

# ***Phyllanthus emblica* Leaves Extract: A Potential Amylase enzyme Inhibitor with antioxidant and antimicrobial activity**

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## **Abstract**

Plants are an important source of chemical constituents, which have potential as functional food and therapeutic agents. The ethanolic extract of *Phyllanthus emblica* leaves exhibited inhibition (61.12%) against  $\alpha$ -amylase at pH 7 and temperature 40°C. The extract acts as a non-competitive inhibitor and reduces turnover number ( $K_{cat}$ ) and catalytic efficiency ( $K_{cat} / K_m$ ). It contains phenol and vitamin C content, 117 and 5.12 mg g<sup>-1</sup> respectively. Antioxidant activity of leave extract (600  $\mu$ g ml<sup>-1</sup>) is equivalent to the 800  $\mu$ g ml<sup>-1</sup> strength of ascorbic acid. The extract has antibacterial effectiveness against bacteria strains *E. coli* and *S. aureus* test organisms.

**Keywords:** *Phyllanthus emblica*; Phytochemical analysis;  $\alpha$ -amylase inhibition

## **1. Introduction**

Traditionally, plants are source for novel drug compounds. Plant derived medicines have made large contributions to human health and well being. Large no of Phytomedicine are used for the treatment of diseases [1]. Plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world [2]. *P. emblica*, also known as amla, has been used in Ayurveda, the ancient Indian system of medicine. It has been used for treatment of several disorders such as common cold, scurvy, cancer and heart diseases [3-5]. The major constituent responsible for this activity is vitamin C which shows antioxidant, anti-inflammatory and antimutagenic properties [6,7]. The aim of the present study was to investigate the amylase modulation, antioxidant and antibacterial effect of the leaf extract of *P. emblica*.

## **2. Materials and Methods**

### **2.1 Collection and preparation of plant materials**

*P. emblica* leaves were collected from the local area of Mata Gujri College Fatehgarh Sahib Punjab India, in the months of January-February. Plant leaves were shade dried and crushed in coarse powder. The dried powder (500 g) of the plant leaves was extracted using water and ethanol in the Soxhlet apparatus. The extract was filtered, and dried by evaporation using steam bath at 40°C. After full evaporation, the yield of the extract was 20.1% (w/w). The extract was then stored in refrigerator at 4°C for further experiments.

### **2.2. In-vitro $\alpha$ -amylase inhibitory assay**

The  $\alpha$ -amylase inhibition assay was performed in well plate method [8]. Starch hydrolysis medium was prepared by mixing, starch and agar 10 and 15 g l<sup>-1</sup> respectively, in phosphate buffer (pH 6.5, 0.1 M). The medium was heated at 85°C to dissolve the agar. The liquid agar medium was poured in the petriplates and allowed to solidify. Wells were made in medium with gel borer. Amylase solution (3 IU ml<sup>-1</sup>) was mixed with ethanolic extract and loaded in the wells. After 30 min incubation at 37°C plates were flooded with iodine solution and clear lyses zone on a deep-purple background was measured in mm. The diameter of lyses zone is indicative of amylase activity. Inhibition (%) was determined by following:

$$\text{Amylase inhibition (\%)} = \frac{\text{Diameter of zone of Control (mm)} - \text{Diameter of zone of plant extract (mm)} \times 100}{\text{Diameter of zone of plants leaves extract}}$$

Further the inhibition potential of leave extract was investigated in liquid medium. Different concentrations of the leave extract were added to a 0.5 ml starch solution (10 g l<sup>-1</sup>) prepared in phosphate buffer (pH 6.5, 0.1 M). The starch hydrolysis reaction was initiated by adding  $\alpha$ -amylase (3 IU ml<sup>-1</sup>). After 30 min incubation, the reaction was stopped by adding 3.0 ml dinitrosalicylic (DNS) reagent (1% 3, 5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na<sub>2</sub>SO<sub>3</sub> and 1% NaOH in

aqueous solution). The reaction mixtures were heated at 100°C for 15 min in order to stop the reaction. Thereafter, 1 ml of 40% potassium sodium tartarate solution was added to the mixtures to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded at 540 nm using a spectrophotometer. Amylase inhibition % by plant leaves extract was determined by the following formula:

$$\% \text{ Relative enzyme activity} = \frac{\text{Enzyme activity with plant leaves extract} \times 100}{\text{Enzyme activity of control}}$$

$$\% \text{ inhibition of } \alpha\text{-amylase activity} = (100 - \% \text{ relative enzyme activity})$$

### 2.3. pH and temperature effect on the enzyme inhibition by *P. emblica* leaves extract:

*P. emblica* leaves extract (100 mg ml<sup>-1</sup>) was preincubated with  $\alpha$ -amylase enzyme solution prepared in buffer of different pH 4, 5, 6, 7 and 8 for 15 min at 37°C. The starch hydrolysis reaction was initiated by adding starch solution (10 g l<sup>-1</sup>). After 30 min incubation, starch hydrolysis rate was determined. To determine the optimum temperature for enzyme inhibition by leaves extract *P. emblica* leaves extract (100 mg ml<sup>-1</sup>) was pre incubated with  $\alpha$ -amylase enzyme solution prepared in buffer (pH 7, 0.1 M). The starch hydrolysis reaction at different temperatures 20, 30, 40, 60, 80 and 100°C was initiated by adding starch solution (10 g l<sup>-1</sup>). After 30 min incubation, the reaction was stopped by adding 3.0 ml dinitrosalicylic (DNS) and % inhibition of  $\alpha$ -amylase activity was determined.

### 2.4. Effect of *P. emblica* leaves extract on the kinetic parameters of amylase enzyme:

Michaelis Menten kinetic constants ( $K_m$  and  $V_{max}$ ) were calculated from double reciprocal Lineweaver-Burk [9] plot for inhibitor and control. The type of inhibition was determined for the prophecy of type of inhibition. The turn over number and catalytic efficiency were calculated.

### 2.5. Antioxidant activity of *P. emblica* leaves extract:

#### 2.5.1 DPPH radical scavenging activity

The ability of plant extract to scavenge DPPH free radicals was determined by the method of [10]. Different concentrations (200-1000  $\mu\text{g ml}^{-1}$ ) of extract were prepared and 0.5 ml of 100 mM methanolic solution of DPPH radical was added. Mixture was mixed and left for 30 min in the dark at ambient temperature. The control contained all the reaction reagents except plant extract. The absorbance was measured at 517 nm. Methanol, DPPH solution and ascorbic acid (AA) were used as blank, control and reference standard respectively. Ascorbic acid (AA) was used as standard and the total antioxidant capacity was expressed as mg of ascorbic acid equivalents (mg AAE g<sup>-1</sup>) of solution. Decrease in the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity and the percentage inhibition activity was calculated as follows:

$$\text{DPPH Radical \%} = (1 - \text{Absorbance of plant leaves extract} / \text{Absorbance of control}) \times 100$$

#### 2.5.2 Reducing power ability

The ferric ions reducing antioxidant power (FRAP) method [11] was used to measure the reducing capacity of leaves extract. Different concentrations of extract (10-100  $\mu\text{g ml}^{-1}$ ); 2.5 ml of phosphate buffer (0.2 M, pH-6.6) and potassium ferricyanide was mixed and incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added and centrifuged at 12000 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared FeCl<sub>2</sub> solution (0.5 ml, 1%). The absorbance was measured at 700 nm. The reducing power of the extracts was represented as mg of ascorbic acid equivalents (mg AAE g<sup>-1</sup>).

### 2.6. Antimicrobial activity of *P. emblica* leaves extract:

For the in vitro antibacterial assay, the human bacterial pathogens *E. coli* and *S. aureus* were grown on nutrient broth at 37°C for 18 h. Bacterial cells were separated with centrifugation at 15000g for 30 min. The cell pellet was suspended in saline (0.85% NaCl) and cell concentration (100 cells ml<sup>-1</sup>) was adjusted. Heat sterilized Muller-Hinton agar medium was poured in sterilized 12 x 12 cm petri dishes and allowed to solidify. Test organisms *E. coli* and *S. aureus* (100 cells ml<sup>-1</sup>) were spread over the medium by a glass spreader. Wells on the medium was made by a sterile gel borer (5 mm diameter). 100  $\mu\text{l}$  of the various concentration of extract and control compound were dropped into labelled well. Inoculated plates were incubated at 37°C for 24 h [12]. The antibacterial activity was determined by measuring the diameter, of the inhibition zone. The minimum inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit the visible bacterial growth. MIC of leaves extract of *P. emblica* was performed by agar well diffusion method. Serial-dilution of the leave extract was prepared in phosphate buffer (pH, 7) to obtain various concentrations 25, 50, 75 and 100 mg ml<sup>-1</sup> and assayed against the test organisms, *E. coli* and *S. aureus*.

### 2.7. Total phenolic content and Vitamin C content

Total phenolic content was determined using Folin-Ciocalteu reagent [13]. Total phenol value was obtained from the regression equation and expressed as mg g<sup>-1</sup> gallic acid equivalent. Vitamin C content of leaves extract was determined by Iodine titration method [14] by using following formula:

$$\frac{X \text{ ml of iodine solution}}{0.250 \text{ g of vitamin C}} = \frac{Y \text{ ml of Iodine solution}}{Z \text{ g of vitamin C}}$$

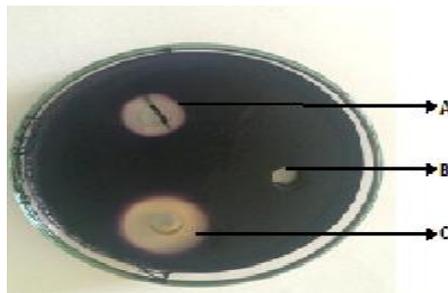
### 2.8. Sugar and protein content:

Total sugar, reducing sugar and protein content in leaves extract was determined using standard methods [15-17].

## 3. Results

### 3.1 Amylase inhibitory effect of *P. emblica* leaves extract:

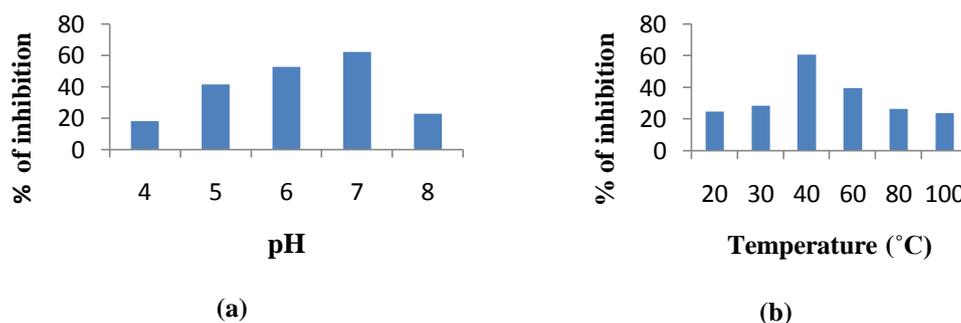
The antidaibatic modulatiatory activity, of *P. emblica* leaves extract was determined by well plate method. The ethanol leaves extract has significantly inhibited the  $\alpha$ -amylase activity. There was 9 mm lysis zone with extract with respect to control, 21mm (Figure 1). It illustrated, 60.14 $\pm$ 2 % of enzyme inhibition. Confirmation of amylase inhibitory activity of plant extract in liquid medium was performed with DNS method [16]. The activity of amylase enzyme decreased, 61.12 $\pm$ 2 % with extract with reference to control.



**Fig. 1:- Amylase activity in the presence of (A) plant extract from *P. emblica* leaves by ethanol extraction (B) buffer and (C) enzyme**

### Effect of pH and temperature on $\alpha$ -amylase inhibition by *P. emblica* leaves extract

Amylase inhibition by leaves extract depends upon the pH and temperature of medium. Maximum inhibition, 61.12 $\pm$ 2 % was at neutral pH 7, but it decreased both in alkaline and acidic conditions (Figure 2a). The enzyme inhibition was 25-61% at 20-80 $^{\circ}$ C, but the maximum inhibition was at 40 $^{\circ}$ C.



**Figure. 2- Effect of pH (a) and temperature (b) on the inhibitory activity of  $\alpha$ -amylase of *P. emblica* leaves extract**

### 3.2. Effect of *P. emblica* leaves extract on kinetic parameters of $\alpha$ -amylase

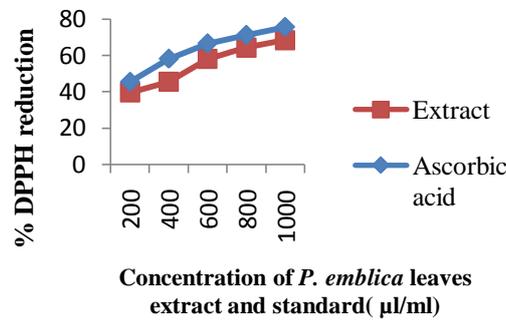
The mode of inhibition of  $\alpha$ -amylase with ethanol leaves extract of *P. emblica* was determined by means of Lineweaver-Burk double reciprocal plot of  $1/v$  versus  $1/[S]$ . There was a non-competitive type of inhibition against  $\alpha$ -amylase. Catalytic efficiency of amylase enzyme decreased by 43% with leaves extract as compare to the control.

### 3.3. Antioxidant and Reducing power ability of *P. emblica* leaves extract

The *P. emblica* leaves extract contains 5.12 mg g $^{-1}$  vitamin C, which is an excellent antioxidant. So it contributed to antioxidant activity. The leaves extract showed considerable radical scavenging activity in a concentration-dependent manner. The extract of leaves (800  $\mu$ g ml $^{-1}$ ) exhibited a good potential to act as a free radical scavenger for DPPH inhibition comparable to that of standard Vitamin C (600  $\mu$ g ml $^{-1}$ ) which are known free radical scavengers (Table, 1). FRAP (Ferric reducing antioxidant power) is one of the fast test for antioxidative activity. Increase in absorbance (Table, 2) caused by the formation of ferrous ions from FRAP reagent depends on the ability of the sample to reduce the ferric complex to ferrous at a low pH. The reducing ability of the extracts was in the range of 10-100  $\mu$ g ml $^{-1}$  comparable to reducing power of standard ascorbic acid.

**Table 1: DPPH reduction (%) Ascorbic acid (standard) and *P. emblica* leaves extract**

Concentration of extract and ascorbic acid ( $\mu\text{g ml}^{-1}$ )	DPPH reduction in the plant extract and Ascorbic acid	
	Ascorbic acid (Standard)	<i>P. emblica</i> leaves extract
200	45.43	39.67
400	58.21	45.64
600	66.67	58.12
800	71.35	64.34
1000	75.71	68.53



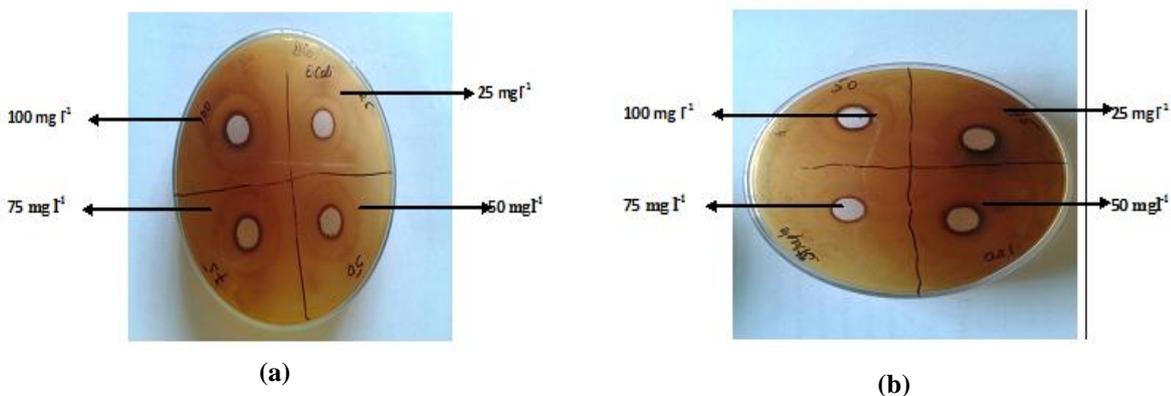
**Figure 3: DPPH reduction (%) in *P. emblica* leaves extract and standard (Ascorbic acid)**

**Table 2:- Absorbance of standard and *P. emblica* leaves extract at various concentrations ( $\mu\text{g ml}^{-1}$ ) in ferric reducing power determination method**

Concentration of extract and ascorbic acid ( $\mu\text{g ml}^{-1}$ )	Absorbance of Standard and plant extract at 700nm	
	Standard (Ascorbic acid)	Plant extract
10	0.195	0.140
20	0.268	0.221
40	0.343	0.300
60	0.411	0.385
80	0.498	0.457
100	0.61	0.511

**3.4. Antimicrobial activity of *P. emblica* leaves extract**

The ethanol extract of *P. emblica* leaves exhibits antimicrobial activity against *E. coli* ( $27 \pm 0.5$  mm) and *S. aureus* ( $2 \pm 0.5$  mm) (Fig.3). The results of antimicrobial activity of extract were comparable with the standard drug (Ampicillin 10 mcg). The minimum inhibitory concentration of extract was  $25 \text{ mg ml}^{-1}$  for *E. coli* and *S. aureus* (Table 4). Below this concentration antimicrobial activity was not reported.



**Figure 3: Zone of inhibition of various conc. (25-100  $\text{mg ml}^{-1}$ ) of *P. emblica* leaves extract against growth of (a) *E. coli* and (b) *S. aureus***

**Table 4: The minimum inhibitory concentration (mg ml<sup>-1</sup>) of *P. emblica* leaves extract against *E. coli* and *S. aureus***

Leave extract concentration (mg ml <sup>-1</sup> )	Microbial zone of inhibition (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
20	Nil	Nil
25	14	12.5
50	19.5	17
75	23	20
100	27	24

### 3.5. Photochemical analysis of *P. emblica* leaves extract:

The vitamin C content in leaves extract was 5.12 mg g<sup>-1</sup>. Ethanol leaves extract of *P. emblica* has total phenol content 117 mg g<sup>-1</sup> GAE. It has total sugar, reducing sugar and protein, 100, 4 and 250 mg g<sup>-1</sup> respectively.

## 4. Discussion

The knowledge of medicinal property of plants has been gained the course of many centuries. Medicinal plants provide accessible relevant resources of primary health care. The remedies based on these plants often have minimal side effect [18]. The antidaibatic modulatiatory activity, of *P. emblica* leaves were screened for the  $\alpha$ -amylase inhibitory property. Ethanol extract inhibited significantly  $\alpha$ -amylase activity. There was 60.14±2 % inhibition of amylase activity with respect to control. Inhibitory activity varies at different pH. Maximum enzyme inhibition was at pH 7. This is similar to inhibitors from the, *Citrullus colocynthis* [19] and opposite to the purified inhibitor from *Colocasia esculenta* and Kidney beans [20,21]. The amylase inhibitory activity by extract depends on temperature of reaction. At lower temperature the inhibitory activity of extract decreased and showed maximum inhibitory activity at 40°C. Two inhibitors extracted from *C. esculenta* and wheat grains [20,22] also showed the optimum temperature of 40°C. The type of inhibition of extract of leaves against  $\alpha$ -amylase was determined by Line weaver-Burk, double reciprocal plot of 1/v versus 1/[S]. It showed a non-competitive type of inhibition against  $\alpha$ -amylase. In the presence of a non-competitive inhibitor, the apparent enzyme affinity is equivalent to the actual affinity. In terms of Michaelis-Menten kinetics,  $K_m^{app} = K_m$ . but, this type of inhibition reduces the maximum rate of a reaction. This can be seen as a consequence of binding of the inhibitor to both the enzyme and the enzyme-substrate complex equally. Catalytic efficiency ( $K_{cat} / K_m$ ) of enzyme decreases with *P. emblica* leaves extract.

The *P. emblica* leaves extract contains 5.12 mg g<sup>-1</sup> of vitamin C. So it contributed antioxidant activity. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [23]. Antioxidant activity of leave extract (600  $\mu$ g ml<sup>-1</sup>) is equivalent to the 800  $\mu$ g ml<sup>-1</sup> strength of ascorbic acid. The reductive potential of extract (80  $\mu$ g ml<sup>-1</sup>) is compatible to the 80  $\mu$ g ml<sup>-1</sup> of standard ascorbic acid. The hydro-methanolic leaf extract of *Embllica officianalis* has DPPH radical scavenging activity and reductive potential as measured by ferric ion reduction [24]. Ethanol leaves extract of *P. emblica* contains 117 mg g<sup>-1</sup> GAE, total phenol content. In the present study, antioxidant activity indicates that phenols are contributing to total antioxidant activity of extract. It is found that antioxidant activity, depends on quantities of total phenols [25,26]. The study indicates that phenols present in the extract of *P. emblica* might be responsible for the antioxidant properties.

Plants have formed the basis of sophisticated traditional medicine system. Natural products make excellent leads for new drug development [27]. Bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents [28]. The antibacterial activities of *P. emblica* leaves extract were carried out. The extract show antimicrobial activity against, *S. aureus* (gm +ve ) and *E. coli* (gm - ve). The result of antimicrobial activity of ethanol extract (100 mg ml<sup>-1</sup>) was equivalent with the standard drug Ampicillin (10 mcg). The inhibition of bacterial growth in-vitro by the extract could be due to the presence of some active compounds in the extracts. These active compounds may act alone or in combination to inhibit bacterial growth. The crude extract contains organic components including flavonoids, tannins, alkaloids, triterpenoids, all of which are known to have antibacterial affects. Leaves extract contain phenols that are very good antimicrobial agent [29]. The extracts of the *P. emblica* is proved to have potential antibacterial activity. The minimum inhibitory concentration (MIC) of ethanolic extract of *P. emblica* leaves for *E. coli* and *S. aureus* was 25 mg ml<sup>-1</sup>. The antimicrobial activity against *E. coli* and *S. aureus* supports the potential, as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Similarly, Ethanol and acetone extracts of *E. officianalis* exhibits antimicrobial activity [30,31] against test organisms including gram positive and gram negative bacteria.

The vitamin C content in *P. emblica* leaves extract was 5.12 mg g<sup>-1</sup> and it act as good antioxidant. It is relevant to results of DPPH radical scavenging activity and reducing power ability. Vitamin C helps to regulate the blood sugar level and prevent heart diseases by preventing free radicals from damaging artery walls, which could lead to plaque formation.

Phenols offer resistance to disease and reduce risk of cancer. Higher the phenol content stronger is the antioxidant activity [32]. Ethanolic leaves extract of *P. emblica* shows total phenolic content 117 mg g<sup>-1</sup> GAE which is equivalent to *P. emblica* leaves extract [33,34] and *Marrubium peregrinum L.* extract [35]. Phenols can protect the human body from free radicals, whose formation is associated with the normal metabolism of aerobic cells [36,37]. Ethanol extract of leaves contains total sugar, reducing sugar and protein, 100, 4 and 50 mg g<sup>-1</sup> respectively. Proteins are essential nutrients for the human body. These are one of the building blocks of body tissue and can also serves as a fuel source.

## 5. Conclusion

This work revealed that ethanol extract of *P. emblica* leaves has effective amylase inhibitory potential. It contains phenols and citric acid that are important for health as antioxidants. Further investigation is taken in this research is the antibacterial effectiveness of extract against pathogenic bacterial strains *E. coli* and *S. aureus* test organisms.

**Ethical Consent:** Authors read this research paper and declared that nothing is done in this research that violates any ethical state.

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