Phytochemical Screening and *in vitro* Antioxidant Activity of Jawarish Amla- A Poly Herbal Formulation

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ABSTRACT

The present research work was carried out to study the antioxidant potential of methanolic extract of Jawarish amla using various *in vitro* tests including Reducing power assay, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, DPPH free radical scavenging assay, and hydrogen peroxide method. These various antioxidant activities were compared to standard ascorbic acid. Preliminary phytochemical screening revealed that the extract of jawarish amla possesses flavonoids, steroids and phenolic compounds. In this study, quantitative determinations of flavonoids and phenols were conducted by colorimetric methods, using aluminum chloride method and Folin Ciocalteu reagent respectively. The results indicated that methanolic extract of Jawarish amla possess antioxidant property.

Key words: Antioxidant activity, Ascorbic acid, DPPH, Jawarish Amla

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) are often generated as byproducts of biological reactions or from exogenous factors.[1] These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells [2] including DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease, and Parkinsons disease.[3-5] Recent investigations have shown that the antioxidants with free-radical scavenging properties of plant origins could have great importance as therapeutic agents in aging process and free radical mediated diseases including neuro degeneration.[6,7] Plant extracts[8] and plant products such as flavonoids and other polyphenolic constituents have been reported to be effective radical scavengers and inhibitors of lipid peroxidation.[9, 10] Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant. In view of its wide use and its chemical composition, the methanolic extract of Jawarish amla was evaluated for its *in vitro* anti oxidative activities.

Biological studies of plant extracts have been carried out to verify the pharmacological properties of the plants. The radical scavenging, reducing capacity and metal chelating properties of antioxidants are known to eliminate and prevent the generation of free radical. The properties have been contributing directly or indirectly in the prevention of pathogenesis and deterioration of food,[11, 12] whereas the ability of plant extract to kill or inhibit the growth of microorganisms is at interest for the development of antimicrobial agent. Thus, such studies add value and provide scientific information to continually validate the potential of the plant known as ethnomedicine.[12, 13]

Jawarish amla name is due to its chief ingredient amla (*Emblica officinalis*) belongs to family Euphorbiaceae. It is extensively found all over India, as well as Sri Lanka, Malaysia, China, Pakistan and Bangladesh.

The fresh (or) the dry fruit is used in traditional medicines for the treatment of diarrhoea, jaundice and inflammations.[14] The pulp of the fruit is smeared on the head to dispel headache and dizziness.[15] Amla leaves and fruit have been used for fever and inflammatory treatments by rural

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populations in its growing areas. The earlier study have demonstrated potent anti-microbial,[16] adaptogenic,[17] anti-tumour[18] and anti-ulcerogenic activities.[19] Amla is one of the richest sources of Vitamin C. In addition to this, potent antioxidant, several active tannoid principles (Emblicannin A, Emblicannin B, Punigluconin and Pedunculagin) have been identified which appear to account for its health benefits.[20, 21] Earlier work on this plant showed the occurrence of tannins, lignans, flavonoids and alkaloids.[22-24]

MATERIALS AND METHODS

Chemical and reagents

Folin Ciocalteu reagent, 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH), Nitro blue tetrazolium (NBT), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. Catechin and rutin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade available commercially.

Preparation of extract

The formulation of Jawarish amla was extracted with mixture of methanol by a soxhlet apparatus at 60ºC. The solvent was completely removed by rotary evaporator (Rotavapor® R-210, BUCHI Corporation) and obtained brownish gummy exudates. This crude extract was used for further investigation for antioxidant properties.

Phytochemical screening

The freshly prepared extract of Jawarish amla was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using standard procedures.[25, 26]

Determination of total phenolic content

Total phenols were determined by Folin Ciocalteu reagent.[27] A dilute extract of formulation (0.5 ml of 10mg/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm (Schimadzu UV-Vis 1601). The standard curve was prepared using 25, 50, 100, 150, 200, 250, 300 µg/ml solutions of gallic acid in methanol.

Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination.[28] Formulation extract (0.5 ml of 10mg/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm (Schimadzu UV-Vis 1601). The calibration curve was prepared by preparing rutin solutions at concentrations 10 to 100 µg/ml in methanol.

In vitro Antioxidant Parameter

Reducing power assay

The reducing power of Jawarish amla was determined according to the method described.[29] Different concentrations of Jawarish amla extract (10 µg/ml – 50 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml. 0.1%) and the absorbance was measured at 700 nm (Schimadzu UV-Vis 1601). Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

Superoxide anion scavenging activity assay

The scavenging activity of the Jawarish amla towards superoxide anion radicals was measured by the method.[30] Superoxide anions were generated in a non-enzymatidc phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of phosphate buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations (10 µg/ml – 50 µg/ml) of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm (Schimadzu UV-Vis 1601) was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction.[31] Reaction mixture contained 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2, and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm (Schimadzu UV-Vis 1601) was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method.[32] Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of Jawarish amla extract at various concentrations (10 µg/ml – 50 µg/ml) and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm. The nitric oxide radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

The free radical scavenging capacity of the extracts was determined using DPPH.[33] DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic extract of Jawarish amla, was mixed with 95% methanol to prepare the stock solution (1 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes then Jawarish amla extract was added followed by serial dilutions (10 µg/ml to 50 µg/ml) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Schimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard and dissolved in double distilled water to make the stock solution with the same concentration (1 mg/ml) followed by serial dilutions (10 µg/ml to 50 µg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging of Hydrogen Peroxide

The ability of Jawarish amla to scavenge hydrogen peroxide was determined according to the method.[34] A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV-Vis 1601). Jawarish amla extract (5 µg/ml – 25 µg/ml) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 2 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 20 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of Jawarish amla and standard compounds was calculated by using the above equation.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening of the extract of Jawarish amla revealed the presence of various bioactive components of which flavonoids and tannins were the most prominent and the result of phytochemical test has been summarized in the Table I.

Total phenolic content

Phenolic compounds are known to be powerful chain breaking antioxidants and are important constituents of plants. Phenolic compounds may contribute directly to antioxidative action. It is suggested that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1.0 gm daily from a diet rich in fruits and vegetables. The total phenolic content of the jawarish amla extract measured by Folin-Ciocalteu

Table 1: Phytochemical screening of methanolic extract of Jawarish Amla

Phytochemical screening of methanolic extract of Jawarish Amla	
Alkaloid	+
Sterol	+
Carbohydrate	+
Phenolic compound	+
Flavonoid	+
Proteins and amino acids	+
Lipid/fat	-
Mucilage	+
Resin	+

Table 2: Total phenolic and flavonoid content of Jawarish Amla extract

Total phenolic content (µg/mL)	Mean± SEM	Total flavonoid content (µg/mL)	Mean ± SEM
102.83		132.25	
98.16	103.33 ± 3.139	135.87	135.83 ± 2.055
109.00		139.37	

reagent in terms of gallic acid equivalent jawarish amla extract was 103.33±3.139µg/mL (Table 2).

Total flavonoid content

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process.[35, 36] Therefore, in the present study, total flavonoid content present in extract was estimated using Aluminum chloride colorimetric method. In Jawarish amla extract, the flavonoid content was found to be 135.83±2.055µg/ml (Table 2).

In vitro Antioxidant Activity

Reducing power assay

Figure 1 shows the reducing power of the jawarish amla extract and ascorbic acid, as a function of their concentrations. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers causes the reduction of the Fe 3+/ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration. The reducing properties are generally associated with the presence of reductones,[37] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[34]

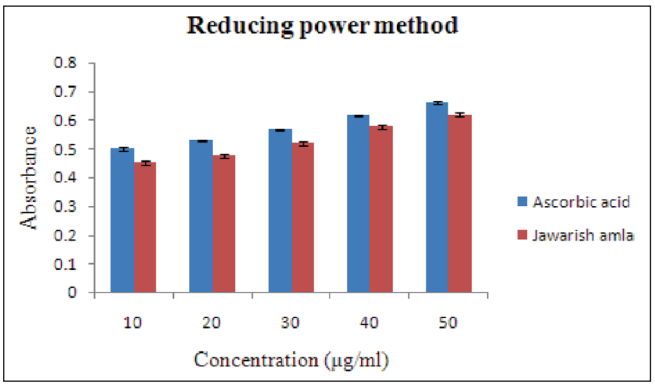

Figure 1: Reducing power assay

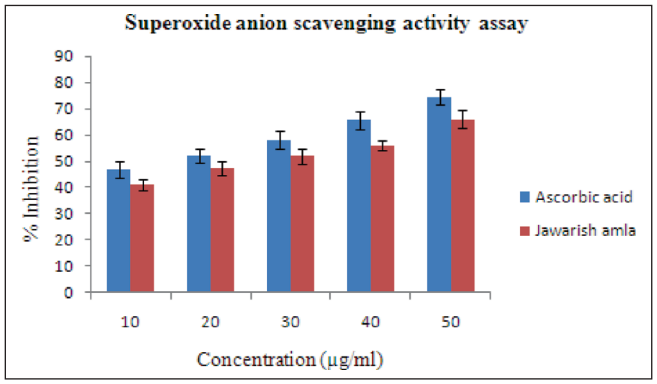

Figure 2: Superoxide anion scavenging activity assay

Superoxide anion scavenging activity assay

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation.[38]The superoxide anion radical scavenging activity of Jawarish amla extract assayed by the PMS-NADH system was shown in figure 2. The superoxide scavenging activity of Jawarish amla extract was increased markedly with the increase in concentrations. Thus, higher inhibitory effects of the rhizomes extracts on superoxide anion formation noted herein possibly renders them as a promising antioxidants. The half inhibition concentration (IC_{50}) of Jawarish amla extract was 45.94µg/ml while IC_{50} value for ascorbic acid was 25.83µg/ml. These results suggested that Jawarish amla extract has a potent superoxide radical scavenging effects.

Hydroxyl radical scavenging activity assay

Activity of the rhizomes extract on hydroxyl radical has been shown in figure 3. Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various

hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule. Jawarish amla extract exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC_{50} value of extract was found to be 6.59µg/ml while IC_{50} value for ascorbic acid was 1.27µg/ml.

Nitric oxide scavenging activity assay

The jawarish amla extract showed a moderate nitric oxide-scavenging activity between 10 and 50 µg/ml in a dosedependent manner (IC_{50} = 22.00 µg/ml) (Figure 4). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions.[39] The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. The extract showed a moderate nitric oxide-scavenging activity. The % inhibition was increased with increasing concentration of the extract. The IC_{50} value of ascorbic acid was found to be 3.90µg/ml.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

The jawarish amla extract showed a concentration-dependent antioxidant activity by inhibiting DPPH radical with an IC_{50} value of 11.07µg/ml (Figure 5). The IC_{50} value of ascorbic acid was found to be 7.79µg/ml, which was used as standard. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine. It has been found that ascorbic acid, reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability.[40] It appears that the jawarish amla extract possesses hydrogen donating capabilities and acts as an antioxidant. The scavenging effect increased with increasing concentration of the extract.

Scavenging of Hydrogen Peroxide

As shown in figure 6, Jawarish amla extract also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 6.05µg/ml while IC_{50} value for ascorbic acid was 2.60µg/ml. Hydrogen peroxide

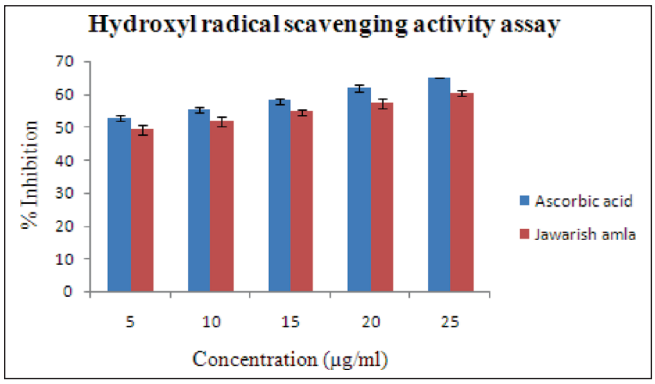

Figure 3: Hydroxyl radical scavenging activity assay

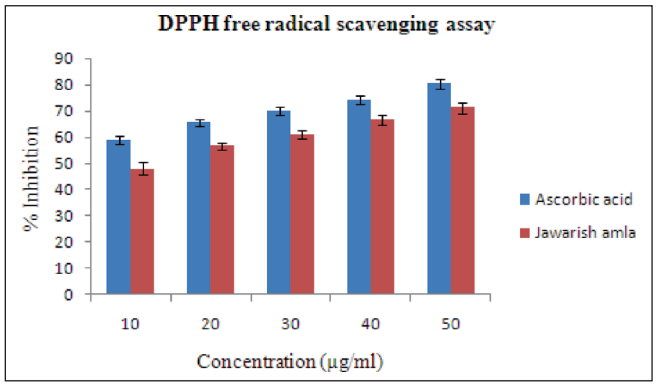

Figure 5: Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

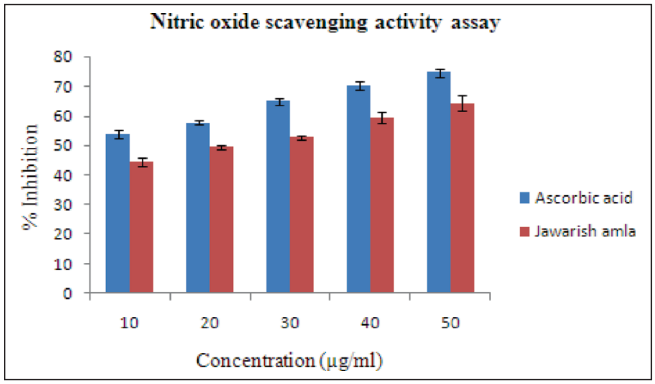

Figure 4: Nitric oxide scavenging activity assay

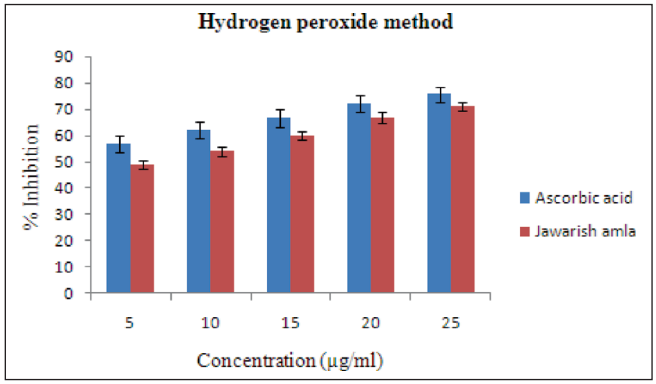

Figure 6: Scavenging of Hydrogen Peroxide

is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.[41] It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by Jawarish amla extract may at least partly result from its antioxidant and free radical scavenging activity.

CONCLUSION

For the purpose of characterizing antioxidant activity of plant extracts, it is desirable to subject it to a battery of tests that evaluates the range of activities such as Reducing power assay, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, DPPH free radical scavenging assay, and hydrogen peroxide method in formulation of jawarish amla. The *in vitro* antioxidant activities of the methanolic extract indicated the efficacy of the formulation as a source of natural antioxidants which will have application towards reducing lipid peroxidation /oxidative stress with consequent health benefits. The activity of jawarish amla might be due to the presence of amla which is a well known plant antioxidant.[39] Further research work is required for isolation and characterization of phytoconstituents responsible for its antioxidant property.

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