Research Article

In Vitro Evaluation of Antioxidant, Antiproliferative and Cytotoxic Properties of Methanol Extract of *Emblica officinalis* Leaves

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ABSTRACT

Oxidative stress is the cause of a number of disorders such as cancer, diabetes and heart problems. Abnormally high levels of reactive oxygen species and the simultaneous decline of antioxidant defense mechanisms lead to the cell and tissue damage. Current pharmacological regimens do not completely normalize cancer cells. Research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. The medicinal value of these plants is related in their phytochemical components which produce definite physiological actions on human body. Hence the present research was conducted to evaluate the compound with antioxidant, antiproliferative & cytotoxic properties. The plant leaves were prepared in methanol solvent using standard procedures. The antioxidant, antiproliferative activity and cytotoxicity evaluations were done by the recommended procedures with some modifications. In the DPPH radical scavenging test the IC50 values ranged from 7.21 to 91.79 µg/mL. Protective effects of the absolute methanol extract may be attributed due to the significantly high content of phenolics and flavonoids. The cytotoxicity assay resulted that the methanolic plant extract has minimum cytotoxicity when tested on brine shrimp. The present analysis will help in establishing its role as a safe chemotherapeutic drug of natural origin however further compound isolation is necessary to confirm the activities of individual compound.

Keywords: Cancer, oxidative stress, antioxidant, chemotherapeutic.

INTRODUCTION

Plants used in traditional medicine are revalued for their therapeutic principles in several laboratories all over the world. Experimental results are suggestive that free radicals and reactive oxygen species are linked with the causation and deteriorating effects of diseases. Reactive oxygen species (ROS) are formed constantly in living organisms as metabolic byproducts or as a result of many different environmental influences. Oxidative DNA damage may be formed through a direct insult on nucleotides in the helix or through the incorporation of damaged nucleoside triphosphate during replication, which leads to the faulty translation of genetic code, a critical event in carcinogenic transformation.[1,2]

Despite all the advances in medical sciences, cancer, a disease as old as humankind, is globally a major health problem. Recent reports from the International Agency for Cancer Research indicate that in 2008, approximately 12.7 million new cancer cases and 7.6 million cancer deaths occurred and of these, 56% of all new cancer cases and 63% of cancer deaths were in the less developed regions of the world.[3] Projections are that by 2020, the incidence of cancer will increase three-fold and that there will be a disproportionate rise in cancer cases and deaths from the developing countries that have limited resources to tackle the problem.[4]

Conventionally, when localized, cancer may be treated with either surgery (if operable), or with ionizing radiation (when inoperable), or by combining both these modalities. However, in the advanced stage, and more importantly, when metastasis is observed, the use of cytotoxic chemotherapeutic agents is obligatory.[5] Unfortunately, the use of chemotherapy and ionizing radiation is associated with deleterious side effects as their cytotoxic effects are unbiased, and in association with neoplastic cells it can also affect normal tissues.[5,6] In addition, the treatment of cancer and its complications is very expensive, and to patients in developing countries, where general health care in itself is beyond the reach of most people, the cost is exorbitant and unaffordable.[7]

In the light of these observations, in recent years the popularity of complementary medicine has increased. Over 50% of all modern clinical drugs are natural product origin and they play an important role in drug development programs of the pharmaceutical industry.[8]

Emblica officinalis Gaertn (*Phyllanthus emblica*, Linn Family Euphorbiaceae) is a medicinal plant described in Ayurveda, which is the oldest medicinal system in the world and the World Health Organization has approved its efficacy.[9] Experimental studies conducted with fruit extracts indicated that they have significant protective effect against a number of disorders.[10]

The present study was aimed to investigate the *E. officinalis* methanolic leaves extract antioxidant, antiproliferative & cytotoxic activities.

MATERIALS AND METHODS

Fresh leaves of *Emblica officinalis* L. for this study were collected from the local area and were authenticated by the institute's botanist. The collected leaves (20g) were dried at room temperature in the shade and away from

direct sunlight for 5d and were kept in hot air oven for 2 days.

Preparation of crude extract

After drying, the leaves were coarsely powdered and extracted by dissolving with methanol for 7 d. The sediments were filtered and the filtrates were dried at 40°C in a water bath. The solvent was completely removed by filtering with Whatmann filter paper and obtained dried crude extract was used for experiment. The supernatant of extract was subjected to various phytochemical tests to determine the activity of constituents present in the crude extracts.[11-14]

Antioxidant Activity

Free radical scavenging activity of the methanol extract of *E. officinalis* leaves, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Braca *et al.*[15] Crude extract (0.1 mL) was added to 3 mL of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated by using the equation:

$$\% \text{ scavenging activity} = [(A_0-A_1)/A_0] \text{ X } 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Lower the absorbance, higher is the free radical scavenging activity. The curves were prepared and the IC_{50} value was calculated using linear regression analysis. Ascorbic acid 10 mg/ml was taken as standard solution.

Cytotoxicity screening

The brine shrimp lethality bioassay was used to determine the cytotoxic compounds using simple zoological organism *Artemia salina* as a convenient monitor for the screening. The eggs of the brine shrimp were hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using the method of Meyer *et al.*, 1982[16] which also concurred with the method used by Hossain *et al.*[17] The test sample was prepared by dissolving them in methanol (not more than 50 µL in 5 mL solution) with sea water (3.8% NaCl in water) to attain concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL. A vial containing 50 µL methanol diluted to 5 mL was used as a negative control. Standard vincristine sulphate was used as positive control. The matured nauplii were applied to each of all experimental vials and control vial. After 24 h, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial was counted. The percent (%) of mortality of the brine shrimp was calculated for each concentration using the formula:

$$\% \text{ Mortality} = N_t/N_0 \text{ X } 100$$

where, N_t=Number of killed nauplii after 24 h of incubation, N_0=Number of total nauplii transferred, i.e. 20. The LC_{50} (median lethal concentration) was then determined using Probit analysis.

Antiproliferative analysis

The extracts (1% w/v, in methanol) of *E. officinalis* leaves were subjected to antiproliferative analysis. For this study *Allium cepa* L. root tip cells were used. Onions were placed with aerated water at room temperature to root for 24 h. Methanol was used as control. The control group was considered as time zero (0-h) until the first root sample was obtained. This root sample was then placed for 24h in extract solution of *E. officinalis* leaves. After this time period a few root tips were removed and the bulbs were returned to water, for further 24h to observe if there was recovery from possible damage. The treated roots were fixed and stained by acetocarmine and mounted on permanent slides. The slides were analyzed under microscope with 40X objective lens. Cells were examined for morphological and structural alterations and the mitotic index and cytolytic index were determined.[18]

Statistical Analysis

All the experiments were conducted in triplicate and statistical analysis of the data was performed by analysis of variance, using the STATISTICA 5.5 (Stat Soft Inc, Tulsa, OK, USA) software. A probability value of difference $p \leq 0.05$ was considered to denote a statistical significance. All data were presented as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

The free radicals are the culprit for a large number of diseases. Free radical damage within cells has been linked to a range of disorders including cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes. There has been some evidence to suggest that free radicals and some reactive nitrogen species trigger and increase cell death mechanisms within the body such as apoptosis and in extreme cases necrosis.[19]

Ayurveda, the traditional Indian system of medicine, is one of the oldest systems of medicine and is practised in the Indian subcontinent. Emphasis in Ayurveda is on disease prevention and promotion of good health by adopting a proper lifestyle and following therapeutic measures, which will rejuvenate the body.[20] The Ayurvedic remedies, which are both preventive and therapeutic, are mostly made of plants and when compared with their synthetic counterparts are either nontoxic or less toxic. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. In this regard, the naturally occurring antioxidants present in the diet and beverages consumed by humans are receiving increased attention.

Antioxidants are helpful in reducing and preventing damage from free radical reactions because of their

ability to donate electrons which neutralize the radical without forming another. Ascorbic acid, for example, can lose an electron to a free radical and remain stable itself by passing its unstable electron around the antioxidant molecule. The antioxidant chemicals found in many foods are frequently cited as the basis of claims for the benefits of a high intake of vegetables and fruits in the diet. In a 2010 survey of 1000 plants, 356 had clinical trials published evaluating their "pharmacological activities and therapeutic applications" available in the Western market.[21]

Preclinical studies have conclusively shown that *E. officinalis* (amla) ameliorates the oxidative and xenobiotic-induced stress, mutagenesis, and carcinogenesis by increasing the antioxidant enzymes. Reports suggest that amla increases the antioxidant enzymes and prevents benzo[a]pyrene, cyclophosphamide, DMBA, Y-radiation, hexachlorocyclohexane, and ethanol-induced toxic effects.[22]

The medicinal effects of plants are often attributed to the antioxidant activity of the phytochemical constituents, mostly the phenolics. Plants having significant medicinal values have often been found to be rich in phenolics and to have high antioxidant potentials.[23] The antioxidant activity of phenolics is due to their redox property which allows them to act as reducing agents, metal chelators and free radical quenchers.[24] The secondary metabolites such as phenolics and flavonoids from plants have been reported to be potent free radical scavangers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark.[25] Natural phenolic exert beneficial effects mainly through their antioxidant activity. These compounds are capable of decreasing oxygen concentration, intercepting singlet oxygen, preventing first chain initiation by scavenging initial radical, such as hydroxyl radical, chelating metal ion catalyst, decomposing primarily product of oxidation to non radical species and breaking chains to prevent continued hydrogen abstraction from substance.[26] The phytochemical screening indicates qualitative presence of alkaloid, glycosides, saponins and flavonoids. The antioxidant effect by absolute methanol extract could be due to the higher concentration of phenolics and flavonoids[11].

DPPH radical scavenging activity of *E. officinalis* L. was found to increase with increasing concentration of the extract. This assay was based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The results and methods of analysis of antioxidant activities concurred with other studies.[27-29]

The cytotoxicity of the crude extract of *E. officinalis* L. leaves to brine shrimp was determined on *Artemia salina* after 24 h of exposure the samples, the negative control and vincristine sulphate. This technique was applied for the determination of general toxic property of the plant extract. The LC_{50} value of the extract was 102.37µg/mL and that for standard vincristine sulphate was 8. 50µg/mL. No mortality was found in the control group, using methanol and sea water.

An assessment of cytotoxic and mutagenic potential is necessary to ensure antiproliferative property. The methanol extract showed low mitotic index of 0.42% whereas cytolytic index was found to be 77%. Angayarkanni *et al.*[30] have reported that infusion prepared from Amorphophallus tuber extracts showed mitotic index of 0.34% whereas cytolytic index was found to be 80% in ethanol. While Teixeira *et al.*[31] have reported that ethanolic extracts of *Psidium guajava* and *Achillea millefolium* showed mitotic index of 1.1% and no activity for *Achillea millefolium* which is comparatively less than the present investigation.

Together, these observations clearly suggest that the presence of phytocompounds like phenols & flavonoids in significant quantity resulted in the desired effects of amla leaves.

CONCLUSION

During the development of a drug the cytotoxicity should be taken into consideration. The experiment shows that the methanol extract of leaves of *E. officinalis* has considerable antioxidant and antiproliferative activities with minimum cytotoxicity.

The phytochemical screening showed the presence of glycosides, alkaloids, flavonoids and saponins. These compounds show these activities because the biological activities of plants may be due to the presence of these diverse groups of chemical compounds. However this study was conducted by crude extract, further advanced studies should be carried out for compound isolation and it is necessary to observe which compounds are actually responsible for specific effects.

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