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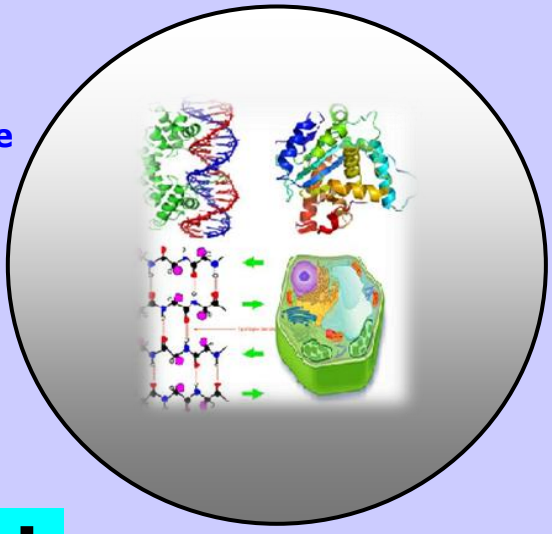

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RESEARCH PAPER

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Effect of *Euphorbia pulcherrima* Leaf Extract on Secretion of Microbial Extracellular Enzymes

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ABSTRACT

Microbes are able to produce extracellular enzymes and secrete them outside the cell membrane to break down complex chemical structures. These enzymes allow microbes to derive energy and nutrients from complex polymers and also catalyze the cycling of organic matter in ecosystems. Plant produces various chemicals which are responsible to inhibit the extracellular enzymes produced by pathogenic microbes. Hence, the present study was done with an aim to study the effect of acetone extract of Euphorbia pulcherrima leaf on α-amylase and protease enzyme production by test pathogens. Results suggests that enzyme activity of microbes was found to be decreased with increasing concentration of the extract and at minimum inhibitory concentration of the extract 100% inhibition in enzyme activity was observed.
Keywords: Euphorbia pulcherrima, α-amylase Enzyme and Protease Enzyme.

INTRODUCTION

Infections or infectious diseases occur as the result of interactions between pathogenic microorganisms and the host. Some pathogens may be capable of growth within the cells of the host, causing severe disruption of normal physiological process. In other infections the pathogen may grow extracellularly and damage body cells as the result of elaboration of poisonous substances by the microorganism (Pelczar et al. 1993).

Microbial adherences to the host, colonization factor, production of invasins are also responsible for their pathogenicity. Host cell wall, membrane and tissue matrices serve as the first level of defense against pathogens. Pathogens secrete several extracellular

enzymes which break these barriers and infect the host (Alexopolous and Mims, 1993; Dubey and Maheshwari, 1999).

Enzymes may be defined as thermolabile catalysts of definite organic nature elaborated by living tissue but capable of action outside these tissues also (Bansal et al., 2005). The cell wall degrading enzymes produced by various pathogens are known to play an important role in the pathogenesis.

Amylase act on starch, glycogen and derived polysaccharides to hydrolyse the α-1, 4 glucosidic linkages. The amylases may be divided into 3 groups: the α-amylase (endoamylases), β-amylases (exoamylases) and glucoamylases (Ajayi and Fagade, 2003). Srivastava and Baruah (1986) reported that some *Bacillus* sp. produce alpha amylase together with β-amylase. Oberai and Kalra (2006) have screened *Aspergillus niger* isolates for glucoamylase production.

Proteases are proteolytic enzymes which catalyse the cleavage of peptide bonds in other proteins or bring about total hydrolysis of proteins, various microorganisms such as bacteria, fungi, yeasts and actinomycetes are known to produce proteases (Steele and Stowers, 1991). Proteases are also a group of protein digesting enzymes produced by various microorganisms such as *Bacillus subtilis* and *Aspergillus oryzae* (Alcamo, 1994). Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*.

The ability of plant molecules to act as bioactive protoxins, enzyme inhibitors, receptor agonists, receptor antagonists and psychoactive agents etc. is linked to plants antimicrobial activities. A variety of chemicals that inhibit amylases and proteases as well as membrane active entities like lipid transfer proteins, defensins, napsins, osmotins and thaumatins that can damage cell wall of pathogenic fungi are produced by plants (Polya, 2003).

Plant derived secondary metabolites have the ability to inhibit various microbial enzyme activities. Hwang *et al*. (2001) studied that seven tannins and related compounds isolated from aerial parts of *Euphorbia pekinensis* inhibits chitin synthase II from *Saccharomyces cerevisiae*. Homer *et al*. (1990) have reported the inhibition of protease activities of periodontopathic bacteria by extracts of plants used in Kenya as chewing sticks. An alkaloid camptothecin was produced by *Camptotheca acuminata* trees is reported to be responsible for the inhibition of eukaryotic DNA topoisomerase I (Bodley and Shapiro, 1995). Amariin (hydrolysable tannin) obtained from *Phyllanthus amarus* and maslinic acid (triterpene) isolated from *Geum japonica* have been found to inhibit HIV-1 protease (Polya, 2003). Mehta and Mehta (2005) studied the effect of plant extracts on production of cell wall degrading enzymes by fungi (*Geotrichum candidum*). Correia *et al*. (2004) have reported that bioprocessed phenolic enriched extracts of pineapple waste are linked to amylase and *Helicobacter pylori* inhibition and also suggested that natural α-amylase inhibitors offer an attractive therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch.

Hence, in the present study effect of acetone extract of leaf of *E*. *pulcherrima* on α-amylase and protease enzyme production by test pathogens was studied because according to screening results acetone extract of leaf was found to be the most inhibitory extract against test microorganisms. Decrease or increase in enzyme activity was studied at all test concentrations including MIC of extract.

MATERIAL AND METHODS

As microorganisms produce extra cellular enzymes in the presence of substrate, test pathogens were cultured in liquid medium containing respective substrate as well as different concentrations of extract at 37±C^{o} for 72 h (fungi) or 24 h (bacteria). The culture filtrate obtained after centrifugation was used for enzyme assay. Extract free medium containing substrate was used as control. Three replicates of experiment were maintained and experiment was repeated thrice. Reagents used for this purpose are listed in Annexure I.

Amylase Enzyme Assay

Amylase activity was assayed by method suggested by Nelson (1944). Freshly prepared 1% starch solution was used as substrate for amylase assay. Fungal and bacterial pathogens were cultured in liquid medium containing different concentrations of extract up till MIC and 1ml of starch solution respectively. Tubes were incubated for 24 h (bacteria) or 72 h (fungi) and centrifuged at 5000 rpm for 30 minutes. The supernatent was used to estimate amylase activity.

Amylase activity was assayed by boiling 1ml of supernatent with 0.5ml of DNSA reagent on a waterbath for 10 minutes. The cooled samples were diluted with 4ml of distilled water. Transmittance of orange-red colour developed was noted at 520nm. Amount of liberated maltose was calculated with the help of standard reference curve prepared by using known concentration of maltose. The results of amylase activity are expressed as mg maltose lib. /ml.

Protease Enzyme Assay

Protease activity was assayed by method suggested by Kunitz (1947). Freshly prepared 1% casein solution was used as substrate for protease assay. Fungal and bacterial pathogens were cultured in liquid medium containing different concentrations of extract up till MIC and 1ml of casein solution respectively. Tubes were incubated for 24 h (bacteria) and 72 h (fungi) and centrifuged at 5000 rpm for 30 minutes.

1ml of 12% TCA (Trichloroacetic acid) was added to the supernatant and once again centrifuged to remove precipitated protein. Supernatant collected at this stage was used for enzyme assay. 0.5ml of each sample was mixed with 4ml of distill water and 1ml of Ninhydrin reagent and heated in water bath for 15 minutes. 1ml of 50% ethanol was added to cooled samples and transmittance of purple/pink colour developed was measured at 570 nm. Amount of liberated tyrosine was calculated with the help of standard curve prepared by using known concentrations of tyrosine. The result of protease activity is expressed as µg tyrosine lib. /ml.

RESULT AND OBSERVATION

Effect of Acetone Extract of *E. pulcherrima* Leaf on α-Amylase and Protease Activity of bacteria

Results of effect of acetone extract of leaf on α-amylase and protease activity of test bacteria suggests that in control, maximum enzyme activity was observed for *B. subtilis* followed by *E. coli*. α-Amylase enzyme activity of *B. subtilis* decreased from 130.1 µg/ml in control to 18.1 µg/ml at 125 µg/ml (sub-MIC) concentration of the extract. 100% inhibition was observed at 250 µg/ml (MIC) concentration (Table1).

6.79%, 16.56%, 21.74%, 54.97%, 65.71%, 74.98%, 86.71% inhibition of enzyme activity was observed for *E. coli* at 3.95 µg/ml, 7.81 µg/ml, 15.62 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml and 250 µg/ml extract concentration respectively. 100% Inhibition was exhibited at 500 µg/ml (Table 2).

Decrease in protease activity to treatment with plant extract was observed for all test organisms. Result suggests that as compared to control amount of protease secreted is inversely proportional to increasing concentration of the extract. Results indicate that in control maximum enzyme activity was observed for *E. coli* followed by *B. subtilis*. The enzyme activity of *B. subtilis* decreased from 0.076 µg/ml in control to 0.010 µg/ml after treatment with extract at 125 µg/ml extract concentration (Table 1). Average enzyme activity of *E. coli* before treatment with extract (control) observed was 0.082 µg/ml whereas after treatment with extract it was decreased to 0.010 µg/ml at 250 µg/ml (sub-MIC) extract concentration. 100% inhibition was observed at 500 µg/ml (MIC) extract concentration (Table 2).

Effect of Acetone Extract of *E. pulcherrima* Leaf on α-Amylase and Protease Activity of test fungi

Results of effect of acetone extract of *E. pulcherrima* leaf on α-amylase activity of test fungi suggests that inhibition of enzyme activity was observed for *C. albicans*. As compared to control there was 49.16% and 82.44% inhibition of enzyme activity at 3.95 µg/ml and 7.81 µg/ml respectively whereas 100% inhibition was observed at MIC i.e. 15.62 µg/ml (Table 3). The average enzyme activity of *A. fumigatus* before treatment with extract (control) was 172.06 µg/ml whereas after treatment at 7.81 µg/ml extract concentration it was reduced to 60.2 µg/ml. Enzyme activity was totally inhibited at 15.62 µg/ml (MIC) concentration (Table 4).

Decrease in protease activity to treatment with plant extract was observed for all test organisms. Result suggests that as compared to control amount of protease secreted is inversely proportional to increasing concentration of the extract. Results of effect of acetone extract of *E. pulcherrima* leaf on protease activity of test fungi indicates that in control maximum enzyme activity was observed for *C. albicans* followed by*A. fumigatus*. In case of *C. albicans* average enzyme activity decreased from 0.080 µg/ml in control to 0.020 µg/ml at 7.81 µg/ml (sub-MIC) extract concentration (Table 3) whereas 30.15% and 82.53% inhibition of enzyme activity of *A. fumigatus* was observed at 3.95 µg/ml and 7.81 µg/ml extract concentration respectively. 100% inhibition was observed at MIC i.e. 15.62 µg/ml (Table 4).

DISCUSSION AND CONCLUSION

Pathogenicity and virulence of bacteria and fungi are associated with α-amylase and protease secreted by the microorganisms thus these enzymes play an important role in a number of microbe-interactions. Present study establishes the phenomenon that as the concentration of the extract increased, secretion of extracellular enzyme decreased. Decrease in enzyme secretion is also correlated with inhibition of growth of test bacteria and fungi.

Park *et al*. (2002) have reported inhibitory effects of Korean medicinal plants and camelliatannin H from pericarp of *Camellia japonica* on human immunodeficiency virus type 1 protease with 0.9 µM IC50 value.

Phenolic compounds like tannins present in the cells of plants are found to be potent inhibitors of many hydrolytic enzymes such as pectolytic macerating enzymes used by plant pathogens (Aboaba and Efuwape, 2001). Athikomkulchai *et al*. (2006) isolated two new compounds from the branches of *Croton hutchinsonianus* (Euphorbiaceae) and reported that one compound exhibited potent but non-selective activity against the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).

Table 1. Effect of Acetone Extract of *Euphorbia pulcherrima* Leaf on α-Amylase and Protease Activity of *Bacillus subtilis.*

S. No.	Extract Concentration µg/ml	α-Amylase activity		Protease Activity	
		Enzyme Activity ((µg/ml)	% Inhibition	Enzyme Activity ((µg/ml)	% Inhibition
1.	Control (Extract Free)	130.1±0.15	-	0.076 ±.0005	-
2.	3.95	68.0±0.15	47.73%	0.055 ±.0005	27.63%
3.	7.81	60.1±0.15	53.8%	0.045 ±.0005	40.78%
4.	15.62	50.0±0.15	61.56%	0.036 ±.0005	52.63%
5.	31.25	40.0±0.05	69.25%	0.025 ±.0005	67.10%
6.	62.5	30.1±0.28	76.86%	0.014 ± .001	81.57%
7.	125	18.1±0.15	86.08%	0.010 ±.001	86.84%
8.	250	00	100%	00	100%

Table 2. Effect of Acetone Extract of *Euphorbia pulcherrima* Leaf on α-Amylase and Protease Activity of *Escherichia coli.*

S. No.	Extract Concentration µg/ml	α-Amylase activity		Protease Activity	
		Enzyme Activity ((µg/ml)	% Inhibition	Enzyme Activity ((µg/ml)	% Inhibition
1.	Control (Extract Free)	120.16±0.15	-	0.082 ±.0005	-
2.	3.95	112.0±0.1	6.79%	0.075±.0005	8.53%
3.	7.81	100.26±0.64	16.56%	0.064±.0005	21.95%
4.	15.62	94.03±0.15	21.74%	0.052±.0005	36.58%
5.	31.25	54.1±0.1	54.97%	0.045±.0005	45.12%
6.	62.5	41.2±0.1	65.71%	0.032±.0005	60.97%
7.	125	30.06±0.05	74.98%	0.023±.0005	71.95%
8.	250	15.96±0.15	86.71%	0.010 ±.001	87.8%
9.	500	00	100%	00	100%

Kim *et al*. (2005) have reported that ethanol extract of pine bark and needle showed competitive inhibition against salivary alpha-amylase and the combination of non-competitive and uncompetitive inhibition against yeast alpha-glucosidase.

Two triterpenoid compounds ursolic acid and uvaol have been isolated from *Crataegus pinnatifida* Bunge leaves, out of which ursolic acid was found to inhibit chitin synthase II of *Saccharomyces cerevisiae* (Jeong et al., 1999). Vallejo *et al*. (2006) have evaluated the enzymatic inhibitions of xanthine oxidase, beta-glucosidase and beta-glucuronidase of microbes by essential oils obtained from cultivated *Salvia canariensis* (Lamiaceae). Anolignan B isolated from ethyl acetate root extract of *Terminalia serica* has been reported to exhibit significant inhibitory activity against both COX-1 and COX-2 enzymes with 1.5mM and 7.5mM IC_{50} values respectively (Eldeen et al., 2006). Tawfik *et al*. (2000) reported that garlic extract suppresses or inhibits amylase, cellulase, phenoloxidase and protease enzyme production by fungi. A 36-kDa α-amylase inhibitor was isolated and characterized from *Lablab purpureus* (AILP) and was found to inhibit α-amylases from several fungi but had little effect on those from animal and plant sources (Fakhoury and Woloshuk, 2001). α-amylase and protease play an important role in a number of microbe interactions and are also associated with virulence and pathogenicity of bacteria and fungi. The above given reasons could be a possible explanation for reduction in enzyme activity.

Table 3. Effect of Acetone Extract of *Euphorbia pulcherrima* Leaf on α-Amylase and Protease Activity of *Candida albicans.*

S. No.	Extract Concentration µg/ml	α-Amylase activity		Protease Activity	
		Enzyme Activity ((µg/ml)	% Inhibition	Enzyme Activity ((µg/ml)	% Inhibition
1.	Control (Extract Free)	228±0.05	-	0.080 ±.0005	-
2.	3.95	115.9±0.05	49.16%	0.045 ±.0005	43.75%
3.	7.81	40.03±0.11	32.44%	0.020 ±.0005	75.0%
4.	15.62	00	100%	00	100%

Table 4. Effect of Acetone Extract of *Euphorbia pulcherrima* Leaf on α-Amylase and Protease Activity of *Aspergillus fumigates.*

S. No.	Extract Concentration µg/ml	α-Amylase activity		Protease Activity	
		Enzyme Activity ((µg/ml)	% Inhibition	Enzyme Activity ((µg/ml)	% Inhibition
1.	Control (Extract Free)	172.06±0.20	-	0.063 ±.0005	-
2.	3.95	100.1±0.2	41.82%	0.044 ±.0005	30.15%
3.	7.81	60.2±0.26	65.01%	0.011 ±.0005	82.53%
4.	15.62	00	100%	00	100%

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