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Bromelain A Cysteine Protease: Helps To Reduce Infection Caused By A*cinetobacter* spp., A Nosocomial Pathogen.

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ABSTRACT

Bromelain is a cysteine protease which is found in the tissues of plant from Bromeliacea family of which pineapple Ananas cosmossus is best known. Bromelain shows antibacterial activity and has been shown kill several forms of bacteria particularly involved in bronchial, respiratory and urinary tract infections in humans. Acinetobacter spp. is a gram negative bacteria widely distributed in nature and commonly found in human flora which is a major nosocomial pathogen involved in respiratory tract and other infections. The Acinetobacter spp. cause infections by colonization by secreting specific carbohydrate binding protein (lectin).The present study was aim to determine the effects bromelain a cysteine protease in controlling Acinetobacter infections in human flora.
The bromelain was extracted and characterized from plant Ananas cosmossus and crude extract was prepared. The Acinetobacter were isolated from upper respiratory tract of healthy humans and characterized for lectin activity and compared with standard Acinetobacter culture. The antibacterial activity of bromelain extract was found against Acinetobacter spp by agar well diffusion method. Again Acinetobacter spp isolates checked for lectin activity.

Key words: Bromelain, Acinetobacter, Proteases, Lectin.

INTRODUCTION

The control of hospital acquired infection caused by many resistant gram negative bacilli has proved to be a particular problem today. Nosocomial infections caused by *Acinetobacter* strains are increasing all over the world.[17] It is now recognized that *Acinetobacter spp*. play a significant role in the colonization of infection of patients admitted to hospital.*Acinetobacter spp*. is responsible nosocomial pathogen causing respiratory tract infections, urinary tract infections, secondary meningitis and many other.[9] *Acinetobacter spp* are Gram negative, strongly aerobic, catalase positive and oxidase negative, non motile encapsulated rods with DNA G+C content of 39-47%.[18]

Acinetobacter is the only gram negative genus found on the human skin.[17]In general *Acinetobacter* species are considered nonpathogenic to healthy individuals.[29]*Acinetobater spp*. are commonly

found as a part normal human flora like skin, respiratory tract, genitourinary tract and conjuctiva. They are able to colonize on the surface. *Acinetobacter species* role as a part of human flora its role is unclear.[17] The colonization of bacteria on human mucosal surface tissue is a prerequisite for an infection.[18] For successful colonization of mucosal surface the organism needs to bind to the epithelial cells of these tissues otherwise they are eliminate by the host defense mechanism. The attachment of bacteria to human require adhesion factor. *Acinetobacter spp*. recognizes glycoconjugate on other cells. The adhesions are called as lectins. [18] Acinetobacter have been shown to aggregate bacteria that otherwise do not form aggregates causing formation of biofilm. Biofilm formations is an important feature of most clinical isolates of Acinetobater spp. and play a role in infections diseases such as cystic fibrosis and periodontitis.29

Acinetobacter species are resistant to many classes of antibiotics including penicillin, chlorampenicol and often aminoglycosidase.[29] The ability of *Acinetobacter* species to adhere to the surfaces ,form biofilm and display antibiotic resistance has becoming the factors responsible for their growth.[29,22]. Therefore there is a need for development of naturally occurring compound as medicine for the treatment of infections caused by this nosocomial pathogen.[15]

Many types of plants produce proteolytic enzymes.The pineapple plant (*Ananas cosmosus*) was shown to contain at least four cysteine proteinase, from them the major cysteine proteinase of pineapple fruit is called as bromelain which is a member of papain superfamily.[24]Bromelain is a crude extract from the stems and immature fruits of pineapple constituting an unusually complex mixture of different thiol-endopepetidase and other.[13] in addition bromelain contain several proteinase inhibitors.[13] Bromelain is a cysteine protease which cleaves glycyl,alanyl and leucyl bonds.[13]Bromelain has various medicinal activities such as anti-inflammatory, anticancerous, antithromotic,immunomodulatory,antiedematous.[5, 7.] The antiviral and antibacterial activity of bromelain has been shown to kill several forms of viruses and bacteria particularly involved in bronchial and respiratory tract infections.play.5,7,13.

In this paper we study the antibacterial activity of fruit bromelain against nosocomial pathogen *Acinetobacter spp.* which is responsible for various tracts infections mainly respiratory tracts infections. As bromelain is a cysteine protease and *Acinetobacter spp*. cause infections by colonizing on tracts of humans by secreting lectins. We also focus on effect of bromelain on this lectin activity.

2. MATERIAL AND METHODS:

Many of the reagents were obtained from the sigma. *Acinetobacter calcoaceticus* (NCIM-2890) culture was obtained from National Chemical Laboratory, Pune. In addition fresh pineapples were purchased from local supermarket.

2.1 Enzyme Extraction and Purification

Fruit bromelain was extracted and purified from fresh pineapple fruit. The leaves and outer husk were removed from the fruit. The main fresh fruit portion weighed around 50 g was diced into small cubes and minced in a blender.[2]This was then mixed with 35ml of cold 0.1M sodium phosphate buffer (p H 7.0) containing 2mg/ml cysteine as a diluent.Homgenize for exactly 1 min. The resultant homogenate was then centrifuged at 2000x g for 10 min. The supernatant was then decanted into cold beaker. The supernatant was then filtered through a piece of filter paper using cold funnel, filtering in the cold room and then filtrate collected in cold 100ml beaker containing prechilled acetone. The solution was then incubated at 4^0 c for overnight. Then next day solution was again filtered and centrifuged at 2000X for 10min .The pellet were taken and dried in vacuum oven at 40^0 c.[4]

2.2 Characterization

2.2.1. Enzyme Assay:

Bromelain is a thiol proteinase, protein digesting enzyme. Many substrates like gelatin, casein, collagen are susceptible to this enzyme. The bromelain enzyme proteolytic activity was checked by assay against gelatin as substrate by gelatin digestion unit method. One gelatin digestion unit

Is the amount of enzyme which will liberate after 20 minutes digestion at 45 C,1 mg of amino nitrogen from a standard gelatin solution at $_{P}$H 4.5 OR 5.[5.10,28,3]. Activity was calculated by finding GDU units. A Bromelain enzyme solution was prepared by adding 8.3ml buffer solution in 50mg bromelain powder. The mixture was diluted to 50ml using distilled water ($_{P}$H 4.5) for 5 min and stirred for about 15 min.25 ml substrate were taken in 100 ml beaker and hold in a water bath at 45^0 C until temperature equilibrium is established. Then 1ml enzyme solution was added and stirred .[3, 10.28] After 20 min digestions at 45^0 c, two drops of 3% Hydrogen Peroxide solution were added and stirred again. Then beaker was removed from water bath, pH was adjusted with sodium hydroxide and 10ml 37% formaldehyde were added. Titrated to p H 7.8.This was taken as blank 25ml substrate[28] with two drops of 3% Hydrogen Peroxide and 1ml enzyme solution was used. The above beaker removed from water bath and adjusted to $_{P}$H 6.0 and 10ml 37% formed were added .Titrated to $_{P}$H 7.8.This was then taken as blank B.GDU unit was calculated by using formula.[3, 10.28]

2.2.2. SDS-Polyacrylamide gel electrophoresis:

SDS-PAGE was carried out using polyacrylamide 15% separating gel and 3% stalking gel was used. The gel was run for 50mA till the dye reached separating gel. The gel was stained with

Coomasseae Brilient Blue G250 .The gel was observed for bands and molecular weighed was determine by using molecular marker in the range of 66,000 Da to 14,300 Da.

2.3. Isolation and identification of Acinetobacter species from upper respiratory tract of humans:

The isolation was done by throat swabbing with the help of sterile cotton soaked in saline. The throat sample from five individuals between the age of 22-24 yrs. were collected.[29] The swabs were immediately streaked on plates containing glucose yeast extract medium [24] and mineral medium with containing crude oil[24].Plates were incubated at 37^0 c for 24 to 48 hrs. Morphological and biochemical identification was carried out on the basis on oxidase activity, motility, Gram staining.[21]

2.4. Screening for lectin activity of *Acinetobacter spp*:

All the isolates were grown separately in Luria Bertaini broth at 37^0 C in 250 ml Erlenmeyer's flask for 120hr and lectin activity was checked after every 24^{th} hrs, by hemagglutination assay.[21]The time course of lectin production by *Acinetobacter spp* was followed by monitoring the protein content as well as hemagglutination activity after every 24^{th} up to 120h.[29]

2.5. Partial purification of lectin from *Acinetobacter spp*:

The culture of *Acinetobacter* spp. was grown overnight in Luria Bertaini Broth (5ml) was inoculated in 500ml Luria Bertaini Broth and incubated at 37^0 C for 120h.After incubation the broth was centrifuged at 10,000rpm at 4^0C for 40 minutes and the culture supernatant obtained was subjected to ammonium sulfate precipitation. A total of four ammonium sulfate saturation were checked, 20%, 40%, 60% and 80% .After centrifugation the precipitate was dialyzed against against 0.15 M NaCl for 24hr at 20^oC. [29] The protein estimation was one by using Folin Lowery Method using bovine serum albumin as standard.[21]

2.6. Characterization of lectin

Hemagglutination assay was performed as follows. From the original partially purified lectin solution a series of decreasing concentration was prepared and agglutination capacity checked by using 3% human erythrocyte suspension.[29]

2.7. Activity of Bromelain against *Acinetobacter spp*:

2.7.1 Preparation of Extract of Bromelain:

1mg bromelain powder obtained from 2.1 weighed and was taken in two separate round bottom flask and 250 ml benzene was added to the powder in the flask. Above flask were subjected to reflux condenser by attaching reflux condenser to the flask. The condensing was carried out for 1hr.The distillate was collected in separate flask, the round bottom flask were washed again and distillate was again collected in it and reflux condensing for 1hr again. The extract was then used for agar well diffusion method.

2.7.2 Agar well diffusion method:

0.1 ml of 24 hr. old culture of isolates was spreaded with sterile nutrient agar plate. 22 ml Agar well was bored with 7mm cork borer. Benzene extract of bromelain were filled in the wells of 25ul in each well with micropipette .Benzene was taken as positive control. Plates were incubated at 37^0 C. The plates were then observed for zone of inhibition.

RESULTS:

2.1 Enzyme Extraction and Purification

Fruit bromelain was extracted and purified from fresh pineapple.Bromelain was extracted in the form of yellowish powder after drying the filtrates in oven at 40^0 c.The crude bromelain was used for further study.

2.2 Characterization

2.2.1. Enzyme Assay:

Generally Bromelain enzyme activity is optimum when it shows GDU between 1100-1500GDU /gm.[28] The values obtained for titer (t) was 5.5ml and for blank was 2ml.Normality of standardized NaOH was 0.100 and weight of enzyme was 2 mg. The value of GDU for bromelain was calculated as per formula and it was found to be 1225 units/g.

2.2.2. SDS-Polyacrylamide gel electrophoresis:

SDS-PAGE was carried out using polyacrylamide 15% separating gel and 3% stalking gel was used. The band pattern and molecular weight was determined. After electrophoresis of fruit bromelain a single band was observed. The molecular weight of bromelain was found 25,000 Da by comparing with molecular marker used which ranged from 14,300 Da to 66,000Da having marker proteins as Bovine serum albumin(66kD), ovalbumin (43kD),carbonic unhydrase (29 kD),lysozyme(14.3 kD).[Fig 1]

2.3. Isolation and identification of Acinetobacter species from upper respiratory tract of humans:

Five healthy human volunteers from the age group 21-23 years were selected**.** Throat sample were streaked on plates containing glucose yeast extract medium [24] and mineral medium with containing crude oil. After 48-56th hrs. incubation colonies were observed on both types of media plates. On both media plates pale yellow color, smooth colonies were observed after 48hrs incubation. Colonies were similar with culture obtained from National Chemical Laboratory after reviving on nutrient agar media.[Fig2] The colonies were checked for characters like gram staining, oxidase activity and motility. Cultures were found gram negative, oxidase negative and non-motile.

2.4. Screening for lectin activity of *Acinetobacter species*:

Acinetobacter calcoaceticus isolates were screened for lectin activity for a period of 120hr.The lectin activity revealed increase in protein content and hemagglutination activity and it was given in table1.The lectin was produced within 48 hr. in isolates and after 24 hr. in culture obtained. [Table 1& Table 2]

2.5. Partial purification of lectin from *Acinetobacter species*:

The precipitate obtained at 60% ammonium sulfate saturation was dissolved in sterile distilled water ad dialyzed against 0.15Msodium chloride for 24 hr. The activity was obtained in the fraction precipitated at 60% saturation which was checked by hemagglutination assay.

2.6. Characterization of lectin

Erythrocyte suspensions from four different blood group samples were checked for agglutination.

The erythrocyte suspension from samples A Rh + were agglutinated by the lectin produced from *Acinetobacter calcoaceticus.* Figure3 & Table 3

2.7. Activity of Bromelain against *Acinetobacter calcoaceticus*:

2.7.1 Preparation of Extract of Bromelain:

The condensate after reflux condensation was obtained. It was collected in separate flask and used for agar well diffusion method.

2.7.2 Agar well diffusion method

The zone of inhibition was observed from the two isolated and also from the culture obtained. The diameter of zone of inhibition was calculated and compared with control. It was found more in the benzene extract of bromelain. The isolate 1 show zone of inhibition of around 20mm. The isolate 1 show zone of inhibition of around 22mm.The highest activity was shown by the culture obtained which was 24mm. Figure 4 & Table 4

DISCUSSION:

Fruit bromelain was extracted and purified from fresh pineapple fruit. Molecular weight of fruit bromelain were determined as 25,000Da from the SDS-PAGE Electrophoresis by using standard marker of known molecular weight. The activities of bromelain were determined by assaying enzyme bromelain against gelatin as a substrate and activity was confirmed by finding the gelatin digestion unit value as 1225 units/g.

Acinetobacter species were isolated from upper respiratory tract of human volunteers and culture revived obtain from National Chemical Laboratory, Pune. The lectin activity of *Acinetobacter species was* found after 20 hrs of incubation and hemagglutination activity was found against human erythrocyte suspension of blood from different blood groups and agglutination was found of Acinetobacter calcoaceticus against blood group a Rh +ve.

The proteolytic activity of bromelain against *Acinetobacter calcoaceticus* was determined by agar well diffusion and bromelain inhibited colonies on media plates and highest zone of inhibition was found around 24mm.

CONCLUSION:

Acinetobacter spp. cause infections by colonizing on tracts of humans by secreting lectins. The fruit bromelain a cysteine protease act on lectin and cause decrease in lectin activity of *Acinetobacter spp* thus reduce colonization of bacteria. Thus bromelain can be good source of natural medicine for treating the *Acinetobacter spp* infections in humans.

REFERENCES:

1. A.Jawad,P.M.Hawkey et al. Description of Leeds Acinetobacter Medium, a New Selective and Differential Medium for Isolation of Clinically important Acinetobacter spp,and comparison with Herellea Agar and Holton's Agar. Journal of Clinical Microbiology.vol.32,No10, Oct.1994.p2353-2358.
2. Andrew D. Rowan,* David J. Buttlet et al. The cysteine proteinases of the pineapple plant. Biochem. J. (1990) 266, 869-875.
3. Arun & co, Mumbai. Bromelain; Activity Assay: Gelatin Digestion Units.
4. Biochemistry 4124.Isolation and characterization of bromelain from pineapple plant.
5. Cathy Wong. Benefits Of Bromelain Enzyme .About.com Guide.october 03,2011
6. Christhoper W. Wharton . The Structure and Mechanism of Stem Bromelain. Biochem. J. (1974) 143, 575-586.
7. Desire Hendricks, What are the function of Bromelain enzymes in pineapple?;eHOw Coimtributor.
8. Dixon.M.and Webb E.C.Enzymes.3rdEdition, Academic Press: New York, an Fransico.p.890(1979).
9. E.Bergogne-Berezin,K.J.Towner. Acinetobacter spp.as Nosocomial Pathogens: Microbiological ,Clinical, and Epidemological Features; Clinical Microbiology Reviews, Apr. vol 9,No 2,1996,p.148-165.
10. Enzyme Development Corporation, New York.; Gelatin digestion unit method(GDU) method;(212) 736-1580.

11. Eric R. Secor Jr1, William Fet al. Oral Bromelain Attenuates Inflammation in an Ovalbumin-induced Murine Model of Asthma, eCAM Advance Access published March 14, 2007; eCAM 2007;Page 1 of 9, doi:10.1093/ecam/nel110.
12. E. SilveiraI; M. E. Souza. Expanded bed adsorption of bromelain (E.C. 3.4.22.33) from Ananas comosus crude extract. Brazilian Journal of Chemical Engineering; Eng. vol.26 no.1 São Paulo Jan./Mar. 2009.
13. H.R.Maurer.Review: Bromelain: biochemistry, pharmacology and medical use. CMLS Cellular and Molecular Life Science;Cell. Mol .Life Sci 58 (2001) 1234-1245.
14. Holton J.A note on the preparation and use of a selective and differential medium for the isolation of the Acinetobacter spp from clinical sources.J.Appl.Bacteriology 1983;54: 141-142.
15. Jerzy M Behnke, David J Buttle et al. Developing novel antithelmintics from plant cysteine proteinases.Parasites&Vectors2008,1:29 September2008;.
16. Ji-Young KIM, Hyun-Jong YANG et al. Partial characterization of a 29 kDa cysteine protease purified from Taenia solium metacestodes. The Korean Journal of Parasitology. Vol. 43, No. 4. 157-160, December 2005.
17. J.R.Patil,B.A.Chopade.Distribution and in vitro antimicrobial susceptibility of Acinetobacter species on the skin of healthy humans. The Natinal Medical Journal Of India,vol.14,No.4,2001.
18. Jyoti R Patil,Neelakshi R Jog,B.A.Chopade. Isolation and characterization of Acinetobacter Spp. From upper respiratory tract of healthy humans and demonstration of lectin activity.Indian Journal Of Medical Microbiology.(2001)19 (1): 30-35.
19. Laishram Rupachandra Singh et al. Enzymological characterization of pineapple extract for potential application in oak tasar (Antheraea proylei J.) silk cocoon cooking and reeling. Electronic Journal of Biotechnology, Vol. 6 No. 3, Issue of December 15, 2003.
20. Ortiz Barbara, Zazil, Nayarit (MX).European Patent Application .International Publication Number :WO2007/148951 (27.12.2007 Gazette 2007/52).
21. P.Gunasekaran.Laboratory manuals in microbiology. New Age International Publishers.
22. Reinhold J.Baues ,Gary R.Gray , Lectin Purification on Affinity Columns Containing Reductively Aminated Diasaccharides.The Journal Of Biological Chemistry.Vol.252,No.1,Jan 10. 57-60,1997.
23. Rokhbakhsh-Zamin Farokh et al. Characetrization of plant growth promoting traits of Acinetobacter species isolated from Rhizosphere of Pennisetum glaucum. J.Microbiolgy and .Biotechnolgy.(2011),21(6).556-566.
24. Ronald M.Atlas**.**Handbook of Enviornmental Microbiology; second edition.236,390.
25. Rosenber lier,Mediwound ltd. International Application Published Under The Patent Corporation Treaty (PCT);World Intellectual Property Organization; International Publication Number WO2006/054309 A2; 26/05/2006.
26. S. Sathivel, K. Niranjan et al. 11A-19. Increasing efficiency of bromelain extraction from pineapple and its wastes.The IFT Annual Meeting1999.
27. Tibor Harrach,1 Klaus Eckert et al. Isolation and Characterization of Two Forms of an Acidic Bromelain Stem Proteinase. Journal of Protein Chemistry, Vol. 17, No. 4, 1998.
28. www.mpbio.com.Bromelain ;Catalog Number;101142.
29. www.wikipedia.com.Acinetobacter.

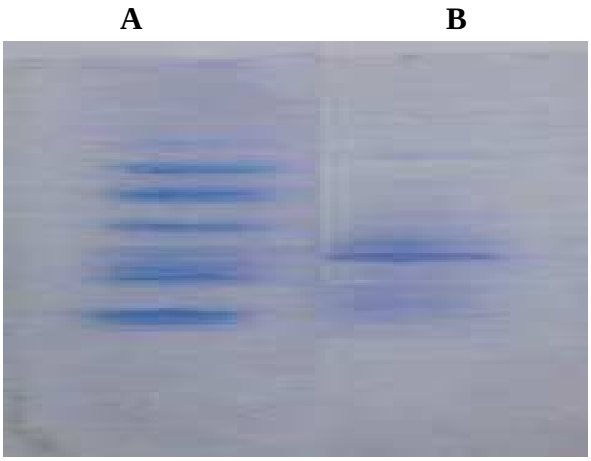

Fig.1 SDS-PAGE pattern of fruit bromelain A) Molecular weight standard marker ranging from 14,300 Da, 29,000Da,43,000Da,66,000Da B) Band observed of fruit bromelai fraction arong 25,000Da.

Fig. a **Fig. b**

Fig.2 a.Colonies of Acinetobcter species on glucose yeast extract medium b. Colonies of Acinetobcter species on mineral medium with containing crude oil c. Colonies of Acinetobcter scalcoaceticus on nutrient medium.

Time (hrs)	Total Protein Content (g/ml)		
	Isolate 1	Isolate 2	Isolate 3
20	6	7	6
48	7	7	9
72	9	8	10
96	10	10	12
120	11	12	12

Table 1. Protein estimation by lowrey method of Acinetobacter Species isolates from 20 hrs of incubation till 120hrs.

Time (hrs)	Hemagglutination Activity (units/ml)		
	Isolate 1	Isolate 2	Isolate 3
20	23	32	32
48	24	36	36
72	25	40	48
96	26	45	48
120	26	48	50

Table 2. Hemagglutination activity of Acinetobacter specied isolates from 20hrs to 120hrs incubation.

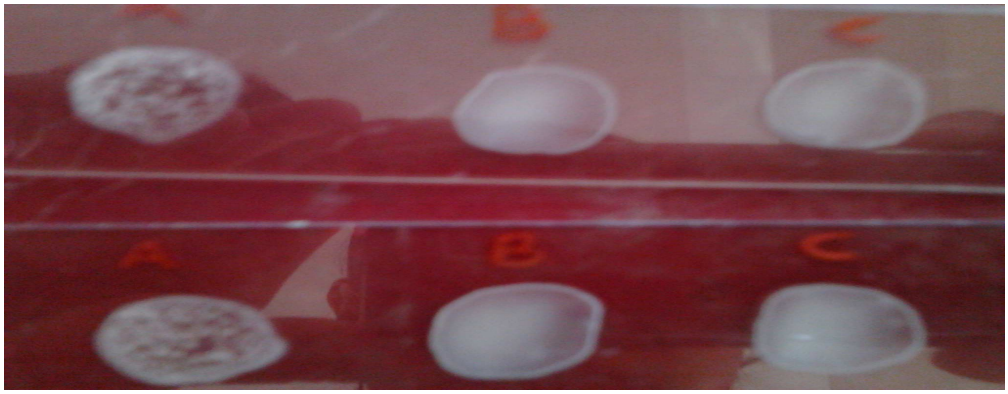

Figure 3. Agglutination of *Acinetoacter calcoaceticus* isolates on four human blood gropus.1) Sample A contains blood group A Rh + 2)Sample B contains blood group B Rh + 3) Sample C contains blood group AB Rh + 4) Slide 2 sample A contains blood group 0 Rh +.

Blood Group	Hemagglutination
A Rh +	_
B Rh +	_
AB Rh +	_
O Rh +	+

\+ Hemagglutination - No Hemagglutination

Table 3. Blood group specificity and hemagglutination of activity of *Acinetobacter calcoaceticus*

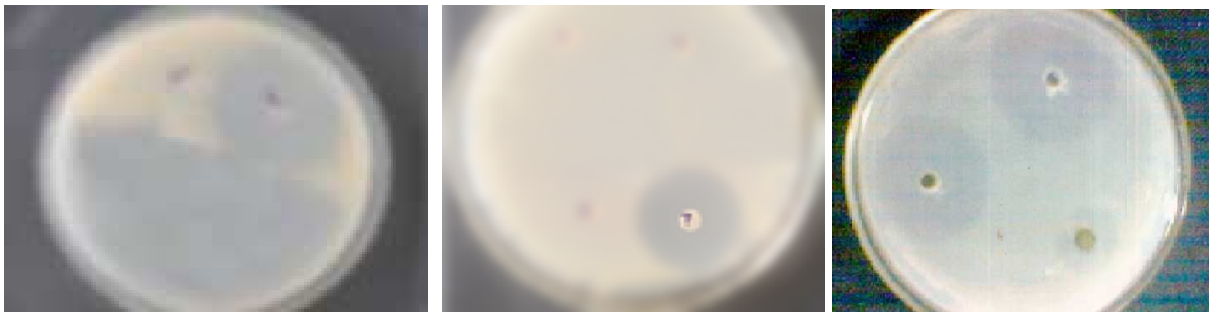

Fig.a **Fig.b** **Fig.c**

Figure 4. Zone of inhibition observed against Acinetobacter calcoaceticus on bromelain extract.a) Islate 1 from human voluteer 1. 2) Isolate 2 from human volunteer 2. 3) Culture Of *Acinetobacter clacoaceticus*.

Sample	Zone Of Inhibition (mm)
Isolate 1	20
Isolate 2	22
Isolate 3	24

Table 4. Zone of inhibition of *Acinetobacter calcoaceticus* isolates (mm).