25
B. subtilis	5	10	17	15	18	17	25
S. aureus	10	12	15	12	15	17	18

Figure 3

Figure 1: DPPH free radical scavenging assay of Amry card powder, ascorbic acid.

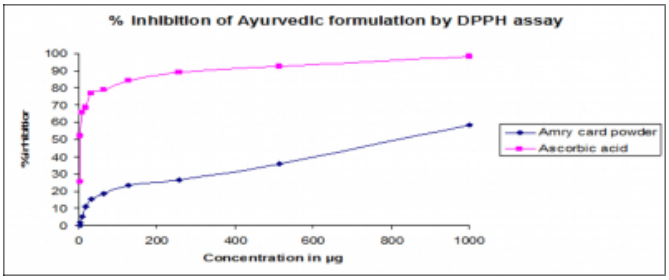


Figure 4

Figure 2: Inhibition of ABTS Radical anion of Amry card powder, ascorbic acid.

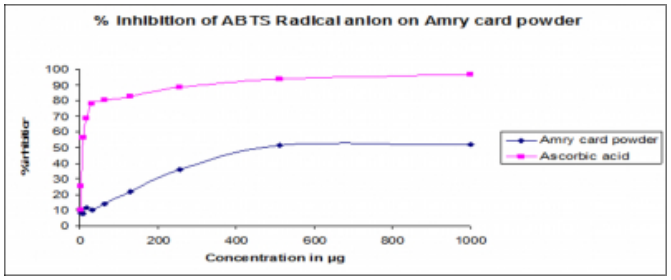
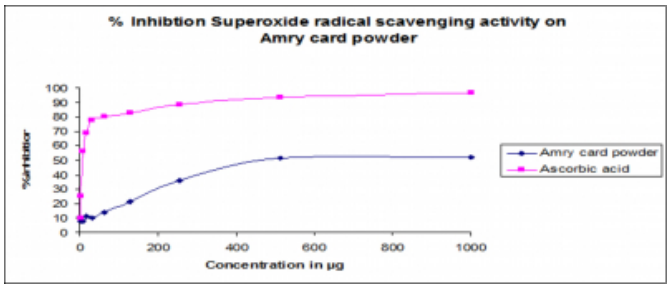


Figure 5

Figure 3: Superoxide radical scavenging activity of Amry card powder, ascorbic acid.



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