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Research Article

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *PHYLLANTHUS EMBLICA* FOR ITS APPLICATION IN TREATMENT OF OPHTHALMIC DISORDERS

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ABSTRACT

The oxidation stress is known to be a reason for various tissue degeneration and plays a role in premature aging. In wake of this, there is need to explore a natural antioxidant which can help body maintain a balance between inbuilt oxidation stress and detoxifying reactions. We present the antioxidant power of various extracts of *Phyllanthus emblica* which was tested by FRAP method. The phytochemical analysis of the extracts was checked by HPLC. Along with the standard compound, gallic acid, the peaks were analyzed. The peaks showed the compound present was mainly, Gallic acid. The antioxidant activity was found to be maximum in the order of Ethyl Acetate extract followed by Acetone, Methanol and Aqueous extract. Along with antioxidant activity the extracts were tested as an anti-microbial agent for the pathogens known to cause various eye infections. The minimal inhibitory concentration was calculated and was found to be 1mg/ml for *S.mutans, S.aureus* and *S.pyogenes* and 0.6mg/ml for *S.pneumoniae. Phyllanthus emblica* is a powerful antioxidant along with its activity to inhibit the growth of the pathogens makes us believe that it can be used as new drug compound for preparation of eye drops and also in various therapeutic.

Keywords: Phyllanthus emblica, HPLC, Antioxidant, Eye infection, Broad-spectrum antibiotic, MIC

INTRODUCTION

Oxidative Stress (OS) is a generally used to describe the sturdy state of oxidative damage in a cell or tissue and can also effect the normal function of organs which is caused by the *reactive oxygen species* (ROS) [1]. This oxidative stress affects either a specific molecule or the organism as a whole. Reactive oxygen species represent a class of molecules that are derived from the metabolism of oxygen and are inherently found in all aerobic organisms in form of free radicals and peroxides [2]. Energy-derived metabolic activity or endogenous source are the primary source of production of such free radicals such as those involving detoxification reactions like liver cytochrome P-450 enzyme system or the energy generation from mitochondria. Enzyme system or the energy generation from mitochondria [3]. Beside these endogenous source the environmental factors that play role in ROS generation includes exposure to cigarette smoke, emission from automobiles and industries, consumption of alcohol in excess, and bacterial, viral or fungal infection [4]. The exogenous factors beside the environmental pollutants are vitamins A, C, E, carotenoids etc [5]. Oxidative stress is resultant of an imbalance between production of the reactive oxygen and a body's ability to naturally detoxify the reactive intermediates. Resultant of this imbalance oxidation stress is involved in most of the major disorders like cardiovascular diseases, arthrosclerosis and Parkinson's and in the minor imbalance results in premature ageing [6]. In serious diseases like, Duchenne Muscular Dystrophy (DMD), progressive muscle cell necrosis occurs due to loss of dystrophin and its demonstrated in mice that muscle cells, under oxidative stress are severely damaged which is generally protected by Stra13 and in absences of Stra13 muscle necrosis progress leading to symptoms of DMD in mice [7].

The level of damage due to oxidative stress is determined by the balance between induced oxidative damage versus the rate at which it is capably repaired and removed by the natural system [8]. Damage can also be determined by the rate of reactive oxygen generated and how rapidly the endogenous agents inactivate ROS like antioxidants and the level of repair enzymes present in the human body in turn decides this. There is involvement of the individual hereditary factors that are unique for each human and these can act as one of determinant factors in fighting the oxidation stress built up in the body. Besides these factors the lifestyle and environment of the each individual can also play a key role in the fighting ability of the body against the oxidation stress at any given point [9].

Present day clinical treatments are having a setback because of the ever-evolving nature of microorganisms, which have developed resistance to them, and hence treatment of infectious diseases is problematic scenario [10]. The infections caused by bacteria, viruses and fungus can also lead to the built up of the reactive oxygen species in the body [11]. Oxidative damage is majorly observed in the eyes particularly affecting the retina and the lens, is an also known contributing factor to age-related ocular degeneration and cataract [12]. The present study in detail describes the extraction procedure, phytochemical analysis, antioxidant power and screening of the antimicrobial activity of *Phyllanthus emblica* against pathogens especially known to cause various eye infections.

MATERIAL AND METHODS

Materials

The plants material (seeds) was collected from the local market in Mumbai. Mueller-Hinton agar was purchased from Hi-Media, Mumbai, Bacterial Inoculum was procured from IMTECH chandigarh, India. Chemicals used for HPLC analysis were of HPLC grade. The microorganisms used in this study are *Streptococcus mutans (S.mutans), Streptococcus pyogenes (S.pyogenes), Streptococcus pneumoniae (S.pneumoniae), Staphylococcus aureus (S.aureus)* and *Pseudomonas aeruginosa (P.aeruginosa)*. These microorganisms were procured from the IMTECH, Chandigarh, INDIA.

Preparation of plant extracts

Dried fruits were powdered in the grinder and stored in the airtight container in the dark until further use. 25g of the powder of seeds of *P.emblica* were taken in a thimble and the extracts were prepared in the series of 500ml of different solvents based on increasing polarity (Ethyl acetate, Acetone, Methanol, and Water) using Soxhlet extraction method. The solvents were evaporated to dryness using rotary evaporator and the extracts were lyophilized at −50°C. The lyophilized extracts powder was stored in airtight bottles at 4°C till further experimentation [13].

High Performance Liquid Chromotagraphy (HPLC)

HPLC analysis was carried out using C18 PCX 500 Dionex analytical column, (WATER 2414 refractive index detector and 515 HPLC pump) with 0.1 M KCl, 0.05 M HCl and 10% acetonitrile as the mobile phase. The detection was carried out at 260nm using UV detector. Peak areas were identified, and gallic acid was used as an external standard [14].

Antioxidant Power by FRAP Method

The Ferric-Reducing Antioxidant Power (FRAP) assay measures the antioxidant potentials of "antioxidants" to reduce the Fe3+/ 2,4,6-tripyidyl-s-triazine (TPTZ) complex present in a stoichiometric excess to the blue coloured Fe2+ form. [15]. The stock solution of various extracts of concentration 0.3mg/ml was prepared. The tubes were incubated at 37ºC was 15minutes. The absorbance was read at 593nm in spectrophotometer, which was set zero with blank (FRAP reagent and distilled water).

The experiment was repeated in triplicates and represented as mean value average + S.D.

Table 1: Gives the composition of the test solutions for FRAP

Extract Concentration (M)	FRAP Working Solution (ml)	Water (ml)
$6x10^{-4}$	1.5	3.49
$12x10^{-4}$	1.5	3.48
$18x10^{-4}$	1.5	3.47
$24x10^{-4}$	1.5	3.46
$30x10^{-4}$	1.5	3.45
$36x10^{-4}$	1.5	3.44
$42x10^{-4}$	1.5	3.43
$48x10^{-4}$	1.5	3.42
$54x10^{-4}$	1.5	3.41
$60x10^{-4}$	1.5	3.40

Determination of antimicrobial activity

For antibacterial activity study, Ethyl acetate, Acetone, Methanol, and aqueous extracts were dissolved in DMSO to a final concentration of 10mg/100µl.

Susceptibility test by well diffusion method

The inoculum size of the test strain was standardized according to the National Committee for Clinical Laboratory Standards [16]. The test bacterial strains were inoculated into Mueller Hinton Broth medium and incubated for 3-6 hours at 37°C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland units. Susceptibility tests were performed using modified agar-well diffusion method. Inhibition Zone was observed after 24hrs and Zone of inhibition was recorded. Inhibition Zone Diameter (IZD) was measured to the nearest millimeter (mm). In this experimental set up DMSO was a negative control. The tests were performed in triplicates for the microorganism evaluated and the final results were presented as the Mean zone of Inhibition and Standard deviations were calculated.

Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MICs) of the extracts of *P.embellica*, was determined against the tested bacteria by macro broth dilution assay method [16]. Two-fold serial dilutions of all the extracts (based on their IZD results), DMSO as negative and Gentamicin as positive control were prepared in well plates with Mueller-Hinton Broth as diluent. The plates were incubated at 37ºC for 24hrs. The least concentration of the extract or standard drug showing no visible growth was taken as the MIC. The results were tabulated after 24hrs of incubation period and its mean MIC value was calculated.

RESULTS AND DISCUSSION

Phytochemical analysis of Plant Extract using HPLC

Plants are essential source of potent compounds for the development of new drug moieties. The major step for this is examining the properties of these plants for being an antibacterial and antioxidant agent [17]. Various groups have evaluated the antibacterial, antifungal, antiviral, and anti-inflammatory properties of plants [18, 19 & 20]. Some of these findings have helped in identifying an active principle compounds that are responsible for such activities and in the developing drugs for the therapeutic use .The HPLC analysis of the various extracts was carried out to identify the phytochemicals, responsible for its antioxidant and antimicrobial activity. Gallic acid was identified as the major compound in Ethyl acetate, Acetone extracts. Where as, in Methanol though Gallic acid was detected to be a major compound, but the presence of other types of compounds has led to enhance the antimicrobial activity though showing variation in a antioxidant property of the extract. In aqueous extract the amount percentage of gallic acid is very less. The HPLC analysis of various extracts shows various peaks with retention times of 6.584, 6.579, 6.667, and 6.447, which corresponded to the retention time of Gallic acid chosen as a standard compound. Gallic acid is a well-known component of natural herbal product with high potency as an antioxidant and antimicrobial in nature. The presence of tannins and flavonoids in all the *P. emblica* extract is likely to be the reason for the free radical scavenging effects that was observed. Flavonoid and tannin is the major phenolic compound present in the plants and are known to act as primary antioxidants or free radical scavengers agents.

Antioxidant activity of *Phyllanthus emblica*

Free radicals are believed to be involved in major disorders like neurodegenerative diseases, muscular dystropin, AIDS etc. Antioxidants agents through their scavenging power are valuable in the management of many such diseases. The Ferric-Reducing Antioxidant Power (FRAP) assay measures the antioxidant potentials of "antioxidants" to reduce the Fe3+/ 2,4,6-tripyidyl-s-triazine (TPTZ) complex present in a stoichiometric excess to the blue coloured Fe2+ form. The free radical scavenging activity in the different plant extracts decreased in the following order: Ethyl acetate > *Acetone* > *Methanol* > *Aqueous*. All the extracts of *P.emblica* at different concentrations exhibited more than 70% scavenging activity (Fig 1-4). This study suggests that the plant extract retained its antioxidant activities during the course of extraction and can counteract the oxidative damage induced by the pathogens responsible for various ophthalmic disorders. These extracts can be designed as drug moiety in treatment of severe disorders.

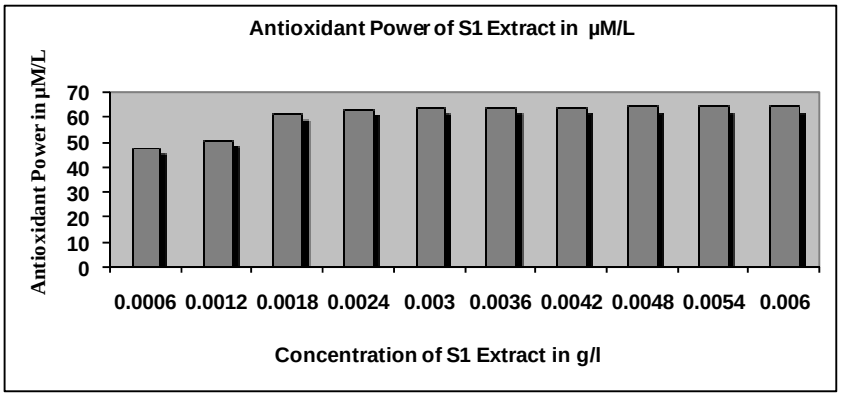

Fig. 1: Represents the antioxidant power (µM/l) of ethyl acetate extract of *P.emblica*

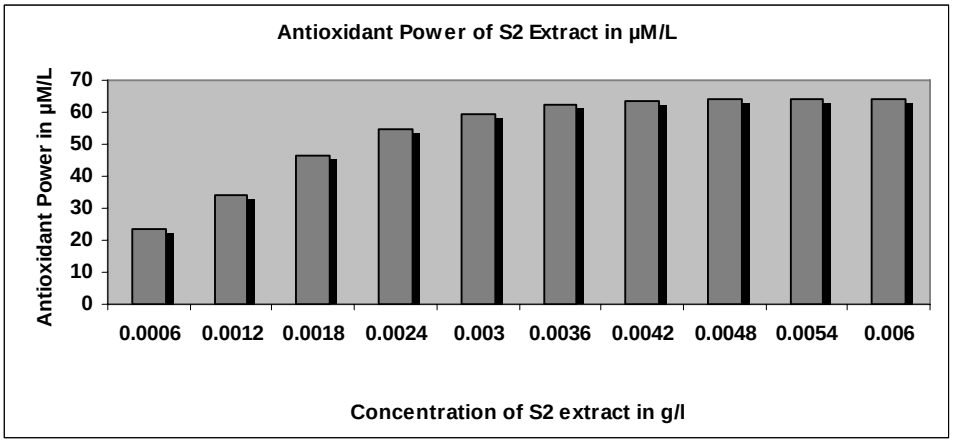

Fig. 2: Represents the antioxidant power (µM/l) of acetone extract of *P.emblica* in

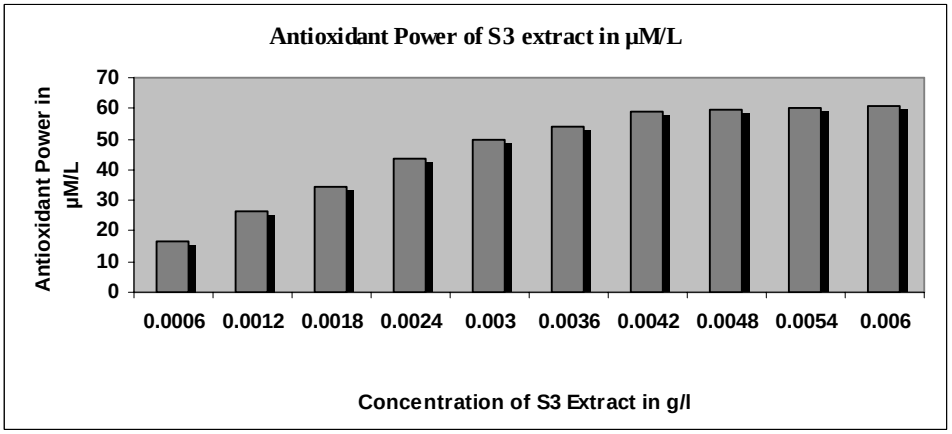

Fig. 3: Represents the antioxidant power (µM/l) of methanol extract of *P.emblica*

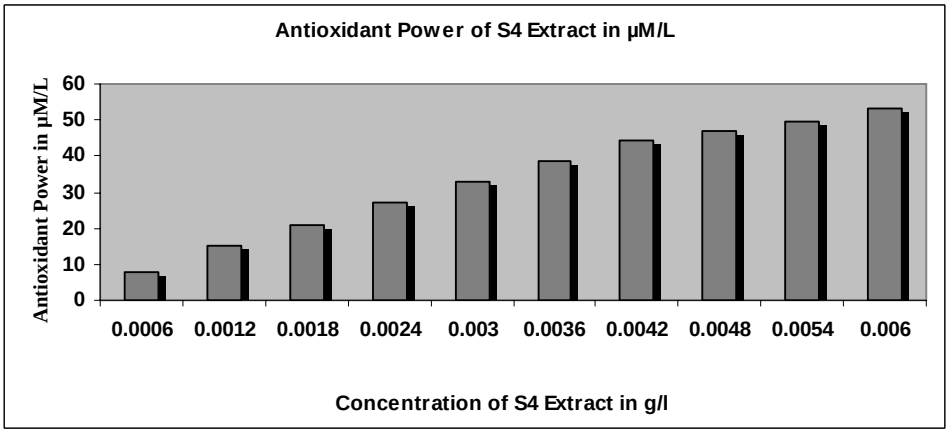

Fig. 4: Represents the antioxidant power (µM/l) of aqueous extract of *P.emblica*

Susceptibility of microbes to *P.emblica*

The microbes chosen in this study were found to be involved in different ophthalmological infections. The results (Table 2 and 3) show all the extract of *P.emblica* having strong inhibiting power on the growth of microorganism. The aqueous extract was found to less effective when compared to other extracts since the average inhibition zone diameter was found to be ranging from 5.33mm to 8mm which was lesser than other three extracts which showed the IZD value to be ranging from 6.67mm to 12mm for various pathogens in study. The extraction procedure does not seem to have any affect in antimicrobial activity.

The crude and extracted form was both found to have high antimicrobial activity. The zone of inhibition ranging more than

12mm was considered to be sensitive and minimal inhibitory concentration was found to be 1mg/ml for all the microbes except for the ethyl acetate extract for *S.pneumoniae*, which had the MIC value to be 0.63mg/ml.

Table 2: Represents the antimicrobial activity of *Phyllanthus emblica*

Microorganisms	Zone of Inhibition (mm)			
	Ethyl Acetate	Acetone	Methanol	Aqueous
Streptococcus mutans	7±0.82	6.67±0.47	7.67±2.1	5.33±0.47
Streptococcus pneumoniae	9.67±0.47	10.67±1.0	6.67±1.3	7±0.82
Streptococcus pyogenes	-	-	8.77±2.1	-
Staphylococcus aureus	10.67±0.47	12	10.33±1.7	8±0.82
Pseudomonas aeruginosa	-	-	-	-

Table 3: Represents the MIC of *Phyllanthus emblica*

Microorganisms	Minimal Inhibitory Concentration (mg/ml)			
	Ethyl Acetate	Acetone	Methanol	Aqueous
Streptococcus mutans	1.00	1.00	1.00	1.00
Streptococcus pneumoniae	0.63	1.00	1.00	1.00
Streptococcus pyogenes	-	-	1.00	-
Staphylococcus aureus	1.00	1.00	1.00	1.00
Pseudomonas aeruginosa	-	-	-	-

*Each value in the table was obtained by calculating the average of five experiments ± standard deviation.

CONCLUSION

The result of the present study shows that the extract of *P.emblica*, which contain highest amount of phenols, tannins, lignins, flavonoids (as reported in early works), possessed the highest antioxidant activity. The scavenging property of extract of *P.emblica* can be due to hydroxyl groups present in the phenolic compounds in the plant extract and this chemical structure helps in providing the necessary component as radical scavenger. Free radicals are often resultant product of biological reactions or due to exogenous factors. Free radicals involvement in the various pathogenesis is well documented and it is also well established that a potent scavenger of free radicals can serve as a possible intercession for the diseases [21]. All of the extracts of *P.emblica* in this study exhibited different range of antioxidant activity along with microbial inhibition. The microbes screened are mostly involved in eye related disorders, *S.aureus* and *S.pneumoniae* are the causative agent of Keratitis and Iritis, *S.pnemoniae* and *S.pyogenes* causes septicaemia and postoperative traumatic. All these pathogens are all responsible for periorbital and orbital cellulites. The susceptibility of these pathogens at low concentration of extract opens up arena of using *P.emblica* as new drug compound for treatment of various ophthalmological disorders.

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