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Evaluation of latex proteins for Lectin, protease, antibacterial activities and protein profiling from the genus *Artocarpus*

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Abstract
In this work, the latex of *A. integrifolia, A. hirsutus, A. lookocha,* and *A. altilis*, one of India's oldest fruits in the Western Ghats region, was examined for lectin, protease, and antibacterial activity, as well as protein profiles, using SDS-PAGE. Partially purified lectins were extracted using ammonium sulphate precipitation and dialysis. Latex from *A. integrifolia* substantially agglutinates pig, goat, and sheep erythrocytes but not human erythrocytes, whereas latex from *A. altilis* exhibits strong lectin activity against trypsinized human and rabbit erythrocytes. The lectin activity of *A. hirsutus* and *A. lookocha* latex is quite low. Despite high protein levels, latex from four different species has different protein compositions with molecular weights ranging from 170 to 10 kDa. Antibacterial activity was investigated using the agar well diffusion method. In the agar well diffusion assay, the partly purified lectins from all four *Artocarpus* species revealed to have antibacterial action against diverse microbial pathogens. The partially purified lectin from *A.atltilis* displayed substantial antibacterial activity against *K. pneumonia, P. aeruginosa, S. typhimurium,* and *B. subtilis,* whereas the lectin from *A. hirsutus* efficiently inhibits *K. pneumonia* and *S. typhimurium* growth. *K. pneumonia, P. aeruginosa,* and *B. subtilis* were all inhibited by the lectins from *A. heterophyllus* and *A. lakoocha.* None of the latex lectins that had been partly purified were ineffective against *E. coli*. The presence of these activities in latex suggesting their significance in phytopathogen and insect resistance as well as predator protection.

Keywords: artocarpus, latex, lectin, casein proteolytic, antibacterial, protein profiling

Introduction
Lectins are a class of carbohydrate-binding proteins or glycoproteins of non-immune origin that are distributed ubiquitously in nature. Lectins provide a variety of biological activities due to their unique carbohydrate recognition. Plants, mammals, fungi, and bacteria all have lectins, which have the ability to interact with carbohydrates and agglutinate cells or precipitate glycoconjugates. They are crucial in the fight against the invasion of bacteria, viruses, and fungi **[1].** Plant-based medications are widely utilized in both traditional and modern medicine, and they are growing increasingly popular. According to the World Health Organization, plant-based medications are used by more than 80% of the world's population in developing nations for basic healthcare [2]. Morus, Ficus, and Artocarpus, for example, are inexpensive food sources and are widely employed in traditional medicine, agriculture, and industry [3]. These genera have piqued scientists' interest because they contain medicinally relevant secondary metabolites with biologically beneficial properties. In Southeast Asia, particularly Indonesia, the western portion of Java, and India, a number of *Artocarpus* species are utilized as food and for traditional folk treatments. Plants of the *Artocarpus* genus are a valuable multipurpose crop that produces both fruits and timber. *Artocarpus* has long been recognized for its exceptional medicinal value, and the genus is economically significant as a source of edible aggregate fruit, such as *Artocarpus heterophyllus* (jackfruit), *Artocarpus altilis* (breadfruit), and *Artocarpus chempeden* (Chempedak), as well as producing reasonably good timber [4].
The genus Artocarpus belonging to the mulberry family (Moraceae) includes approximately 50 species and are widely distributed in tropical and subtropical regions. The propensity of *Artocarpus* to leak profuse yellow to white latex from its damaged sections is one of its distinguishing morphological traits. [5]. It is noted that the latex from Artocarpus is an abundant source of lipids, rubbers, resins, carbohydrates, falvaonoids, phenolics, many proteins, enzymes and other substances. Plant latex has also been used to treat inflammation, cirrhosis, hypertension, malarial fever, diarrhea, and tuberculosis in traditional folk medicine, as well as to manage blood sugar levels in diabetic patients. [6]. For example, fig tree latex has been used in short-term therapy to treat wars with no reported negative effects [7]. In human homeostasis, plant latex contains clot-inducing and -dissolving characteristics [8-9]. *Carica papaya* latex has been used to treat burn wounds in mice [10]. Furthermore, in all gastrointestinal models, ethanolic and dichloromethane extracts of *Mammea americana* latex were revealed to have excellent anti-secretary and/or gastroprotective activities [11]. The evidence shown thus far supports the use of plant latex in a variety of treatments. There are just a few papers on the biological properties of jackfruit latex,

particularly latex proteins and proteases, at this time. From jackfruit latex, a 79.5-kDa serine protease has been identified and purified. [12]. this protein's therapeutic properties, however, have not been studied. The latex of several plants has a broad spectrum of antibacterial effect due to a variety of biologically active compounds. Hence latex producing plants may provide the good source of antimicrobial compounds. The defeating of chemotherapeutics and the birth of antibiotic resistance in microbial infectious pathogens has prompted researchers to test the latex of numerous plant species for antimicrobial effectiveness. Petroleum ether fraction of different plant latex namely; *Carica papaya, Calatropes procera, Artocarpus heterophyllus, Jatropa carcass* and *Thevetia peruviana* revealed the antimicrobial activity against different human pathogenic bacterial and fungal strains. In the present study, proteins were partially isolated from the latex of four *Artocarpus* species using ammonium sulphate fractionation and dialysis in order to gather fresh information on the biological features of latex proteins. Partially purified lectins were further investigated for lectin, protease and antimicrobial activity.

Materials and Methods

Collection of plant latex

The plants *A. integrifolia, A. hirsutus, A. lookocha,* and *A. altilis* were collected from Hosanagara, Thirthalli of Shivamogga District and Shringeri, Koppa of Chikkamagalur District Karnataka. Fresh latex was obtained from these plants by making cuts on the tree trunks using a big knife and pouring the latex into a 15 mL falcon tube drop by drop. The latex was then diluted 1:1 (v/v) in PBS (50 mM, pH 7.2, 1 mM PMSF, 0.5 g polyvinylpyrrolidone, and 0.5 g sodium dithionite) and kept at 4°C until extraction.

Extraction of latex protein

The diluted whole latex (10 ml) samples were centrifuged at 10,000 rpm for 20 min at 4 °C temperature. Among the resultant three layers, white topmost sticky layer and the bottom debris layer were discarded. Middle clear layer was used for extraction of lectin using ammonium sulphate precipitation (90%) and the precipitated proteins were removed by centrifugation 10,000 rpm, 20 min at 4 °C temperature. The obtained protein pellet was dissolved in minimum volume of 50mM PBS, pH 7.2, followed by dialysis overnight against distilled water. Dialyzed samples were stored at 4˚C till further studies.

Protein estimation

Protein content was measured according to the Bradford **[13]** procedure with Bovine Serum Albumin as the protein standard.

Hemagglutination Assay

Hemagglutination activity in protein fraction of the latex samples was determined by serial two fold dilution method using trypsinised human as well as animal erythrocytes in 96 well micro titer plates as described by Liener and Hill [14]. The titer was defined as the maximum dilution of the extract that caused visible hemagglutination, and one unit of hemagglutination activity was defined as the minimum concentration of protein necessary for agglutination (MCA). The specific hemagglutination activity is measured in milligrams per gram of protein.

SDS PAGE

SDS-PAGE was used to investigate the protein profile of the latex protein fraction using the Laemmli method [15] on a 15% polyacrylamide gel containing 0.1 percent SDS and Tris-glycine buffer at pH 8.8. Coomassie brilliant blue G-250 was used to visualize protein bands.

Protease Activity Testing

The method published by Satake *et al* (1963) [16] was used to measure proteolytic activity. In a total volume of 1 ml, fat free casein (0.4 ml, 2% in 0.2 M Tris HCl buffer, pH 7.6) was incubated with different quantities of protein fraction (2, 4, 8, 16, and 20 g) of latex samples from *A. altilis, A. integrifolia, A. hirsutus,* and *A. lakoocha* for 2 hours at 37 °C. Undigested casein was precipitated by adding 1.5 ml of 0.44M trichloroacetic acid (TCA) and allowing it to sit for 30 minutes before centrifuging it at 2000 g for 10 minutes. In 1 ml of supernatant, sodium carbonate (2.5 ml, 0.4M) and Folin-reagent Ciocalteu's (1:2) were added successively, and the color generated was read at 660 nm. The amount of enzyme necessary to generate an increase in optical density (OD) at 660nm was defined as one unit of enzyme activity.

Detection of proteases by Caseinolytic Zymography

As previously detailed by Gangaraju *et al.,* (2015) [17], protease activity was measured using casein as a non-specific enzyme substrate. Briefly, each protein fraction (30 µg) of respective latex samples were prepared under non-reduced condition and applied to a 15% resolving gel containing 0.1% SDS and 2% casein. Gels were washed with 10 mM sodium phosphate buffer containing 2.5 percent Triton X-100 for 1 hour with constant agitation to remove SDS after electrophoresis. In a Tris 50 mM HCl buffer (pH 7.6) containing 50 mM $CaCl_2$ and 40 mM NaCl, the gel was incubated overnight at 37 °C. After then, the gel was dyed to reveal the translucent activity bands.

Maintenance of Bacterial Culture

Cultures of five pathogenic bacterial strains *S. typhimurium* (ATCC no: 27853), *Pseudomonas aeruginosa* (ATCC no: 9027), *Klebsiella pneumonia* (ATCC no: 25923), *Bacillus subtilis* (ATCC no: 6633) and *Escherichia coli* (ATCC no: 25922) were procured from Department of Microbiology, Kuvempu University, Karnataka and were maintained on nutrient agar medium at 37°C.

Anti-bacterial activity

The agar disc diffusion susceptibility method was used to assess the antibacterial activity of the protein fraction of latex samples, and inhibition zone diameters were assessed according to Bauer *et al*. (1966) [18]. The initial stage in this evaluation was to add a bacterial inoculum of approximately 2 x10^8 CFU/mL to the surface of a large Mueller-Hinton agar plate (150 mm in diameter). Five pathogenic bacterial strains *S. typhimurium* (ATCC no: 27853), *Pseudomonas aeruginosa* (ATCC no: 9027), *Klebsiella pneumonia* (ATCC no: 25923), *Bacillus subtilis* (ATCC no: 6633) and *Escherichia coli* (ATCC no: 25922) were used in this study. Protein fractions of 50.0 and 100.0µg/per disc unit were produced on paper filter discs (0.5 mm in diameter) and then deposited on the inoculated agar surface. Negative and positive controls were utilized, respectively, with sterile water and ampicillin discs. The plates were incubated at 37°C for 24 hours before the results were determined by measuring the growth inhibition zones (millimeters) around each disc. All of the tests were done in triplicate, with the average being used as the final result.

Results and Discussion

Analysis of salt fractionated latex proteins for haeagglutination activity

The data of haeagglutination assay demonstrate that the salt fractionated proteins of different latex samples exhibit varying reactivities and affinities towards human and animal erythrocytes, thus confirming lectins with different agglutination properties (Table 1). Lectins are classified into several classes depending on their capacity to prevent lectin-induced haeagglutination in presence of monosaccharides or glycosides. However, lectins with the same carbohydrate specificity have been reported to exhibit various reactivities toward distinct oligosaccharide chains and affinities to animal cells, implying that they have their own binding specificity that extends beyond the monosaccharide unit) [19-20]. Hence, a panel of 7 different sources of red blood cells was used to screen the lectin activity in this study, ensuring the detection of lectin activity in all the samples. *A. altilis* exhibits non-specific agglutination towards all types of erythrocytes and highest agglutination was found to be with pig, horse, rabbit, sheep and human erythrocytes.

In contrast, *A. integrifolia* showed maximum lectin activity towards pig erythrocytes followed by goat and sheep erythrocytes. However, latex protein fraction from *A. hirsutus* and *A. lakoocha* demonstrate least lectin activity. Among the different types of erythrocytes, pig, horse and human B strongly agglutinated by the latex protein fraction indicating the differences in the composition of cell surface glycocalyx. Similar studies using a battery of erythrocytes, 54 lectins from 450 species of Indian plants were revealed by their hemagglutinating activities [21].

Table 1: Prevalence of lectin activity in latex samples of different *Artocarpus* species as determined by haemagglutination activity.

Artocarpus Species	Haemagglutination Activity (Titer)								
	Erythrocytes								
	Human			Cow	Horse	Pig	Goat	Rabbit	Sheep
	A	B	O						
A. altilis	512	2048	512	32	2048	8192	1024	1024	1024
A. hirsutus	Nil	Nil	Nil	Nil	Nil	08	Nil	Nil	Nil
A. integrifolia	Nil	Nil	Nil	16	32	262144	1024	32	1024
A. lakoocha	16	Nil	64	Nil	64	64	Nil	16	Nil

Genetic polymorphism and post-translational modification of the lectins, which probably occur in plants of the same species but grow in different geographical areas, have been shown to play an important role in the discrepancy of properties of lectins extracted from the same species [22-24].

Protein profiling of latex of *Artocarpus* species

Electrophoretic pattern of protein fraction of latex sample of four species of Artocarpus exhibited dissimilar protein banding pattern ranging from low to high molecular weights (10-170kDa), suggesting the presence of varied proteins with different molecular weights (Figure 1). These proteins signify the storage, structural, lectins, enzymes inhibitors and biologically active proteins including enzymes associated with the hydrolysis of stored proteins [25]. In addition to this, proteins in latex play defensive role in plants against insects and herbivores.

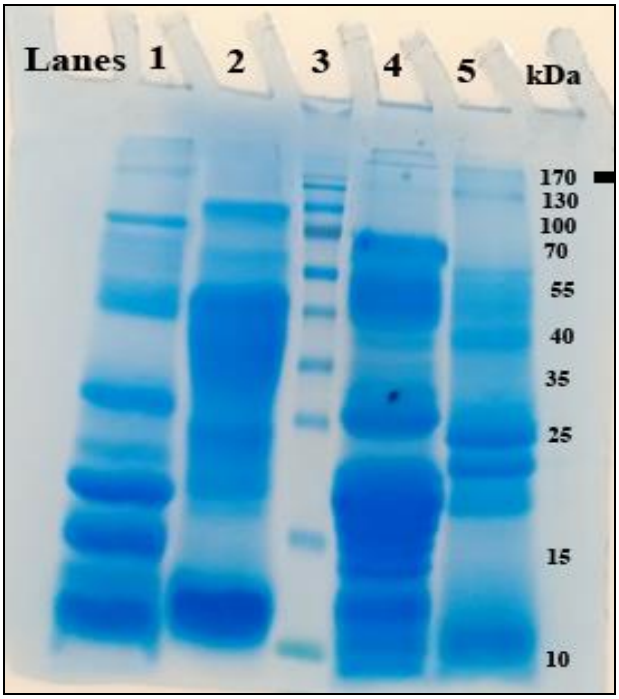

Fig 1: SDS-PAGE of protein fraction of latex of four *Artocarpus* species. Lane. 1: *A. Altilis*; Lane. 2: *A. lakoocha*; Lane. 3: Molecular weight marker (10 – 170 KDa; Lane. 4: *A. integrifolia (heterophylllus)* and Lane 5 *A. hirsutus.*

Proteolytic activity

Table 2 depicts the protein content data and proteolytic activity of the four examined species. In the latex, all plants showed substantial proteolytic activity. With protein levels of 2.39 and 2.17µg/µl, and proteolytic activity of 2.45 and 2.0 Units/mg/min respectively, the latex of *A. integrifolia* and *A.altilis* demonstrated high proteolytic activity.

Table 2: Protein content and Proteolytic activity of latex of four *Artocarpus* species.

Species	Protein content (µg /µl)	Proteolytic activity Units/mg/min
A. altilis	2.17	2.0
A. lakoocha	1.77	0.83
A. hirsutus	2.41	1.23
A. integrifolia	2.39	2.45

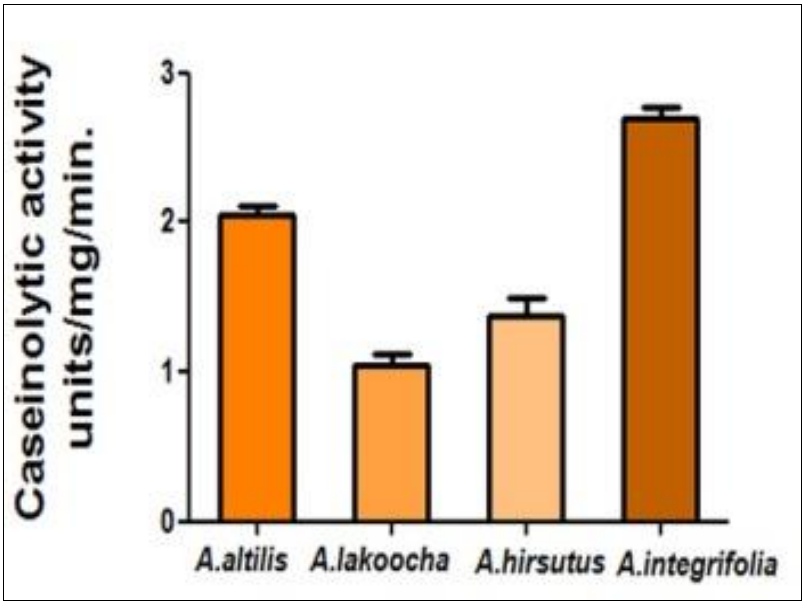

Fig 2: Proteolytic activity and specific activity of the latex samples of *A. altilis, A. hirsutus, A. integrifolia* and *A. lakoocha*

Caseinolytic Zymography

Caseinolytic zymography was used specifically to detect the presence of protease in protein fraction of the latex samples.

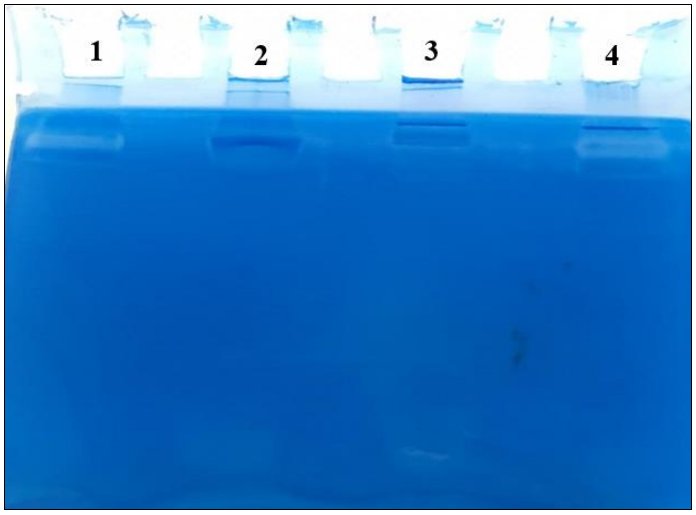

Fig 3: Zymography analysis of the protein fraction of the latex of *A. altilis* (Lane 1), *A. lakoocha* (Lane 2), *A. hirsutus* (Lane 3), and *A. integrifolia* (Lane 4).

Figure 3 shows a substantial breakdown of casein in the upper part of the gel in the *A. altilis and A. integrifolia* samples (near the wells). This significant disintegration appears to be associated with a protein band, but it's also likely that it's associated with two or more proteolytically active proteins. The findings revealed the latex of *A. altilis* and *A. integrifolia* has considerable proteolytic activity. The zymogram for *A. lakoocha* and *A. hirsutus* samples had a similar profile. The significant degradation of casein appears to be minimal in this case. Even when repeating the zymogram with varied sample concentrations, it was not feasible to get the bands properly resolved. These findings indicated the existence of proteins with proteolytic activity in the latex of *A. altilis* and *A. integrifolia*, corroborating the colorimetric results of caseinolytic activity.

Antibacterial activity

The threat of antimicrobial resistance (AMR), the creation of new resistant microbial strains, and undesirable drug reactions, as well as the requirement for protracted therapy in some infectious illnesses, necessitate continued antimicrobial research into medicinal plants and their derivatives. As a result, the goal of this study was to see how effective the protein fraction of latex samples from four *Artocarpus* species were against four different pathogenic bacterial strains.

Table 3: Zone of inhibition of protein fraction of latex of *Artocarpus* species at different concentrations against bacterial strains.

Bacterial Strains	***Artocarpus* species**											
	A. altilis			***A. hirsutus***			***A. integrifolia***			***A. lakoocha***		
	Std.	**100 µg**	**50 µg**	**Std.**	**100 µg**	**50 µg**	**Std.**	**100 µg**	**50 µg**	**Std.**	**100 µg**	**50 µg**
	Diameter of zone of inhibition (mm)											
E. coli	22	Nil	Nil		Nil	Nil	24	Nil	Nil	26	Nil	Nil
K. pneumonia	26	12	8	25	14	10	26	24	12	26	8	Nil
P. aeuroginosa	24	10	Nil	27	Nil	Nil	24	10	Nil	26	10	Nil
S. typhimurium	28	11	7	28	20	12	26	Nil	Nil	24	Nil	Nil
B. subtilis	24	10	7	27	Nil	Nil	24	12	8	27	12	8

The protein fraction of latex samples had various degrees of antibacterial activity in this investigation, but it was less than the positive control. The antibacterial effect of the protein fraction of latex samples agar well diffusion increased with dose as shown in Table 3 and Figure 4, 5, 6 and 7. *A. altilis, A. lakoocha, A. integrifolia* and *A. hirsutus* exhibit strong antimicrobial activity against *K. pneumonia*, whereas moderate antimicrobial activity against *P. aeuroginosa* was seen for *A. altilis, A. integrifolia* and *A. hirsutus.* The growth of *S. typhimurium* was strongly inhibited by the latex of *A.altilis* and *A. lakoocha.* In contrast, *A. altilis, A. integrifolia* and *A. hirsutus* showed significant growth inhibitory effect against *B. subtilis.* No activity was seen for the latex samples against *E.coli.* The antimicrobial activity could be due to the presence of several bioactive compounds including lectins and proteases. Recently, a 48 kDa anti-microbial protease from *A. heterophyllus* latex has been reported [26]. Two novel chitin binding lectins isolated from the genus Artocarpus demonstrated strong antifungal activity [27].

These reports from the literature strongly argue that many plants in their latex possess antimicrobial compounds as one of the defensive mechanism to protect from the microbial pathogens.

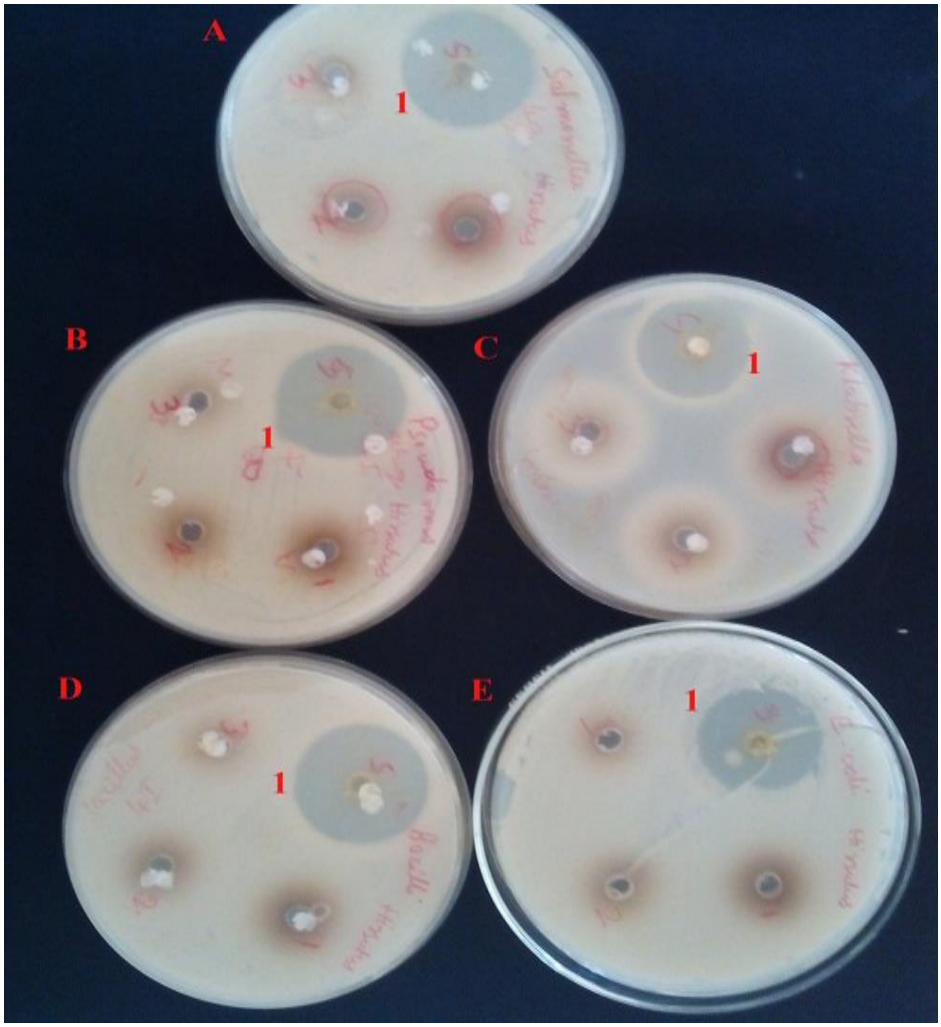

Fig 4: Antibacterial activity of *A. hirsutus* latex where A) *S. typhimurium* B) *P. aeruginosa*, C) *K. pneumonia*, D) *B. subtilis*, E) *E. coli.* 1= Positive control (Ampicillin).

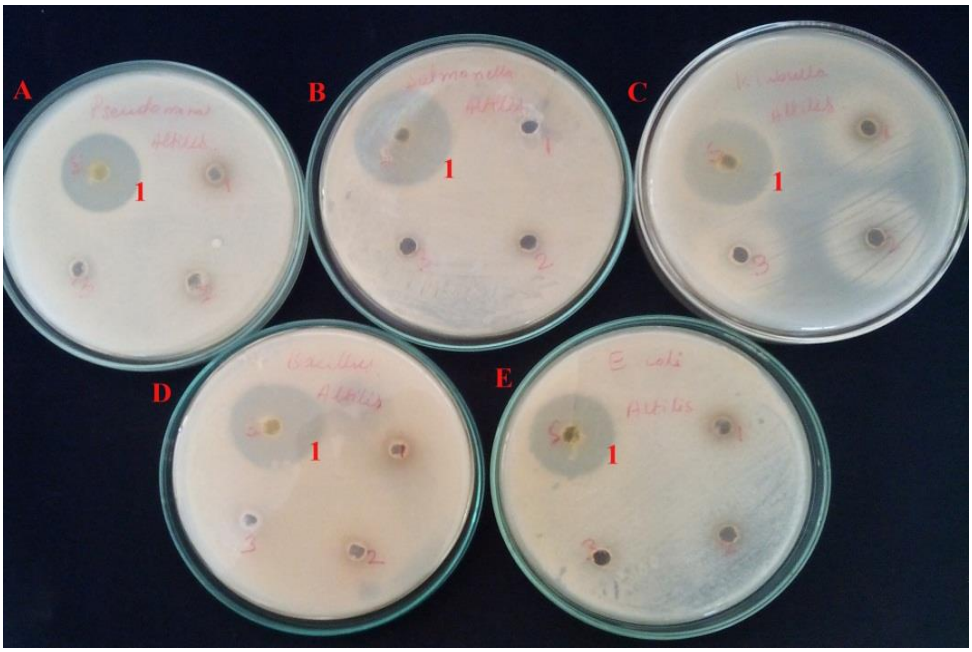

Fig 5: Antibacterial activity of *A. altilis* latex where A) *P. aeruginosa*, B) *S. typhimurium*, C) *K. pneumonia*, D) *B. subtilis*, E) *E. coli*, 1= Positive control (Ampicillin).

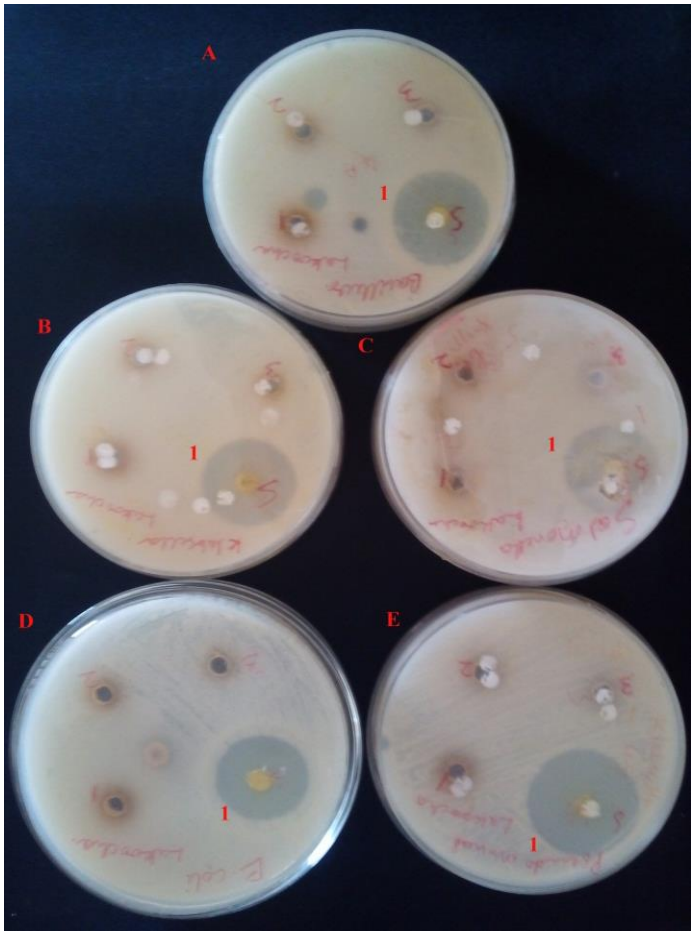

Fig 6: Antibacterial activity of *A. lakoocha* latex where A) *B. subtilis*, B) *K. pneumonia*, C) *S. typhimurium*, D) *E. coli*, E) *P. aeruginosa*, 1= Positive control ((Ampicillin).

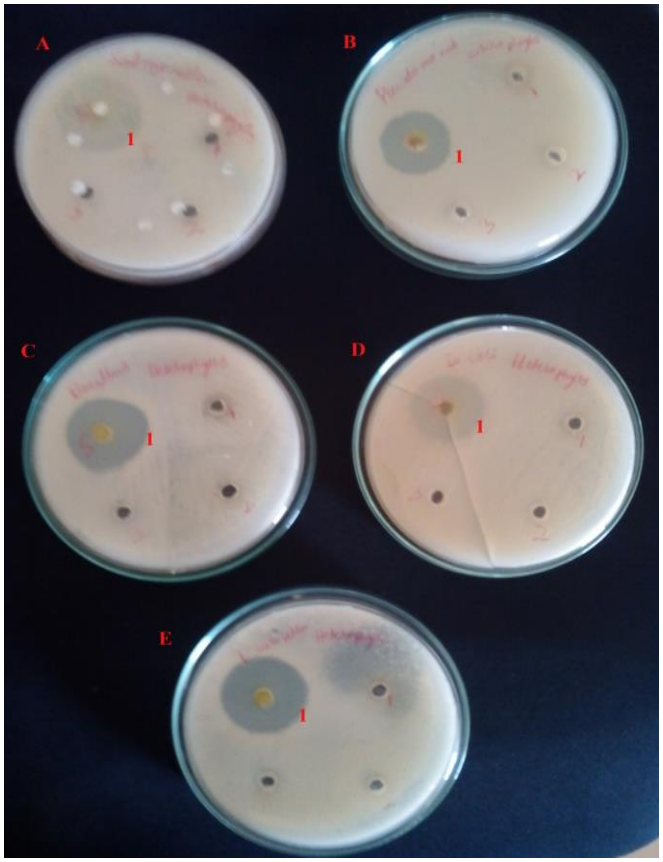

Fig 7: Antibacterial activity of *A. intetegrifolia* latex where A) *P. aeruginosa*, B) *S. typhimurium*, C) *K. pneumonia*, D) *B. subtilis*, E) *E. coli*, 1= Positive control (Ampicillin).

Conclusion

Protein fraction prepared from latex of four Artocarpus species have shown strong lectin, protease and antibacterial activity which attributed for their medicinal values. Proteins, proteases, chitinases, osmotin, alkaloids, glycosides, diterpenes, and saponins are some of the latex components that can act as effective medicinal agents. Due to the presence of considerable active principles, latex samples showed modest growth inhibition and bactericidal effectiveness against harmful bacterial strains when compared to typical antibiotic drugs. If latex components are found, they may undoubtedly be used for a variety of therapeutic and food preservation purposes. Electrophoretic analysis of latex proteins can be used as prognostic tool for genetic variation and relation in germplasm and also to sort out mutatants from their parent genotype.

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