International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 2| ICAM-2019 | 109-124
Proceedings of International Seminar on "Agricultural Microbiome"
Held at Hindustan College of Arts and Science, behind Nava India, Coimbatore, Tamil Nadu 641028, India, 22nd February 2019
| Research Article | Biological Sciences |Open Access |MCI Approved|
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In Vitro Evaluation of Fungicidal Activity of Bromelain from *Ananascomosus* against Fungal Phytopathogens Infecting *Musa paradisiaca*

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Received: 25 Feb 2019 / Accepted: 20 Mar 2019 / Published online: 01 Apr 2019
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Abstract
Bromelain from *Ananascomosus*is a mixture of proteolytic enzymes which are cysteine protease inhibitors containing thiol endopeptidases employed as a phytomedical compound. The present study focussed on extraction and characterization of bromelain and to determine its protein concentration and protease enzyme activity. The effect of temperature and pH on the extracted enzyme was also determined. The extracted enzyme was determined for its molecular weight by SDS and characterized for the presence of their functional groups by FT-IR spectroscopy. The purified bromelain of stem showed highest concentration of protein with maximum protease activity when compared to that of fruit bromelain and on characterization by FT-IR was found to contain thiol containing amino groups. The extracted and characterized bromelain was treated against isolated fungal pathogens of *Musa paradisiaca.* The isolated bromelain was assessed for their enzyme activity and protein concentration *in vitro* against the isolated fungal spores and determined for its activity. The determined two – way ANOVA indicated that there were significant differences at different concentrations after incubation at varying time period and the effective concentration and time were determined through Turkey post-hoc analysis which showed stem bromelain possess more effective antifungal activity *in vitro* against fungal phytopathogens of *Musa* paradisiaca.

Keywords
Bromelain, FT-IR,*Musa paradisiaca,* fungal phytopathogens, *in vitro* assessment.

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INTRODUCTION
Bananas (Musa spp.) represent the fourth most significant staple fruit with a worldwide production of over one hundred million metric tons and rank initially among all sorts of fruits created (FAO 2015). Phyto-pathogenic fungi are the most reason for communicable disease in plants and crops and need the continued use of chemical fungicides for management moreover on meet world food desires. Among them square measure their lack of specificity, the augmented incidence of resistance development upon prolonged application and therefore the adverse impact on human health and

setting. Indeed, negative long-run effects on human health and setting have resulted in deregistration of some vital fungicides [1].

Plants have evolved a classy innate system to intercept incursive pathogens. The plant defence response involves a spread of mechanisms like the fast generation of reactive Oxygen species (ROS), the induction of the supposed hypertensive response (HR) and production of little molecules (phytoalexins) and proteins with antimicrobial activity [2] [3]. The induction of genes secret writing endo-proteases with totally different chemical action mechanisms, particularly Metallo-, serine-, aspartic- or amino acid endo-proteases, in response to infective agent infection has been delineated [4].

Amino acid proteases are noted to be concerned in several aspects of plant physiology and development, together with senescence, embryogenesis, flower development and response to varied kinds of environmental stress [5]. Different works have incontestable the involvement of amino acid proteases within the management of programmed necrobiosis or time unit in several plant species [6] [7]. On a different hand, the medicine action of plant amino acid proteases has long been recognized. Papain, the primary amino acid proteolytic enzyme to be characterized in papaya latex, is getting used as a possible drug for microorganism and flora diseases in medicine analysis [8] [9].

Bromelain is the collective name of connected chemical enzymes found within the tissues of species belonging to family Bromeliaceae, of that pineapple is that the best noted [10] [11]. Two distinct kinds of pineapple bromelain recognized: Stem bromelain and fruit bromelain [12]. Besides enzyme, bromelain is most generally used proteolytic enzyme with the therapeutic application [13]. Pure stem bromelain possesses antifungal activity against agronomically vital crops. The aptitude of bromelain to inhibit flora growth is said to its chemical action activity [14] [15].

MATERIALS AND METHODS

ISOLATION OF FUNGUS AND CHARACTERIZATION

The plantlets of *Musa paradisiaca* were used for study, which was obtained from Spic Agro Biotech Centre, Coimbatore. Infected leaves and pseudostem were carefully selected, surface sterilized and each of 1x1 dimensions were inoculated on PDA with antibiotic and incubated for 96 hrs at 27℃ for fungal growth.The respective single spores of fungal cultures grown on PDA were transferred to PDA after complete growth, sub cultured and maintained at 27°C for 3 to 5 days. The pure isolate after growth was maintained on Potato Dextrose broth at 27°C for 3-5days for revival of cells and stored for further use.

Morphological characterization of fungus

Mycelia were mounted in 0.01% lacto phenol cotton blue and observed under light microscope at 40X magnification. The fungi were identified based on their mycelia and spores characteristics.

Isolation of fungal genomic DNA

The genomic DNA was extracted from five to seven days old fungal cultures grown in Potato Dextrose Broth and isolated [16]. The DNA pellets were air dried and dissolved in 1X TE buffer and stored at 4°C.

Quality and quantity determination of DNA

The quantity of extracted DNA was determined by measuring the absorbance at 260nm, 280nm and 320nm using UV spectrophotometer. The quality of extracted DNA was accessed on 0.8% agarose gel followed by staining with ethidium bromide.

EXTRACTION AND CHARACTERIZATION OF BROMELAIN

Sample preparation

The fruit pulp and stem of *Ananascomosus* are used in the present study. The samples were rinsed well under running tap water to remove impurities and surface sterilized with 0.05% Tween 20 followed by rinsing in distilled water for 3-5 times to remove environmental debris from the sample.

Crude extract preparation

About 80g of samples respectively were weighed and rinsed with distilled water. The samples were homogenized with 100ml of 1M Sodium acetate buffer (pH 7), filtered and centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was collected in respective tubes, added 0.6g Benzoic acid and stored at 4°C[17] [18]. This was used as crude extract for assays.

Ammonium sulphate precipitation

The precipitation was carried out using 120 ml of crude extract of pulp and stem extract respectively. Precipitation was carried out at 50% and 60% saturation with ammonium sulphate salt and allowed for attainment of equilibrium between dissolved and aggregate proteins for 24hrs at 4°C. The further saturated salt enriched solution was centrifuges at 8,000 rpm for 15 min at 4°C and the pellet so obtained is dissolved in 0.1M sodium acetate buffer and stored for further use [17] [18].

Dialysis

The dialysis membrane with molecular cut off of 10,000 -12,000KDa was purchased from Precision pharming. The membrane was subjectedto activation by pre-treatment. And dialysed against 500ml of 0.01M sodium acetate buffer. The respective salt precipitated pellet dissolved in 0.1M sodium acetate buffer was dialysed against 0.01M sodium acetate buffer for 48hrs at 4°C. The dialysed sample so obtained is centrifuged at 8,000 rpm for 10 mins at 4°C and the pellet is dissolved in 0.1M sodium acetate buffer (pH 6.8-7).

Thin layer chromatography (TLC)

TLC was performed with silica as stationary phase n-propanol: water (7:3) as mobile phase. The amino acid of unknown was identified with the obtained R_f value by comparing with the standard known value.

Estimation of protein-Lowry's method and protease enzyme activity

The protein concentration of crude, salt precipitated pellet and dialysed samples were determined by Lowry's method with BSA as standard by measuring optical density at 660nm. The concentration of unknown samples were estimated using the calibrated graph.

The protease enzyme concentration was determined by modified method of Lowry's method with tyrosine as standard and 1.5% casein as substrate by measuring the optical density at 660nm. The protease concentration of unknown samples was estimated using calibrated graph. The specific activity was calculated as the relation between the enzymatic activity and protein concentration (U/mg of protein).

Gelatin Digestion Unit (GDU) ASSAY

One gelatin digestion unit can be defined as the amount of enzyme that will hydrolyse 1mg of amino nitrogen from gelatin after 20 mins of digestion at pH 4.5 at 45°C. Prior to this assay the enzymes were allowed to stand for 60 mins at 4°C.

Gelatin + H_2O_2 ⟶ Amino acids + Oligopeptides

5% gelatin serves as a substrate which is equilibrated to 45°C followed by addition of test enzyme and control enzyme respectively and 3% hydrogen peroxide by incubating at 45°C for 20mins. Titrated against 0.05N NaOH, pH adjusted to 6.9 and incubated at room temperature for 2-3 mins. Added 37% formaldehyde to all the tubes and pH adjusted to 7.2 and optical density reading measured at 660nm [18]. The GDU can be calculated using the formula,

GDU/mg = (T-B) x 14 x Normality of NaOH x 1000 **/** Conc. of enzyme

Determination of molecular weight by SDS-PAGE

SDS-PAGE was performed with dialysed extract enzyme. The samples were pre-prepared and loaded onto 12% resolving gel and 5% stacking gel with medium range protein molecular marker and subjected to electrophoresis for 3-5 hrs at 50V

Staining and destaining

The SDS-PAGE gel was carefully placed in staining solution overnight at 37°C with constant agitation. The stained gel was subjected to destaining with constant agitation in distilled water followed by destainingsolution for 3-4 hrs at 37°C or until staining solution disappears. The obtained respective bands were observed under white Trans-illuminator and photographed.

Activity staining

Activity staining for bromelain was done by casein gel electrophoresis. The completely destained gel was carefully placed in 2% Casein substrate solution for 45 min at 4°C. After incubation, the 2% casein substrate gel was subjected to continuous agitation in freshly prepared 2% casein substrate solution for 30-45 min at 37°C.

Determination of optimum pH and temperature

Sodium acetate buffer of varying pH (2, 4, 6, 8, 10 and 12) were used to determine the optimum pH and optical density reading was measured at 660nm. The optimum pH was determined using calibrated graph.

The optimum temperature of bromelain was determined by modified method of Lowry's estimation by incubating the unknown samples at varying pH (4°C, 20°C, 40°C, 60°C, 80°C and 100°C). The optical density reading was measured at 660 nm. The optimum temperature was determined using calibrated graph.

Fourier Transform Infra-Red spectroscopy (FTIR)

FTIR is useful for identification of organic molecular groups and compounds. FTIR absorption spectra of the purified protease (Bromelain) was obtained. The spectrum was scanned from 400 to 4000 cm^{-1}, at a resolution of 7 cm^{-1} and the data was analysed by using PE-GRAMS/32 1600 software.

ASSESSMENT OF ANTIFUNGAL ACTIVITY OF BROMELAIN

***In vitro* proteolytic activity**

The proteolytic activity of bromelain was assayed in 50% PDB, to determine if the proteolytic activity was found to be affected in presence of bromelain in PDB. The reaction mixture contained enzyme solution with 1% casein as substrate in 50% PDB. The mixture was incubated at 37°C for 20 min and the reaction was stopped by addition of 10% TCA. The activity was determined by following the Anson's method (Anson 1938) and the absorbance was read at 440nm. Varying concentrations of tryptophan was used as standard and the concentration of unknown protein *in vitro* was estimated using the calibrated graph.

***In vitro* protein estimation**

The protein concentration of the extracts was determined by Lowry's method of protein estimation. The reaction mixture contained 50% PDB with extracts. The protein concentration was compared with standard tryptophan of varying concentration. The optical density reading was read at 660nm and the concentration of unknown protein *in vitro* was estimated from the calibrated graph.

Antifungal activity of *Ananascomosus* extracts on fungal isolates

The crude, salt precipitated pellet and dialysed sample of fruit pulp and stem extracts of *Ananascomosus* were assessed for their antifungal property by agar well diffusion method. The test specimens (isolated fungus)

of 7-10 days culture maintained on PDB were swabbed with sterile cotton on PDA plates. Wells were made with gel puncture and 25µl of extracts were loaded onto the wells. A positive control was maintained with commercially available fungicide – 0.1% Dazomet. The plates were incubated at 28°C for 3-5 days. The incubated plates were examined for the interruption of growth over the inoculum. The size of the clear zone was measured to evaluate the inhibitory action of the extracts.

Antifungal activity of *Ananascomosus* extract at varying concentrations

The dialysed sample of fruit pulp and stem extracts of *Ananascomosus* were assessed for their antifungal property by agar well diffusion method at varying concentrations. 0.1%Dazomet of varying concentration was maintained as positive control. Concentrations of 100%, 75%, 50% and 25% were prepared and used for assay. The test specimens (isolated fungus) of 7-10 days culture maintained on PDB were swabbed with sterile cotton on PDA plates. The plates were incubated at 28°C for 3-5 days. The incubated plates were examined for the interruption of growth over the inoculum. The size of the clear zone was measured to evaluate the inhibitory action of the extracts.

ASSESSMENT OF *In vitro* ANTIFUNGAL ACTIVITY

Preparation of fungal spore suspension

The fungal spore suspension was prepared from fungal culture maintained on PDA for 12-15 days at 28°C.The spores of 12-15 days cultures were collected by removal of mycelia with 0.01% Tween20 and collection of spores with sterile distilled water. The mycelia were removed and discarded. The spores collected were made up to 50ml and stored.

***In vitro* antifungal activity**

The *in vitro* antifungal activity was determined by broth microdilution assay as described (Lopez-Gracia *et al.,* 2010). The assay mixture contained 40µl of spore suspension made up to 170µl with 50% PDB and Bromelain and positive control (at varying concentrations). About 40µl spore suspension serves as a control and sodium acetate buffer as blank and stem and fruit bromelain as test sample and added 10µl leupeptin to all the wells and incubated at 28°C. The fungal growth and inhibiting activity were monitored for 3-5 consecutive days by measuring the optical density at 492nm. The OD reading obtained were ANOVA (Analysis of Variance). If there is any significant variance the least and maximum possessed fungicidal activity were calculated for their percentage of fungicidal activity.

$$\text{Percentage of fungicidal activity} = \frac{\text{Control} - \text{Test}}{\text{Control}} * 100$$

Analysis of Variance (ANOVA) and Turkey *post hoc* analysis

The fungicidal activity was calculated using statistical technique, two-way ANOVA to test whether the means of more than two quantitative populations are equal. The determined values indicate if there is any significant difference in the variance both within and between the samples.

Turkey's honestly significant difference (HSD) post hoc test is used to analyses data that were not specified in ANOVA which is used in conjugation to find means that are significantly different from each other. This can be used to assess the fungicidal activity at specific day to determine the effective concentration.

RESULTS

ISOLATION AND CHARACTERIZATION OF FUNGUS

Isolation of fungus

The infected leaves and pseudo stem which showed symptoms of wilting and black streak diseases were inoculated on PDA and incubated at 28°C for 4 days and checked for the growth of fungal isolates. There was a characteristic growth of mycelia, carbon black spores and pigment producing mycelial fungus (Plate - 1).

Isolation of pure fungal colonies

Pure isolates were obtained by specific selection of fungal culture and identified for their morphology after sporulation. Three distinct fungal colonies were obtained which showed varied pattern of growth such as carbon black spore producing mycelia, pigment producing mycelial fungi and colony forming fungi on macroscopic observation (Plate-2).

Morphological characterization

The pure isolates were observed under light microscope at 40X magnification by lacto phenol cotton blue staining which showed characteristic growth of conidia, hyphae, macrospore, microspore and chlamydospore each of which showed distinct characteristics on PDA. (Plate - 3, 4 & 5).

Molecular characterization

Quantity analysis-UV-spectrometry

The quantity of isolated DNA was determined by UV-Spectrometry at Abs (260nm, 280nm and 320nm). The concentration of isolated DNA was determined and tabulated (Table-1).

The total yield of genomic DNA of respective fungal isolates were found to be around 110-365 ng/µl. On quantitative determination the ratio of respective fungal isolates was found to be ≤1, which indicates that the isolated DNA contains protein/phenolic contaminants which can be identified by qualitative analysis on 0.8% agarose gel.

Table – 1. Quantity determination of fungal genomic DNA

S.NO.	Sample	Ratio	Concentration(ng/µl)
1.	*Fusarium oxysporum*	1.001	110
2.	*Nigrospora spp.,*	1.003	365
3.	*Aspergillus niger*	0.952	200

Quality analysis-agarose gel electrophoresis

The proposed method yielded good quality of DNA from fungal isolates on agarose gel electrophoresis, which on comparison with standard 1 kb DNA maker was evident that the fungal isolate contained high molecular weight DNA. The molecular weight of isolated fungus (*Fusarium oxysporum,* and *Aspergillus niger*) was found to be 8 kb (Plate - 6).

Plate-1. Isolation of fungus

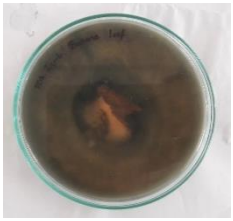


Plate-1(a). Infected banana leaf on PDA

Plate-1(b). Infected banana pseudo stem on PDA

Plate-2. Isolation of pure fungal isolates

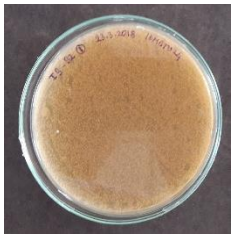


Plate-2(a). *Fusarium oxysporum* on PDA

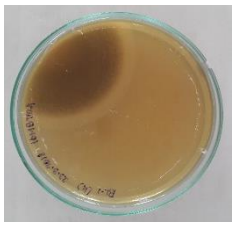


Plate-2(b). Fungal isolate

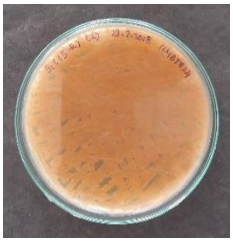


Plate-2(c). *Aspergillus niger*on PDA

Morphological characterization of isolated fungus

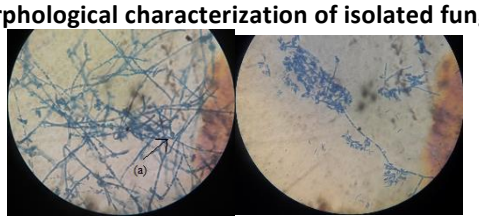


Plate-3(a). Single terminal chlamydospore Plate-3(b). False head monophiliades

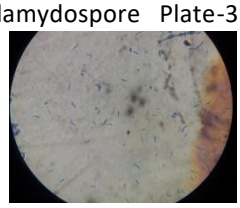


Plate-3(c) & (d). Microconidia and macroconidia

Plate – 3. Microscopic view of *Fusarium oxysporum* under light microscope at 40x magnification, (a) Single terminal chlamydospore (b) False head monophialides
(c) Microconidia (d) Macroconidia.

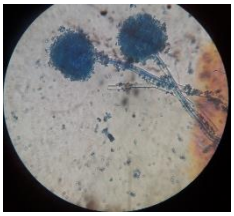


Plate-4(a) & (b). Conidia and Conidiophore

Plate-4(c). Hyphae

Plate – 4. Microscopic view of *Aspergillus niger* under light microscope at 40X magnification (a) Conidia (b) Conidiophore (c) Hyphae (d) Vesicle

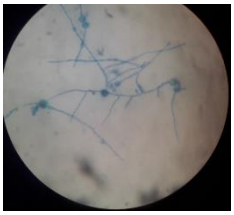


Plate-5(a). Immature chlamydospore and hyphae

Plate – 5. Microscopic view of fungal isolate *Nigrospora spp.,* under light microscope at 40X magnification (a) septate hyphae and conidiophore with inflated apex and black conidium

Plate-6. Quality analysis-isolation of dna

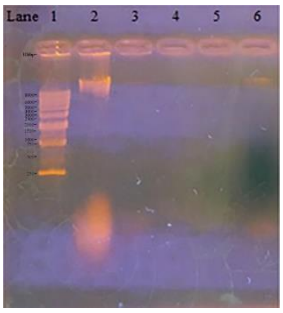


Plate-6. Bands of DNA on 0.8% agarose gel. Lane-1. DNA marker-1 kb ladder;
Lane-2. *Fusarium oxysporum;* Lane-3. *Nigrospora spp.,* Lane-6. *Aspergillus niger*

EXTRACTION AND CHARACTERIZATION OF BROMELAIN

Thin Layer Chromatography (TLC)

The R_f value was calculated from the distance travelled by the solute through the mobile phase solvent. The R_f values of unknown samples (a) fruit pulp – 0.41 and (b) stem – 0.40 on comparison with standard amino acid table were found to be similar to that of Cysteine which shows the extracted sample contains Cysteine (Plate-7).The R_f value of fruit pulp and stem extract, on comparison with standard TLC table of amino acids were found to be Cysteine (R_f = 0.4).

Plate-7. Thin Layer Chromatography (TLC)

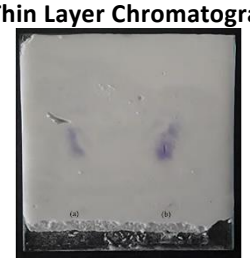


Plate – 7. TLC of (a) Fruit pulp and (b) Stem extract of pineapple showing distance travelled by solute

Estimation of protein-Lowry's method

The protein concentration of unknown samples of fruit pulp and stem of *Ananascomosus*was determined by Lowry's method by comparison with a standard protein BSA. The concentration of protein was found to be high in dialysed samples of fruit pulp and stem of *Ananascomosus* (Fig.-1).The concentration of protein was found to be high in dialysed sample of stem than fruit pulp with crude extract containing less amount of protein concentration due to interfering impurities.

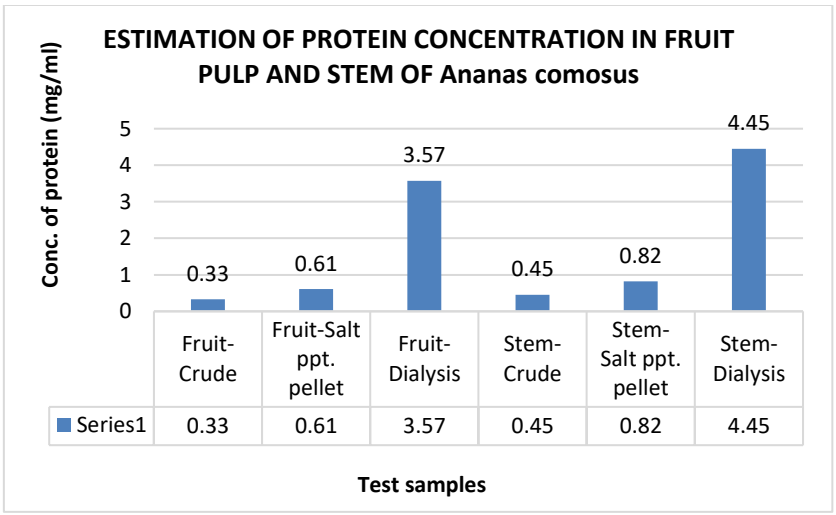


Fig. – 1. Estimation of protein concentration in fruit pulp and stem of *Ananascomosus* Estimation of protease enzyme activity

The enzyme activity of fruit pulp and stem extract of *Ananascomosus* was determined by modified method of Lowry's method by comparison with a standard amino acid Tyrosine. The enzyme activity was found to be high in dialysed samples of fruit pulp and stem of *Ananascomosus* (Fig.-2).The enzyme activity was found to be high in dialysed sample of stem than fruit pulp with crude extract containing less amount of activity due to interfering impurities.

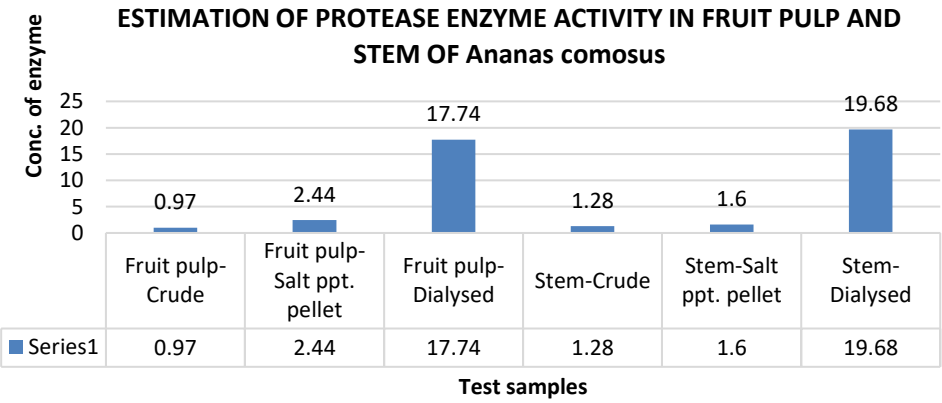


Fig.-2. Estimation of protease enzyme activity in fruit pulp and stem of *Ananascomosus* Gelatin Digestion Unit (GDU)

The GDU was found to be high in Ammonium sulphate salt precipitated sample of fruit pulp and stem extract due to presence of nitrogen containing ammonium group. (Fig-3)

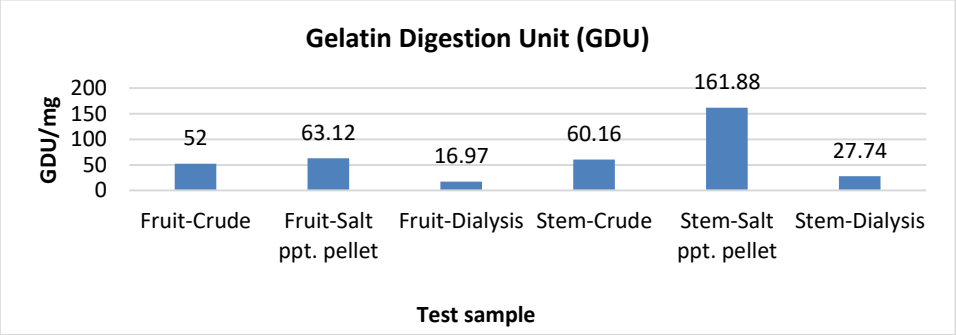


Fig 3 - Gelatin digestion unit of fruit pulp and stem extracts of *Ananascomosus* Determination of optimum pH of enzyme

The extracts were found to stable at a pH range of 6-8 indicating the stable pH of the enzyme (Fig.- 4 & 5). The activity of enzyme declined at a pH range from 10-12 which showed that the extracts were unstable above pH 10.

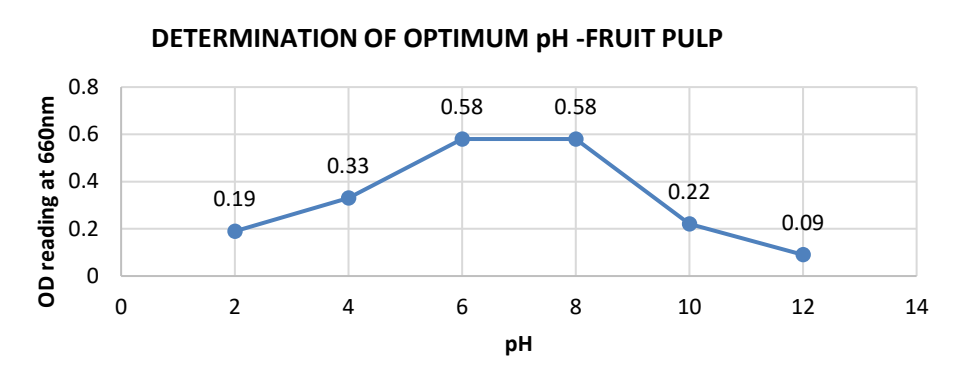


Fig.-4. Determination of optimum pH-Fruit pulp

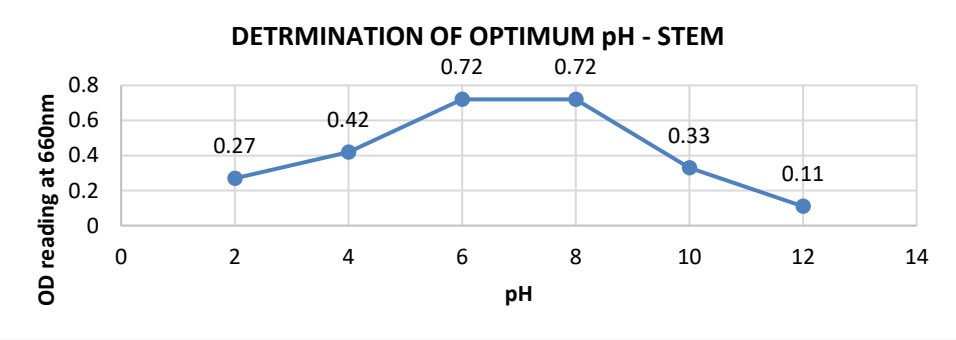


Fig.-5. Determination of optimum pH-Stem

Determination of optimum temperature

The stability of extracts was determined by incubating the extracts at varying temperatures of 4°, 20°, 60°, 80° and 100°. The extracts exhibited activity up to 60°C, after which there was a drastic decline in the activity which indicates that the extracts are stable up to 60°C (Fig.-6 & 7).

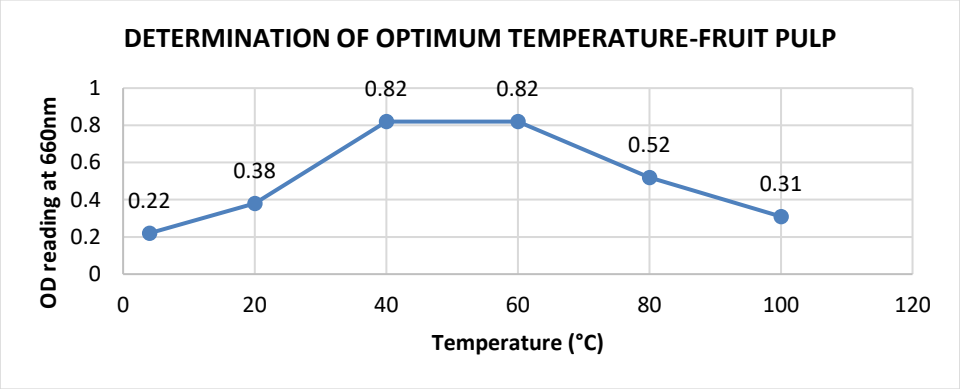


Fig. 6 – Determination of optimum temperature – Fruit pulp

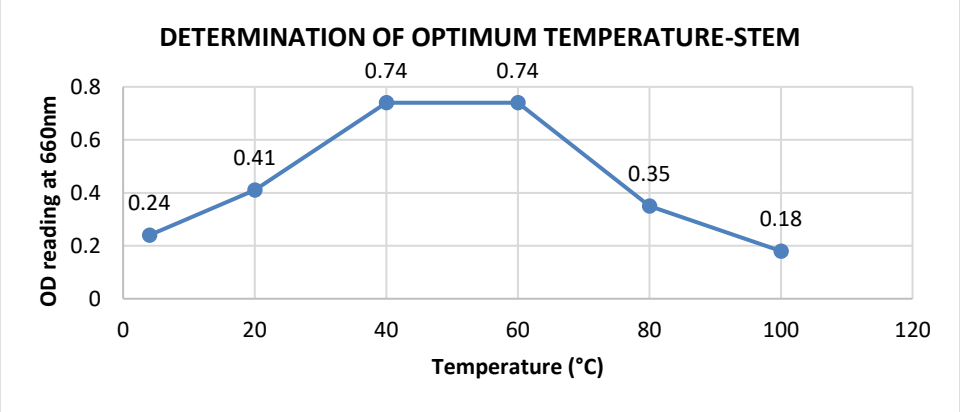


Fig. 7 – Determination of optimum temperature - Stem

Molecular weight determination by SDS – PAGE

The SDS-PAGE (Plate-8) showed that the molecular weight of fruit pulp and stem extracts of partially purified dialysed sample were of molecular range 29 kDa.Bromelain, The fruit pulp and stem dialysed sample (Lane 2&3) -PAGE, on comparison with molecular marker of medium range (94 kDa) had an intense band around 29 kDa, indicating that the molecular weight of unknown protein as 29 kDa which is bromelain.

Activity staining

The SDS-PAGE gel was subjected to activity staining to determine the presence/absence of Bromelain. The dark and intense bands on activity staining with 2% Casein substrate indicated formation of clear and intense band indicating the presence of bromelain (Plate-9).

Plate-8. Molecular weight determination by sds-page

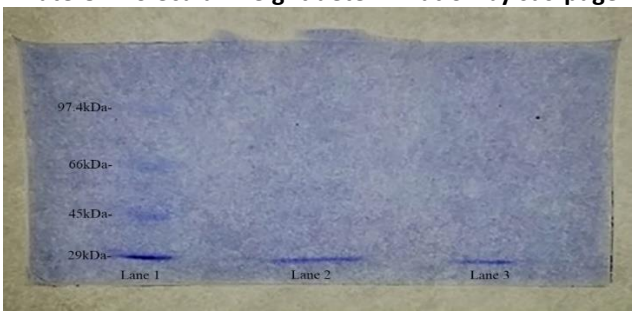


Plate - 8. SDS – PAGE of Dialysed protein samples on 12% resolving gel of fruit pulp and stem compared with medium range protein molecular marker – Lane 1- Medium range protein molecular marker; Lane 2 – Stem; Lane 3 – Fruit pulp

Plate-9. Activity staining by casein-gel electrophoresis

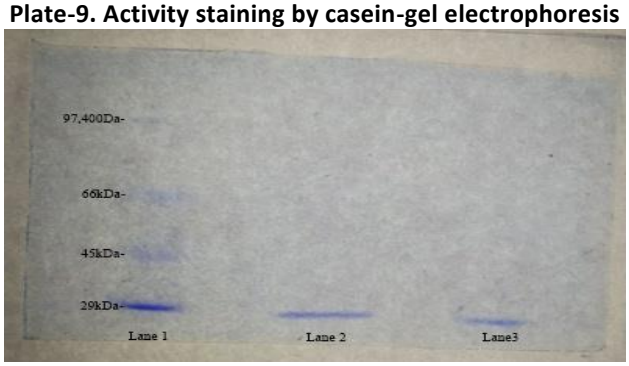


Plate – 9. SDS-PAGE gel after activity staining with 2% Casein substrate. The intense band and formation of clear zone around the bands indicate the presence of bromelain.
Lane -1. Protein molecular weight marker (94 kDa); Lane-2. Stem bromelain; Lane-3. Fruit bromelain.

Characterization of functional groups of bromelain by FT-IR

The functional group common to fruit pulp and stem were S-H stretch and secondary amide NH_2 stretch indicating the presence of functional group of cysteine which is a thiol peptidase.

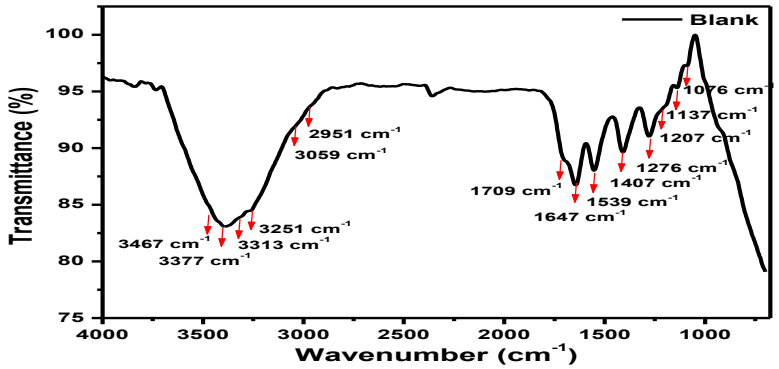


Fig.8 – FTIR spectra of blank-Sodium acetate buffer

Table – 2. FT-IR spectra peak of Sodium acetate buffer

Wavelength (cm^{-1})	Functional group	Peak characterization
1276	Aliphatic C-N	Stretching
1407	Azo compound N=N	Stretching
1539	Secondary Amide N-H C-N	Bending Stretching
1647	Primary Amide NH_2	Bending

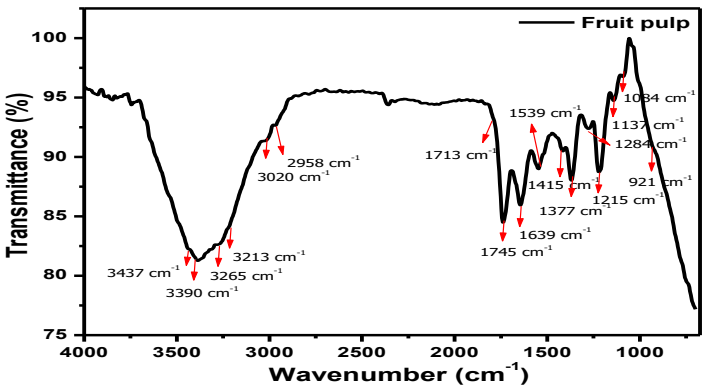


Fig. 9 – FT-IR spectroscopy of dialysed fruit pulp of *Ananascomosus*

Table – 3. FT-IR spectra peak of Fruit pulp of *Ananascomosus*

Wavelength (cm^{-1})	Functional groups	Peak characteristics
1215	Aromatic C-O	Stretching
1377	Aliphatic nitro compound NO_2	Symmetric Stretching
1539	Secondary Amide	
	N-H	Bending
	C-N	Stretching
1639	Primary amide NH_2	Bending
1745	C-H	Stretching
2958	S-H	Stretching
3390	Secondary amide N-H	Stretching

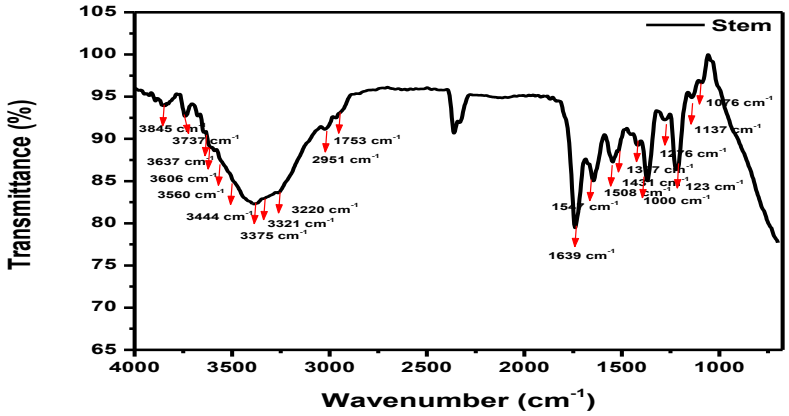


Fig. 10 – FTIR spectroscopy of dialysed stem of *Ananascomosus*

Table – 4. FT-IR spectra peak of stem extract of *Ananascomosus*

Wavelength (cm^{-1})	Functional groups	Peak characteristics
1137	Amines N-C	Stretching
1276	Aliphatic C-N	Stretching
1431	Azo compound N=N	Stretching
1508, 1547	Secondary Amide N-H C-N	 Bending Stretching
1639	Primary amide NH_2	Bending
2360, 2951	S-H	Stretching
3375	Secondary amide N-H	Stretching

***In vitro* ANTIFUNGAL ACTIVITY OF BROMELAIN AGAINST THE FUNGAL PHYTOPATHOGENS**

Antifungal susceptibility test – Agar well diffusion method

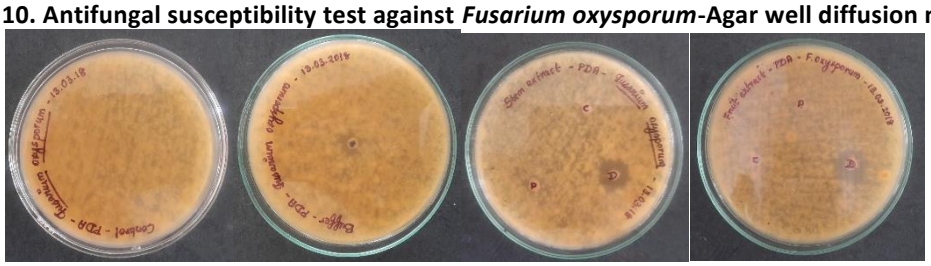
The fruit pulp and stem extracts of crude sample, Ammonium sulphate precipitation (pellet) and dialysed sample were assessed for their antifungal activity by agar well diffusion method. The extent of zone of inhibition indicate the antifungal activity of each of the extracts. The dialysed sample of stem extract containing bromelain and crude extracts possessed antifungal activity against *Fusarium oxysporum, Aspergillus niger* and *Nigrospora spp.*. The extent of zone of inhibition against the isolated plant phytopathogens are tabulated (Table-5).

Table – 5. Antifungal susceptibility test – agar well diffusion method – zone of inhibition

S.No.	Sample	Test sample	*F.oxysporum* (Plate-10)	*A.niger* (Plate-12)	*Fungal isolate* (Plate-11)
1		**Crude**	0.00	0.9	1.6
2	**FRUIT**	**Amm.SO_4 pellet**	0.00	0.00	0.00
3		**Dialysis**	1.9	0.9	1.5
1		**Crude**	0.00	1.2	2.0

	STEM	**Amm.SO_4 pellet**	1.1	0.00	0.9
2					
3		**Dialysis**	1.8	0.9	1.7
	Positive control	**Dazomet**	2.5	0.00	0.7
1					
2	**Blank**	**Bufffer**	0.7	0.7	1.5

Plate – 10. Antifungal susceptibility test against *Fusarium oxysporum*-Agar well diffusion method



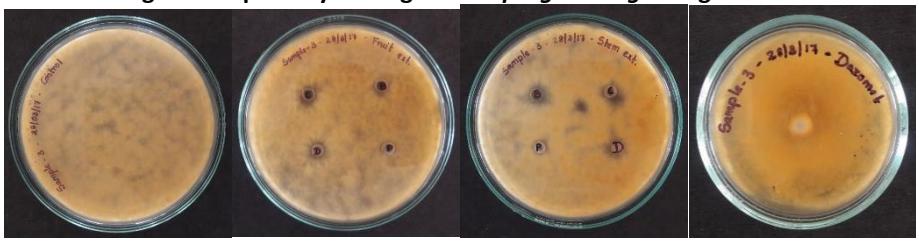
(a)Control (b) Blank-Buffer (c) Stem extract (d) Fruit pulp extract

(e) Positive control-0.1% Dazomet

Plate – 11. Antifungal susceptibility test against *Nigrospora spp.,*–agar well diffusion method

(a)Control (b) Blank-Buffer (c) Fruit extract (d) Positive control-0.1% Dazomet

Plate – 12. Antifungal susceptibility test against *Aspergillus niger*– agar well diffusion method



(a)Control (b) Blank-Buffer (c) Fruit extract (d) Positive control-0.1% Dazomet

B – Buffer-1M Sodium acetate buffer; C – Crude extract; P – Salt precipitation pellet; D – Dialysed sample

***In vitro* estimation of protein**

The protein concentration of fruit pulp and stem extracts (Fig.-11) were estimated *in vitro* with std. BSA. The so obtained concentration was compared with previously estimated protein concentration to determine if 50% PDB possess any inhibitory action. On comparison, it was observed that they possess inhibitory mechanism when supplemented in PDB medium.

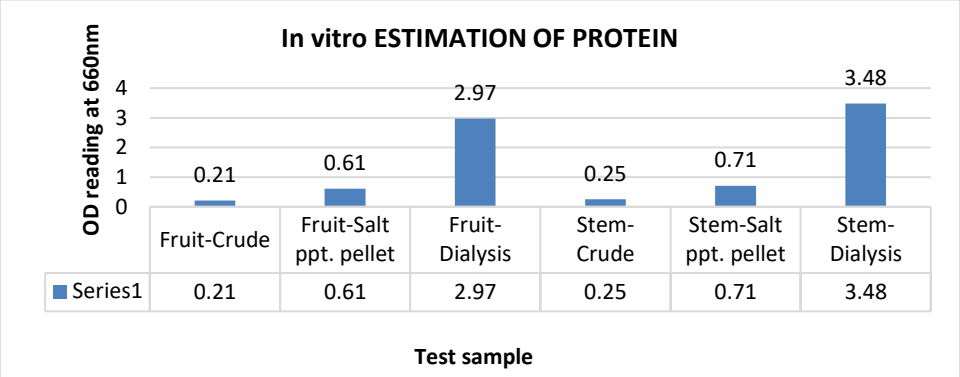


Fig. 11 – *In vitro* estimation of protein in fruit pulp and stem extract of *Ananascomosus* *In vitro* protease enzyme activity

The protease enzyme activity of fruit pulp and stem extracts (Fig.-12) were estimated *in vitro* with std. tyrosine. The so obtained concentration was compared with previously estimated protein concentration to determine if 50% PDB possess any inhibitory action. On comparison, it was known that they possess inhibitory mechanism when supplemented in PDB medium.

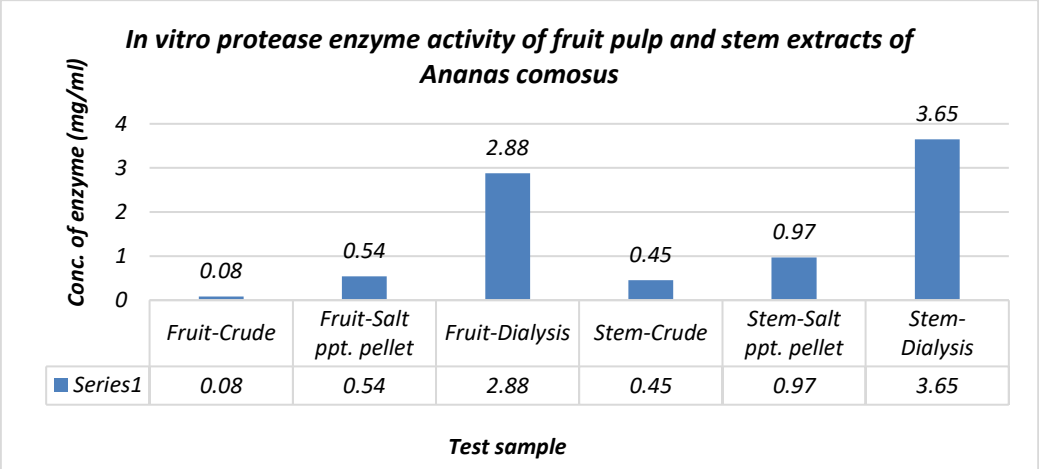


Fig. 12 - *In vitro* protease enzyme activity of fruit pulp and stem extracts of*Ananascomosus*

Comparative analysis on protein concentration and protease enzyme activity of Lowry's assay and *in vitro* assay of fruit pulp and stem extracts of *Ananascomosus*

The protein concentration and enzyme activity of fruit pulp and stem extracts were compared to determine if PDB possess any inhibitory action. On comparison,*In vitro* proteolytic enzyme activity with protease assay, the enzyme activity was found to be less in *in vitro* proteolytic assay which indicates that PDB possess inhibitory action toward bromelain when incorporated into the medium. To overcome this, protease inhibitor specific for cysteine proteases are added which preserve protein integrity after membrane lysis.

***In vitro* ANTIFUNGAL ASSAY**

The antifungal assay was carried out in varying concentration to determine the effective fungicidal property of bromelain by Broth micro dilution assay. The fungicidal activity of fruit pulp and stem bromelain with buffer as negative control and dazomet as positive control was assessed *in vitro* against *Fusarium oxysporum, Aspergillus niger*and *Nigrospora spp.,* (Table – 6, 7 & 8).

Table – 6 - 9. Percentage of fungicidal activity

Table – 6(a) *In vitro* fungicidal activity against *Fusarium oxysporum*(after 24 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	95.65	93.41	92.58	91.7	90.97	86.91
Fruit pulp	89.16	87.74	85.01	82.76	75.54	58.79
Stem	87.40	82.81	74.12	74.51	54.69	20.56
Dazomet	73.83	37.89	28.96	10.74	5.27	1.17

Table – 6(b) *In vitro* fungicidal activity against *Fusarium oxysporum* (after 120 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	99.76	99.36	99.24	99.20	99.08	98.96
Fruit pulp	99.64	99.36	98.80	98.68	98.44	98.36
Stem	99.80	98.32	97.84	96.42	96.16	99.08
Dazomet	99.08	99.40	98.40	96.72	96.24	95.60

Table – 7(a) *In vitro* fungicidal activity against *Aspergillus niger* (after 24 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	96.55	95.56	94.62	94.1	92.51	91.38
Fruit pulp	92.95	91.42	88.27	88.17	87.58	74.08
Stem	97.19	88.81	84.67	84.67	64.47	20.89
Dazomet	99.16	46.08	28.98	12.46	6.26	1.01

Table – 7(a) *In vitro* fungicidal activity against *Aspergillus niger* (after 120 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	99.68	97.65	97.36	97.21	96.5	96.3
Fruit pulp	95.53	94.34	92.98	92.15	91.87	81.69
Stem	96.45	95.45	94.53	93.86	87.00	41.92
Dazomet	91.51	80.22	76.47	70.73	64.4	98.47

Table – 8(a) *In vitro* fungicidal activity against *Nigrospora spp.,* (after 24 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	97.28	96.80	96.52	96.23	95.61	52.31
Fruit pulp	96.95	96.61	96.28	96.14	95.422	46.63
Stem	74.68	70.2	68.19	62.28	59.42	43.92
Dazomet	44.35	27.94	87.73	7.53	4.72	3.2

Table – 8(b) *In vitro* fungicidal activity against *Nigrospora spp.,* (after 120 hrs)

	% of fungicidal activity					
Sample	**20µl**	**40µl**	**60µl**	**80µl**	**100µl**	**120µl**
Buffer	99.96	99.51	99.25	99.22	99.17	9910
Fruit pulp	99.81	99.25	98.58	98.54	98.47	98.13
Stem	99.93	99.81	98.39	97.42	97.09	95.29
Dazomet	99.85	99.78	97.94	97.94	96.73	95.32

Analysis of Variance

The variance was determined at 0.05% significance which showed that the test samples possessed significant variance between each column (Concentration) and rows (days).

TURKEY *post hoc* TEST

The Turkey *post hoc* test was used to determine the significant difference between each sample number within each concentration for multiple comparison. This determines the variance within each groups, between each samples and significance among each samples (Table – 9, 10 & 11).

Table – 9. Turkey *post hoc* analysis of *Aspergillus niger*

S.NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	120	72 -120+
2.	**Fruit pulp**	120	72 – 120+
3.	**Stem extract**	120	120+
4.	**Dazomet**	120	120+

Table – 10. Turkey *post hoc* analysis of *Fusarium oxysporum*

S.NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	100	72 -120+
		120	24-120+
2.	**Fruit pulp**	100-120	24 – 120+
3.	**Stem extract**	120	72-120+
4.	**Dazomet**	40-120	24-120

Table – 11. Turkey *post hoc* analysis of *Nigrospra spp.,*

S.NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	120	24-120+
2.	**Fruit pulp**	120	24 – 120+
3.	**Stem extract**	20-80	24-120+
		100	120+
		120	72-120+
4.	**Dazomet**	20-120	24-120+

CONCLUSION

Bromelain has been widely used as a novel therapeutic substance due to its diverse proteolytic mechanism of action which still is unknown. The use of this cysteine protease as a fungicidal agent against plant pathogens has not been much known. The *in vitro* evaluation study herein shows that there is a significant difference in the growth of fungal pathogens infecting *Musa paradisiaca* after 3 days of supplementation of bromelain at a concentration range of 100-120µl which contains nearly 0.5mg of protein indicating that bromelain possess inhibitory action against *Fusarium oxysporum, Aspergillus niger* and *Nigrospora spp.,*. The highest fungicidal activity was exhibited after 5 days on supplementation of bromelain, which indicates that bromelain from *Ananascomosus* can be used as a substitute for chemical fungicides in order to retain the natural environmental conditions and overcome the hazards caused due to chemical fungicides.

ACKNOWLEDGEMENT

We would like to thank the Management, Principal and Head of the Department of our institution for their timely help and constant support to complete this research work.

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