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Research Article

PRELIMINARY STUDY ON ANTIXANTHOMONAS ACTIVITY, PHYTOCHEMICAL ANALYSIS, AND CHARECTERIZATION OF ANTIMICROBIAL COMPOUNDS FROM *KAPPAPHYCUS ALVAREZII*

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ABSTRACT

Bacterial Leaf Blight (BLB) was reported to have reduced Asia's annual rice production by as much as 60%. In India, millions of hectares were severely infected, causing yield losses from 6% to 60%. BLB of rice caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) was one of the most economically serious diseases of lowland irrigated rice and can cause yield losses up to 50%. The synthetic chemicals such as carbendazim, thiram, dithane M-45, propicanozole, sterpomycin sulphate, and monochrotophos were used to control this plant pathogen. Most of the synthetic fungicides used to control such pathogens, have long residual effect and also causes severe health and environmental hazards. Mitigation of all these problems has necessitated a search for alternate means of pest control that are raw incubated cow urine, biodegradable and environmentally benign too. In the present investigation, cow urine extract, ethyl acetate, methanol, and aqueous fractions of a red algae *Kappaphycus alvarezii* were tested against *Xanthomonas oryzae* pv.*oryzae* for its antibacterial activity. Streptomycin sulphate (30 µg) and DMSO (15 µl) were used as positive and negative control respectively. All the extracts and fractions were effective and showed 7 to 13 mm zone of inhibition. Phytochemical and TLC analysis of the extracts showed the presence of terpenoids, flavonoids, coumarine, tannin, saponin, inulin and lignin. Column chromatography, Thinlayer Chromatography, FTIR, GC-MS, and NMR analysis were performed to identify the secondary metabolite, and 2, 3 Dihydro-7-methy-1, 4-benzoxazine-3- one was identified as the active principle.

Key words: Rice, Seaweed, *Kappaphycus alvarezii,* Cow urine extract, Antixanthomonas activity.

INTRODUCTION

Rice has potential in a wide range of food categories. Rice bran is used as an excellent source of Vitamin B, minerals, proteins, amino acids, and carbohydrates. It is gluten free. Asia is the biggest rice producer around 90% of world's production and consumption of rice.

Every year all over the world the yield is affected by natural calamities, pests, insects and microbes' viz., fungi, bacteria and viruses. BLB is caused by *Xanthomonas oryzae* pv *oryzae* (*XOO*). When rice is affected by this disease up to 20 to 50% of yield loss were reported[1].

A method of hot water treatment involving eight hours soaking of seed in 0.1% ceresan and streptocycline (0.3 g/12 liters) has been reported to reduce the BLB [2]. Carbendazim, thiram, dithane M-45, propicanozole, sterpomycin sulphate, and monochrotophos were used to control plant pathogens [3].

These chemical pesticides have caused health hazards in animals and humans due to their residual toxicity [4]. Mitigation of all these problems has necessitated a search for an alternative means of pest control that are effectively biodegradable and ecofriendly too. Cow urine has been used for a long time in India to control pathogens. In traditional formulations botanicals were soaked in cow or cattle's urine and sprayed to control the insects and pests, plant pathogens.

Seaweeds have become alternative sources to the synthetic pesticides. Ethanol extract of *Sargassum asperum*, *S. swatzii* methanol and chloroform soluble fractions of *S. Variable* have been shown to inhibit the growth of bacteria[5].

It has been identified that less work has been performed in the antibacterial activity of red algae *kappaphycus alvarezii* especially in combination with cow urine. Hence as a novel approach, in the present investigation seaweed red algae *kappaphycus alvarezii* and cow urine together were incubated in a mud pot for 20 days under the soil and the extract was tested for its antibacterial activity against the plant pathogen *Xanthomonas oryzae* pv. *oryae*.

MATERIALS AND METHODS

Bacterial Leaf Blight (BLB) infected paddy leaves were collected from the plant pathology department of agriculture college, Ramji Nagar Thiruchirapalli, in Zip lock covers. A loopful of macerated sample was inoculated on the Nutrient agar and *Xanthomonas* selective medium and incubated at 37°C for 24 hours. Characteristic colonies were subjected to standard biochemical screening.

Collection of Sample

Sea weed *Kappaphycus alvarezii* (Red algae) was collected from Mandapum at Ramanathapuram district in Tamil Nadu and identified by the Department of Botany, National College, Thiruchirapalli, Tamil Nadu, India.

Cow urine extraction

One and a half kg of fresh seaweed was surface sterilized with sterile distilled water and 0.1% mercuric chloride and placed in an earthen pot filled with 10 liters of fresh cow-urine collected from a single cow fed with the same type of feed throughout the study. The pot was incubated in a pit dug in the soil for 20 days. At every 24hrs interval, 500ml of crude extract was collected and condensed into a dry powder using water bath at 40°C. Samples were collected continuously for 20 days.

Organic solvent extraction

Hundred grams of shade dried seaweeds was coarsely powdered and was mixed with 300ml of organic solvents viz., ethyl acetate, methanol, alcohol and water based on increasing polarity. The extracts so collected were evaporated on a water bath at atmospheric pressure and the solvents were completely removed in *vaccuo*.

Agar well diffusion assay method Assay

Antibacterial activity of different forms of cow urine, organic fractions and cow urine extract of *Kappaphycus alvarezii* were assayed using the well diffusion method. Petriplates containing 20ml of nutrient agar medium was seeded with 18 hour old culture of bacterial strain isolate.

To analyse whether the cow urine itself has any effect on pathogen, condensed cow urine (10ml of fresh cow urine was condensed into 1ml using water bath), fresh cow urine, chloroform extract of both fresh and incubated cow urine (20 days in earthen pot) was tested on the pathogen. Different concentrations like 5µl, 10µl, 15µl were added in the well and incubated for 24hrs at 37ºC.

The extracts and organic fractions were dissolved in Dimethyl sulfoxide (DMSO) and sterilized by using sortorious syringe filter of pore size 0.22µm. (Stock solution (0.04g/1ml)).

Various concentrations of the extracts viz 160µg, 240µg, 320µg and 400µg were added into 6mm diameter well. Streptomycin sulphate (30µg) and DMSO (15µl) were used as positive and negative control. Incubation was made at 37ºC for 24hrs. The inhibition zone diameter (IZD) was measured by antibiotic zone reader to nearest mm[6].

Phytochemical Analysis

All the extracts were subjected to preliminary phytochemical screening as per the standard method.

Separation of phytochemical constituents

Extract which showed maximum bactericidal activity was subjected to standard separation techniques. 25 grams of crude cow urine extract was packed in the column made up of silica gel (Acme's mesh; 60 – 120 µl Hi- media). Hexane, chloroform, ethyl acetate, methanol, alcohol and water were used in the order of increasing polarity.

All the fractions obtained were assayed for antimicrobial activity by the standard method. Fractions obtained in ethyl acetate: methanol (70: 30) showed maximum antimicrobial activity. Hence this fraction was subjected to HPLC, GC-MS, FT-IR and ^{1}H NMR spectral analysis.

RESULTS AND DISCUSSION

In the present investigation antimicrobial activity of cow urine extracts and organic fractions of red algae *Kappaphycus alvarezii* were determined against the plant pathogen. From the infected paddy leaves the pathogen was isolated and identified as *Xanthomonas oryzae* pv *oryzae* (*XOO*) by standard techniques.

Coarse powder of *Kappaphycus alvarezii* (100g) was successively extracted using ethyl acetate, alcohol, water and assayed for their antixanthomonas activity. These extracts were not effective in controlling the plant pathogen. All the fractions showed negative result as antibacterial agent (Table 1).

In contrary methanolic extract of the brown seaweed *Sargassum wightii* has been showed to inhibit the growth of *Xanthomonas oryzae* pv. *oryzae* which causes bacterial blight of rice [7, 8, 9]. Fig.1 depicts the antimicrobial activity of incubated cow urine, organic fractions and cow urine extract of *kappaphycus alvarezii* against the *Xanthomonas oryzae* pv *oryzae* at different concentration.

Incubated cow urine exert maximum zone of inhibition followed by cow urine extract of *Kappaphycus alvarezii*.

Cow urine extracts of fresh seaweed *Kappaphycus alvarezii* were tested against *XOO* in invitro condition by well diffusion method.1-10 days (Table 2) extracts were not effective against the plant pathogen, but 11th-20th days extracts showed antixanthomonas activity and the zone of inhibition was measured as 9mm to 13mm at 400µg concentration. 20th day extract was more effective.

Two sesquiterpenes, zonarol and isozonarol reported from brown seaweed, which possess antifungal activities against plant pathogenic fungi[10]. Lipid extract of seaweed inhibited the growth of *Xanthomonas malvaciarum,* which was closely related to *XOO*[11]. Several reports were available regarding the antibacterial activity[12] and antifungal activity [13].

Acrylic acid was the common antibacterial component occurring in many red, brown and green seaweeds. Dimethyl sulfide, acrylic acid and CH_2= CH-COOH were isolated from the seaweeds displayed antibacterial property[14]. A novel long chain fatty ester, pentyl hentriacontanoate one and an orange red pigment, caulerpin two and a pigment caulerpin hitherto were isolated and characterized from a red alga[15].

Preliminary phytochemical analysis was performed for both aqueous and cow urine extract of *Kappaphycus alvarezii*. Saponin was the only metabolite shown to be present in aqueous extract whereas terpenoids, flavonoids, coumarin, tannins, saponins, inulin and lignin were present in cow urine extract of *Kappaphycus alvarezii*. (Table 3). Quantitative analysis of secondary metabolites in cow urine extract was performed by HPLC method.

Presence of terpenoids, flavonoids, coumarine, tannins, saponins, inulin and lignin were observed. Among which flavonoids (1.45 mg kg^{-1}) were found in higher quantity. (Table 4), Saponin and Tannin were present (0.26mg/Kg and 0.23mg/Kg) in relatively smaller amounts. Presence of green colour in 70% EtOAc 30% CH_3OH with Rf value of 0.827 were characteristic of flavanoids and diethyl phthalate.

The yellow colored extract in alcohol fractions with Rf values of 0.89 0.75, 0.53 may be due to saponins, tannins and terpenoids which were of high molecular weight. Presence of bioactive tannins, tannic acid, brominated phenols, poly phenols were reported from seaweeds[16, 17]. Terpenes isolated from seaweed inhibit the growth of many bacteria[18, 19, 20].

A diterpenoid showing antimicrobial properity was isolated from the brown seaweed *Dictyota baratayresii*[21]. Antibacterial activity of methanolic extract of the brown seaweed *Stoechospermum marginatum* was active against human pathogen. The active constituents were spatane, diterpenoids, 19-acetoxy, 5, 15, 18- trihydroxyspata-13, 16-diene[22].

FT-IR spectra (Fig 2) of the cow urine extract of *Kappaphycus alvarezii* exhibited stretching vibrations at 3427cm^{-1} due to N-H groups. At 2076 cm^{-1} C=N vibrations can be found. At 1760 cm^{-1} the –C=O vibrations and at 1641 cm^{-1} CONH vibrations were observed. At 1383cm^{-1} C=C vibration was found. At 1243 cm^{-1} CN bending vibrations occur.

Two very close vibrations occur at 1051 & 1018 cm^{-1} due to C-O groups. At 654cm^{-1} substituted C-C and δ CH vibrations were observed. The GC-MS (Fig 3) spectra of ethyl acetate; methanol (70;30) fraction of the cow urine extract of *Kappaphycus alvarezii* showed the presence of phenyl propanedioic acid ($C_9H_8O_4$) to 34.98% 2, 3-Dihydro-7-methyl-1,4-benzoxazine-3-one ($C_9H_9NO_2$) was present to 33.33%, benzylmalonic acid ($C_{10}H_{10}O_4$) was present to 6.78% and Diethyl Phthalate **(**$C_{12}H_{14}O_4$) about 0.4% was present (Table 5).

Synthesized benzoxazin from quinazolinone and assayed for it was antibacterial activity against human pathogens. They reported that synthesized compounds exhibited mild to good antibacterial activity[25]. Reports were available on antiviral activity of benzoxazin[26]. 1, 3 benzoxazine were reported to have antibacterial activity against plant pathogens like *Xanthomonas citri, Xanthomonas malvecearium* and *Ervinia carotovora*[24].

In the ^{1}H NMR spectra(Fig 4) , the quartet at δ 4.32 ppm was assigned to the two hydrogen atoms in the 2nd position in the oxazine ring, the quartet at 4.02 ppm was assigned to the hydrogen atoms in 9th & 10th position at the benzofusion. A doublet 3.15 ppm was assigned to the hydrogen atoms at 6th and 9th position. At 2.5 ppm and 1.9 ppm the $CHCH_3$ group was found. The spectral analysis reveals the presence of oxazine skeleton in the ethyl acetate; methanol (70:30) fraction.

The active component in the cow urine extract of *Kappaphycus alvarezii* was the oxazine derivative(Fig 5) dihydro 1,3 oxazine condensed with aromatic ring have been found to be posses antibacterial activity [23, 24, 25, 26]. flavanoids (Fig 7) were present in higher proportion in the HPLC analysis. All the various phytochemial analysis indicated that the cow urine extract of *Kappaphycus alvarezii* may contain the active component (Fig 6).

During the incubation in cow urine for 20 days the compound (Fig 6) has degraded into 2, 3-dihydro-7-methyl-1, 4-benzoxazine-3-one (Fig 5)

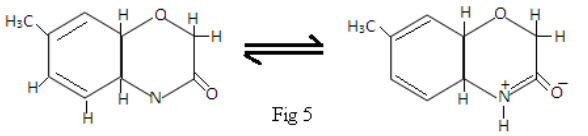

Fig 5

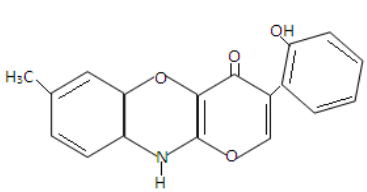

Fig 6

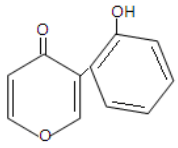

Fig 7

Table 1: Antimicrobial activity of organic fractions of *Kappaphycus alvarezii* against *xanthomonas oryzae.* pv. *Oryzae*

S.no	Extract & fraction of *Kappaphycus alvarezii*	Concentration of extract (µg) / zone of inhibition(mm)					
		Positive control	Negative control	160µg	240µg	320µg	400µg
1	Ethyl acetate	21	0	0	0	0	0
2	Ethanol	21	0	0	0	0	0
3	Water	21	0	0	0	0	0

Table 2: Antimicrobial activity of *Kappaphycus alvarezii* cow urine extract against *Xanthomonas oryzae.* pv. *Oryzae*

S.NO	Days of incubation	Concentration of extract (µg) / zone of inhibition (mm)					
		Positive control	Negative control	160µg	240 µg	320 µg	400 µg
1	Days 1-10	21	0	0	0	0	0
2	Day 11	21	0	7	7	8	9
3	Day 12	21	0	7	8	9	9
4	Day 13	21	0	8	9	9	10
5	Day 14	21	0	8	8	9	10
6	Day 15	21	0	8	8	9	10
7	Day 16	21	0	8	8	8	10
8	Day 17	21	0	9	9	10	11
9	Day 18	21	0	10	10	11	12
10	Day 19	21	0	10	10	10	11
11	Day 20	21	0	11	11	12	13

Table 3: Phytochemical screening of cow urine extract of *Kappaphycus alvarezii*

S.No	Test	Aqueous extract of *kappaphycus alvaerzii*	Cow urine extract of *Kappaphycus alvaerzii*
1	Terpenoids	_	+
2	Flavones	_	+
3	Sugar	_	_
4	Alkaloid	_	_
5	Quinine	_	_
6	Coumarin	_	+
7	Tannin	_	+
8	Saponin	+	+
9	Glycoside	_	_
10	Phenols	_	_
11	Steroids	_	_
12	Phlobotannins	_	_
13	Phytosterol	_	_
14	Starch	_	_
15	Inulin	_	+
16	Lignin	_	+

Table 4: Qualitative analysis of Secondary Metabolites by using Atomic Absorption Spectroscopy

S.No	Name of the parameter (mg kg^{-1})	Sample details
1	Total Terpenoids	0.12
2	Total flavonoids	1.45
3	Total saponin	0.26
4	Total Tannins	0.23
5	Total Inulin	0.05
6	Total Lignins	0.12
7	Total Coumarin	0.03

Table 5: Compound identification in *Kappaphycus alvarezii* (GC=-MS) incubated cow urine.

No.	RT	Name of the compound	Molecular Formula	MW	Peak Area %
1	3.09	Phenol, 4-methyl	C_7H_8O	108	0.33
2	3.51	Cyclohexanecarboxylic acid	$C_7H_{12}O_2$	128	0.57
3	4.00	Heptanediamide, N,N'-di-benzoyloxy-	$_{21}H_{22}N_2O_6$	398	0.78
4	4.19	Benzeneacetic acid, methyl ester	$C_9H_{10}O_2$	150	0.43
5	4.47	Methyl Salicylate	$C_8H_8O_3$	152	1.46
6	4.59	Cyclopentanepropanoic acid	$C_8H_{14}O_2$	142	2.93
7	5.25	Propanedioic acid, phenyl-	$C_9H_8O_4$	180	34.98
8	6.26	Benzylmalonic acid	$C_{10}H_{10}O_4$	194	6.78
9	7.44	L-Arabinitol	$C_5H_{12}O_5$	152	0.76
10	8.12	Spiro[4.5]dec-6-en-8-one, 1,7-dimethyl-4-	$C_{15}H_{24}O$	220	1.33
11	9.3	Diethyl Phthalate	$C_{12}H_{14}O_4$	222	0.40
12	10.31	1,2-Epoxy-5,9-cyclododecadiene	$C_{12}H_{18}O$	178	1.16
13	10.75	1-Decalone (cis-trans)	$C_{10}H_{16}O$	152	2.02
14	11.46	2,5,5,6,8a-Pentamethyl-trans-4a,5,6,7,8,8a-	$C_{14}H_{24}O$	208	2.94
15	11.75	Cyclohexanecarboxaldehyde, 3,3-dimethyl-5-oxo-	$C_9H_{14}O_2$	154	1.85
16	12.74	N-Ethyl-3-methoxyaniline	$C_9H_{13}NO$	151	2.17
17	13.32	2,3-Dihydro-7-methyl-1,4-benzoxazine-3-one	$C_9H_9NO_2$	163	33.33
18	15.39	9-O ctadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	2.58
19	21.45	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	3.20

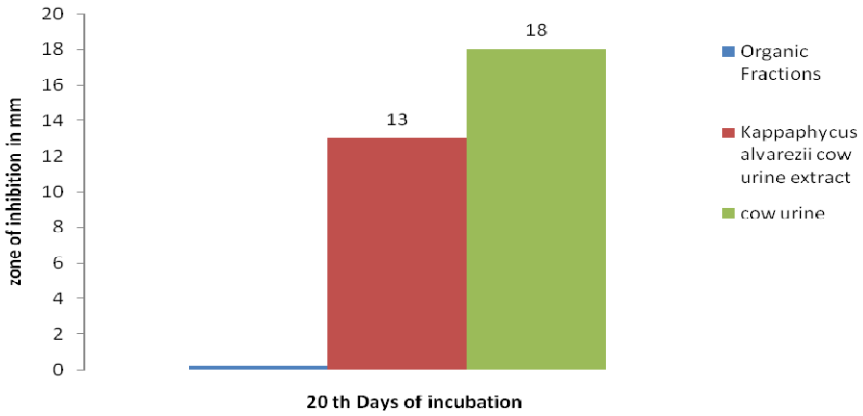

Fig. 1: Antibacterial activity of *Kappaphycus alvarezii* and cow urine under different condition on *Xanthomonas oryzae* pv *oryzae.*

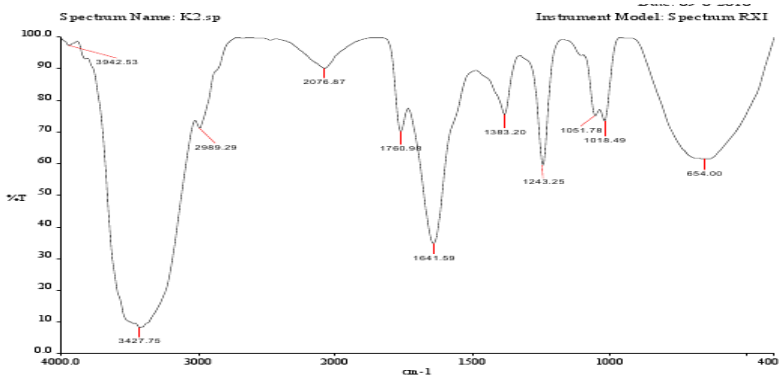

Fig. 2: FTIR spectrum of Ethyl acetate: Methanol (20:80) fraction of *Kappaphycus alvarezii* incubated in cow urine

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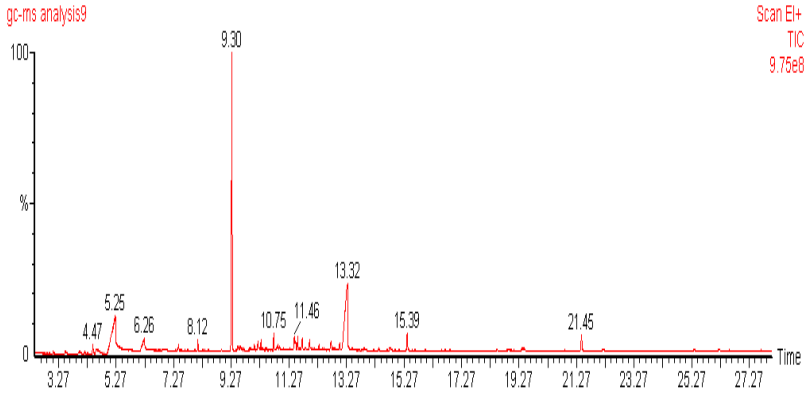

Fig. 3: GC-MS Analysis of Ethyl acetate: Methanol (70; 30) fraction of *Kappaphycus alvarezii* incubated in cow urine

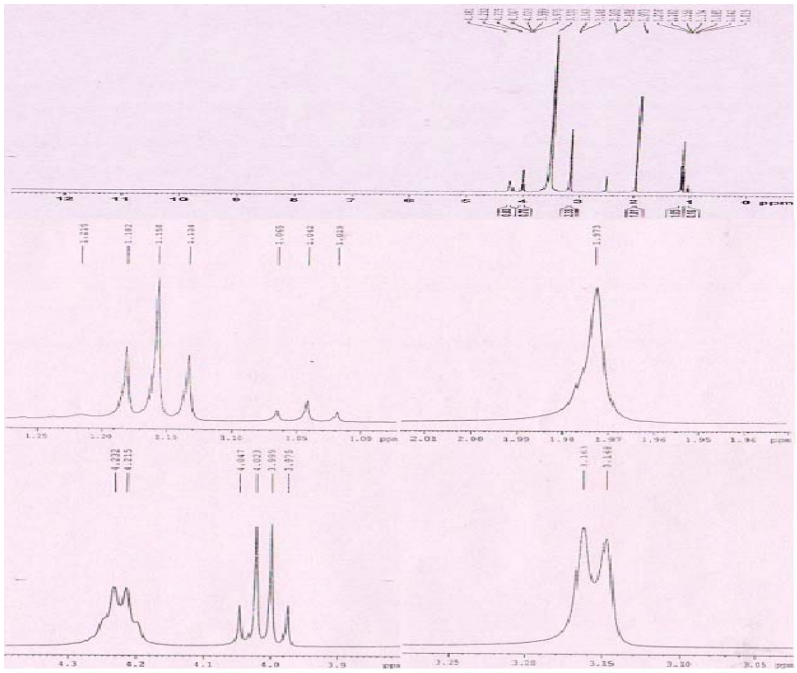

Fig. 4: 1 H-NMR spectra of *Kappaphycus alvarezii* incubated in cow urine

CONCLUSION

Isolation and identification of the plant pathogen *Xanthomonas oryzae* pv *oryzae*, was carried out. organic fractions and cow urine extract of red algae *Kappaphycus alvarezii* were tested for their antimicrobial activity against the isolated, the most effective extract was subjected to purification and identification of the active principle through, TLC, GC-MS, ^{1}H NMR, and HPTLC studies. It has been concluded from this study that incubated cow urine followed by the cow urine extract of *Kappaphycus alvarezii* showed best antixanthomonas activity. The active principle was identified as 2,3-Dihydro-7-methyl-1,4-benzoxazine-3-one. Instead of using hazardous chemicals to control the plant diseases, we may shift our forming towards these natural cost effect ecofriendly new formulations.

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