Bioenhancing Efficacy of Plant and Animal Origin Biproducts against *Xanthomonas Campestris* in Citrus Plant

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ABSTRACT: Bioenhancers are substances that increase the bioavailability and the uptake of drugs in combination therapy. It has been found that cow dung, cow urine and its distillate also possess bioenhancing ability. They act through several mechanisms of action affecting mainly absorption process, drug metabolism or action on drug-target. Drugs from the plants are easily available, less expensive, safe, and efficient and rarely have side effects. In the present study we investigated, the bioenhancing role of cow urine on antibacterial activity of organic extract of *leucas aspera* Linn, Antibacterial activity of organic fraction alone and in the combination of cow urine was determined against *Xanthomonas campestris* pv *citri.* All extracts and fractions were effective and showed 7 mm to 14 mm zone of inhibition. The Chloroform extract (12 mm) and cow urine extract (14 mm) showed great antibacterial activity. The antibacterial effect cow urine combination of extract was higher than the inhibition caused by extract alone and is suggestive of the bioenhancing role of cow urine. Results of Minimum Inhibitory Concentration (MIC) Alcohol and aqueous extracts had lowest value 5.55 µg/ml while highest 16.85 µg/ml shown by Ethyl acetate extract respectively. The cow urine extract had 6.15 µg/ml. Phytochemical analysis revealed the presence of secondary metabolites like alkaloids, flavones, steroids, flavones, tannins, terpinoids, quinines, phenols, steroids, phlobotanins and glycoside. These results indicate that the cow urine has antimicrobial activities, which supports the claim of traditional practitioners. Further studies on mechanism involved in bioenhancing effect of cow urine are under investigation.

Keywords: Alkaloids, Citrus plant, Cow urine extract, *Lamiaceae,* Phytochemical analysis, *Xanthomonas campestris* pv citri.

1 INTRODUCTION

Leucas aspera Linn. (Family: *Lamiaceae)* commonly known as 'Thumbai' in Tamil Nadu grows as a weed on wastelands and roadsides all over India from the Himalayas down to Ceylon. The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, expectorant, aperient, diaphoretic, insecticide and emmenagogue. Leaves are considered useful in chronic rheumatism, scabies, psoriasis, wound healing and other chronic skin eruptions and their juice is used as antibacterial agent and cobra venom poisoning [1, 2]. The efficacy of whole plant extracts of *L. aspera* has been proven on larvicidal and pupicidal activities against the malarial vector *Anopheles stephensi* [3]. Ethyl acetate extract of this plant has been evaluated for *invitro* activity against *Plasmodium falciparum* and assessed for cytotoxicity against HeLa cell line [4]. The ethanol soluble fractions of L.aspera showed good antioxidant activity [5] and its anti-inflammatory activity has been shown in animal models through prostaglandin inhibition. Chemical components like diterpenes, tannins, saponins, sterols, oleic, linoleic, palmitic, stearic, oleanolic and alkaloids [6] and sterols-sitosterol, stigmasterol, campesterol and a novel phenolic compound, long-chain aliphatic compounds triterpenes have been isolated from this plant. An alcoholic extract of the leaves shows anti-bacterial activity against Micrococcus pyogenes and Escherichia coli [7]. The organism like *Xanthomonas campestris* was reported to be severe phytopathogen, causing damage in carrot, potato, tomato, leafy greens, onion, green pepper, squash and other cucurbits. Furthermore, these phytopathogen cause disease in any plant tissue it invades [8]. From the ancient period cow's urine has been used as a medicine. In India, drinking of cow urine has been practiced for thousands of years. It is also used along with herbs to treat various diseases like fever, epilepsy, anemia, abdominal pain, constipation, etc by the traditional healers [9]. Immunomodulatory [10], [11] respiratory effects of cow urine were established scientifically and it shows excellent agricultural application in the form of biofertilizer, vermicompost, biopestisides which improves soil fertility and provide food grains free from diseases while dung has been used as organic fertilizer and in the production of biogas to generate electricity and heat [12]. Bioenhancers are substances which promote and augment the bioactivity or bioavailability or the uptake of drugs in combination therapy. Such bioenhancers have been earlier isolated only from plant sources. Several investigations have reported synergistic antibacterial effect of plant extracts, with significant reduction in the minimum inhibitory concentration of the antibiotic, against many pathogenic bacteria. It has been found that cow urine and its distillate also possess bioenhancing ability and helps reduce the dose of antibiotics and increase the efficacy of antibiotics against infectious agents [13]. When used in combination with number of drug classes such as antibiotics, antituberculosis, antiviral, antifungal and anticancerous drugs they are quite effective by the oral absorption of vitamins, minerals, herbal extracts, amino acids and other nutrients. The invention has direct implication in drastically reducing the dosage of antibiotics, drugs and anti-infective agent while increasing the efficiency of absorption of bio-active molecules, thereby reducing the cost of treatment and also the side-effects due to toxicity. Bioenhancing property of cow urine on antibacterial activity of plant extracts has not been much investigated. In the present study, we investigated the bioenhancing effect of cow urine on antibacterial activity of *Leucas aspera* Linn., plant extract against the pathogen *Xanthomonas campestris* pv citri. The main aim of this product development is to provide employment to the rural youth and to use safe disinfectant for cleaning floors etc.

2 MATERIALS AND METHODS

2.1 Collection and Identification

Citrus canker infected leaves were collected from the Depart-

ment of Plant Pathology, Agriculture College and Research Institute, Ramji Nagar, Trichy, in zip lock cover and transported to the laboratory within 2 hours. Leaves were surface sterilized with sterile distilled water followed by 0.1 % mercuric chloride and then rinsed with distilled water thrice. Cankered area alone was taken out and macerated into a smooth paste. A loopful of culture was transferred into Nutrient agar (Himedia) and *Xanthomonas* selective medium (Himedia) and incubated at 37 ^{o}C for 24 hours. Physiological and biochemical screening were used to identify the isolate. *Leucas aspera* Linn, plant leaves were collected from herbal garden and authenticated by herbal division of Srimad Andavan Arts and Science College, Tiruchirappalli, Tamil Nadu, South India. Plant leaves were washed thoroughly three times with running tap water and once with sterile distilled water and then dried under shade. Fresh and shade dried leaves were used for the study.

2.2 EXTRACTS PREPARATION

Cow urine extraction:
3 kg of plant leaves were surface sterilized with sterile distilled water and cut into small pieces and placed in an earthen pot separately with 10 liters of cow urine which was sufficient to sink all the leaves. The pot was kept in a pit dug in the soil incubated for 1-20 days. At the end of every 24 hrs the extracts were taken out and condensed at 40 ^{o}C into a paste and stored at 4 ^{o}C for further use.

Aqueous extraction:
100 g of shade dried leaves were coarsely powdered and added with 300 ml of sterile distilled water and boiled for 30 minutes. Filtered through three layered muslin cloth and condensed in to solid form at 40 ^{o}C using hot air oven.

Organic solvent extraction:
100 g of shade dried leaves were coarsely powdered and added with 300 ml of Organic solvent viz., Hexane, Chloroform, Ethyl acetate, Alcohol based on increasing polarity. Duration of incubation was 3 days at each solvent. The extracts so collected were evaporated on a water bath at atmospheric pressure and the solvents were completely removed in vacuum and the remaining matter was quantified.

2.3 Screening Anti bacterial

Antibacterial activity of organic fractions and cow urine extract of *Leucas aspera* leaves were assayed using the well diffusion method [15]. Appropriate quantity of extracts were dissolved in DiMethyl Sulfoxide (DMSO) and sterilized by using Sartorious syringe filter of pore size 0.22 µM (stock solution (0.04 g/1 ml). Sterile Petri plates containing 20 ml of Nutrient agar medium were seeded with 0.01ml of 18 hours old test bacterial strain isolate. Cow urine extracts and organic fractions were added at different concentration 400 µg, 600 µg, 1200 µg and 1800 µg were added into 6 mm diameter well. Incubation was made at 37 ^{o}C for 24 hours. The assessment of antibacterial activity was based on the measurement of diameter of the inhibition zone formed around the well, using Himedia scale. Streptomycin sulphates (30 µg) and DMSO (15 µg) were used as a negative control respectively.

2.4 Determination of Minimum Inhibitory-Concentration:

Agar dilution method was used to find out Minimal Inhibitory Concentration. Nutrient agar was prepared, sterilized and kept ready in molten condition. 20 ml of the molten media was taken and was mixed with known concentration of different extracts/fractions and were added in different tubes. This mixture was swirled carefully for complete mixing of extract and media and poured onto the plate. After getting solidified it was inoculated at 37 ^{o}C for 24 hours. After incubation the tubes were then examined for microbial growth by observing for turbidity.

2.5 Phytochemical Screening:

All the extracts were subjected to preliminary phytochemical screening to detect the presence of secondary metabolites as per the standard methods [16].

3 RESULT AND DISCUSSION

Recently the use of herbal medicines has been increased all over the world due to their therapeutic effects and fewer adverse effects as compared to the modern medicines. Plants consist of a complex mixture of a wide variety of compounds which can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells [17] and also cow urine has natural disinfectant and antiseptic qualities and it was consumed as an effective and simple medicine. From above the combination therapy of investigation, the antimicrobial potential of various extracts were compared according to their zone of inhibition against the plant pathogenic organism. The cow urine extracts of *Leucas aspera* showed good results. Maximum zone was exhibited on 6th day extract (14 mm) followed by, 7th day, 8th day, 9th day 10th day and 16th to 20th day extracts showed negative results. Variation of zone diameter was observed during the period of study. As the concentration of extract is increasing the zone of inhibition also increases (Table-1). A recent work on cow urine extract of *Vitex negundo* has been shown to effectively control the *Xanthomonas campestris* pv *citri* showed 16 mm zone of inhibition and its chemical constituents may be responsible for the antibactericidal activity [18]. Among all the fractions chloroform and alcohol fractions were found to be effective at 1600 µg concentration and produced 12 mm zone of inhibition which is more or less in par with the positive control. Aqueous extract also obtained the same diameter of inhibited value. Hexane and Ethyl Acetate extract showed 10 mm and 11 mm respectively.[19] done the Ethanol extract of *Leucas aspera* against both Gram positive and Gram negative bacteria and it exhibited the zone of inhibition against the *B. megaterium* [(13.00±1.50) mm] and *P. aeroginosa* [(13.00±1.00) mm]. He concluded, Gram positive strains were found more sensitive than Gram negative organisms to the extract on an average. Similarly [20], found out in our studies that fresh cow urine was more effective antimicrobial agent than photo activated urine this may be because fresh urine is more acidic in nature. The positive control streptomycin sulphate has shown 21.00 mm at 30 µg concentration. DMSO was used as negative control. (Table.2). When compared to both extracts, the cow urine extract was showed to be highly effective in controlling the pathogen. By using agar dilution method the minimum inhibitory concentration was determined as 6.15 µg/ml for cow urine extract and 5.55 µg/ml for alcohol and aqueous extracts respectively. (Table-4) Our results are supported by [21] who worked on the antibacterial activity of organic fraction of *A.aspera* (belongs to the same family). He reported that at 1.25-1.5 mg/ml it could control *E.coli, Bacillus subtilis, Vibrio cholerea, Salmonella typhi* and *Staphylococcus aureus*. The secondary metabolites existed in the plant extract play the key role in the pharmacological ac-

tions of the whole plant parts. This study was conducted to make a better logical approach in ascertaining the mentioned biological functions of *L. aspera* extract. Preliminary phytochemical screening revealed the presence of various bioactive components which include alkaloids, terpinoids, flavones, quinine, steroids, phenols, Phlobotannins in cow urine extracts (Table.5) and the organic fractions of *Leucas aspera* had terpinoids, flavones, Glycoside, tannin, saponin, alkaloids, quinine, phenols, steroids. (Table.6). These organic fraction screened results were consistent with the previously conducted partial studies shown the presence of alkaloids, flavonoids, terpenoids, steroids, tannins, phlobatannins, saponins and glycosides. [19] and these secondary metabolites responsible for antibacterial activity are greatly dependent on solvent system for their release in extractive. Moreover, growth area also affects the chemical components of the plants and leads to the activity difference.

4 CONCLSION

Considering hazardous effects of chemicals used for control of stored insect pests, it is necessitated to use the ecofriendly approaches such as botanicals and animal origin products for their management. However, it is a novel approach to use animal waste products i.e. cow urine with plant product as a grain protectants against *Xanthomonas campestris* pv *citri* on citrus plants. However, it could be concluded that the cow urine with plant extract highly effective in controlling the pathogen because of some active compounds may be responsible for biotransformation of constituents due to cow urine treatment. These studies clearly demonstrated the potential and possibilities of using indigenous bio products for the control of bacteria in citrus plant. It is totally environment friendly and is ideal for use in our modern world.

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.6 Figures and Tables

Table No 1.
Antibacterial activity of Lucas aspera cow urine extract against Xanthomonas campestris pv citri

S.No	*Lucas aspera* cow urine extract	Concentration of extract (µl) /zone of inhibition (mm)					
		+ve	-ve	40 µl	60 µl	80 µl	100 µl
1.	Day 1	25	Nil	7	7	8	9
2.	Day 2	25	Nil	7	7	8	9
3.	Day 3	27	Nil	8	8	9	11
4.	Day 4	27	Nil	10	10	11	12
5.	Day 5	25	Nil	10	10	10	11
6.	Day 6	26	Nil	11	11	12	13
7.	Day 7	26	Nil	10	10	10	12
8.	Day 8	25	Nil	10	11	11	12
9.	Day 9	26	Nil	10	10	11	11
10.	Day 10	25	Nil	11	11	11	12
11.	Day 11	25	Nil	10	10	10	11
12.	Day 12	26	Nil	10	10	11	11
13.	Day 13	29	Nil	8	9	9	10
14.	Day 14	28	Nil	8	8	9	10
15.	Day 15	27	Nil	-	-	9	11
16.	Day 16	26	Nil	-	-	-	-
17.	Day 17	28	Nil	-	-	-	-
18.	Day 18	28	Nil	-	-	-	-
19.	Day 19	27	Nil	-	-	-	-
20.	Day 20	26	Nil	-	-	-	-

***Key:** (+) means presence of constituent, (–) means absence of constituent.*

Table No 2.
Antibacterial activity of Lucas aspera organic fraction against Xanthomonas campestris pv citri

S.No	*Lucas aspera* fractions	Concentration of extract (µl) / zone of inhibition (mm)					
		+ve	-ve	40 µl	60 µl	80 µl	100 µl
1.	Hexane	27	Nil	8	8	9	10
2.	Chloro-form	28	Nil	10	11	11	12
3.	Ethyl acetate	27	Nil	9	9	10	11
4.	Alcohol	27	Nil	9	10	11	12
5.	Aqueous	28	Nil	10	11	12	12

Table No 3. Minimum Inhibitory Concentration of Lucas aspera

S.No	**Extracts**	**Concentration of Extracts µg/ml**
1.	Hexane	16.65
2.	Chloroform	16.65
3.	Ethyl Acetate	16.85
4.	Alcohol	5.55
5.	Aqueous	5.55
6.	Cow urine extract	16.85

Table No 4. *Phytochemical screening of cow urine extracts of Lucas aspera*

S.No	Days of incubation	Cow urine extract of *Lucas aspera*																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	Terpe-noids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
2.	Flavones	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.	Sugar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	Alkaloid	+	+	+	+	+	+	+	+	+	+	+	+	-+	+	+	+	+	+	+	+
5.	Quinone	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
6.	Coumarin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	Tannin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8.	Saponin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Glycoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.	Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11.	Steroids	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
12.	Phlo-botannins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
13.	Phy-tosterol	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
14.	Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key: (+) means presence of constituent, (–) means absence of constituent.

Table No 5. Preliminary phytochemical analysis of Lucas aspera

S.No	***Test***	***Hexane***	***Chloroform***	***Alcohol***	***Ethyl ace-tate***	***Aqueous***
1.	*Terpenoids*	+	+	+	+	+
2.	*Flavones*	+	+	+	+	+
3.	*Sugar*	-	-	-	-	-
4.	*Alkaloid*	+	+	+	-	-
5.	*Quinone*	+	+	+	+	+
6.	*Coumarin*	-	-	-	-	-
7.	*Tannin*	+	+	+	+	+
8.	*Saponin*	-	-	+	+	+
9.	*Glycoside*	+	+	+	+	+
10.	*Phenols*	-	+	+	+	-
11.	*Steroids*	+	+	+	+	+
12.	*Phlobotannins*	+	+	+	+	+
13.	*phytosterol*	+	-	-	-	-
14.	*Starch*	-	-	-	-	-

(+) means presence of constituent, (–) means absence of constituent.

REFERENCES

[1] G. Adwan and M. Mhanna, “Synergistic Effects of Plant Extracts and Antibiotics on Staphylococcus aureus Strains Isolated from Clinical Specimens”, Middle-East Journal of Scientific Research. 3 (3): 134-139, 2008.

[2] R. N. Chopra, S.L. Nayar and I.C. Chopra, “In; Glossary of Indian Medicinal Plants”, NISCAIR, CSIR, New Delhi, 153, 2002.

[3] P. Damiao De Sousa, S.Ivandilson Santos, C. Tamires Lima, T. Marília de Santana, R. V. Márcio dos Santos, S. S. Juliana Quintans, Lucindo and J. Quintans-Júnior. “Phytochemical and pharmacological evaluation from leaves of Costus spicatus Swartz. (Costaceae”), Journal of Chemical and Pharmaceutical Research, 2014, 6(10):1-8, 2014.

[4] D. K. Abdullahi, O. O.Michael, I. I. Indabawa, “Antibacterial Activities and Phytochemical Screening of Aloe Vera (A Babardensis), Garlic (A Sativum) and Ginger (Z Officinale)”. Journal of Emerging Trends in Engineering and Applied Sciences. 5(3): 172-178. 2014.

[5] B. Latha, Y. Rumaisa, C. K. Soumya, Shafeena Shahul and N. Sadhiya, “Phytochemical studies on Leucas aspera”. J. Chem. Pharm. Res. 2013, 5(4):222-228. 2013.

[6] M. S. Prajapati, J. B. Patel, K. M. B. Modi Shah, “Leucas aspera: A review”, Pharmacogn Rev. 4(7):85–87, [PubMed], 2010.

[7] K. R. Kirtikar and B. D. Basu, “Indian Medicinal Plants, Vol. III, International Book Distributors”, Dehradun, India p 2019, 2005.

[8] J. C. Setubal, L. M. Moreira da Silva, “Bacterial phytopathogens and genome science”, Oct; 8(5):595-600, 2005.

[9] K.Krishnamurthi, D.Dutta, S.S.Devi and T. Chakrabarti, “Protective effect of diatillate and redistillate of cows urine in human polymorphonuclear leukocytes challenged with established genotoxic chemicals”, Biomed. Environ. Sci. 17: 57-66, 2004.

[10] R.S. Chauhan, B.P.Singh and L.K.Singhal, “Immunomodulation with kamdhenu Ark in mice”, J. Immunol. Immunopathol. 71: 89-92, 2001.

[11] J.A. Ojewole and S.O. Olusi, “Effects of cow’s urine concoction on plasma glucose concentration in fasted rats”, Trans. R. Soc. Trop. Med. Hyg. 71: 241-245, 1976.

[12] A. S. A. Leonardo, “J. Sci. 7. 1, 2005.

[13] G. Adwan and M. Mhanna, “Synergistic Effects of Plant Extracts and Antibiotics on Staphylococcus aureus Strains Isolated from Clinical Specimens”, Middle-East Journal of Scientific Research. 3 (3): 134-139, 2008.

[14] C. Perez, M. Paul and P, Bazerque, “Antibiotic assay by agar-well diffusion method”, Acta Biologiae Ethnomedicine Experimentalist. 15: 113–115, 1990.

[15] J. B. Harborne, “Phytochemical methods”, Chapmen and Hall co New York, 1984.

[16] M. M. Cowan, “Plant products as antimicrobial agents’, Clin. Microbiol. Rev. 564-582, 1999.

[17] V. Nithya Devi and S. Pradeep Kumar, “Evaluation of the Antibacterial Potential, Preliminary Phytochemical Screening of Medicinal Plant against Plant Pathogen”. Res.J.Phytochem. 8(3):92-101, 2014.

[18] Md. Atiar Rahman and Md. Saiful Islam, “Antioxidant, antibacterial and cytotoxic effects of the phytochemicals of whole Leucas aspera extract”, Asian Pac J Trop Biomed. 3(4): 273–279, 2013.

[19] Anami Ahuja, Pushpander Kumar, Ankit Verma, S. Ranjeet Tanwar, “Antimicrobial activities of cow urine against various bacterial strains”. Int J Recent Adv Pharm Res. 2(2):84-87. 2012.

[20] M.T .Alam, M.M, Karim, S.N. Khan, “Antibacterial Activity of Different Organic Extracts of Achyranthes Aspera and Cassia Alata”, J. of Scientific research, 1 (2): 393-398, 2009.

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Figure 1. *Antimicrobial activity of organic fraction of Leucus aspera on Xanthomonas citri pv citri.*

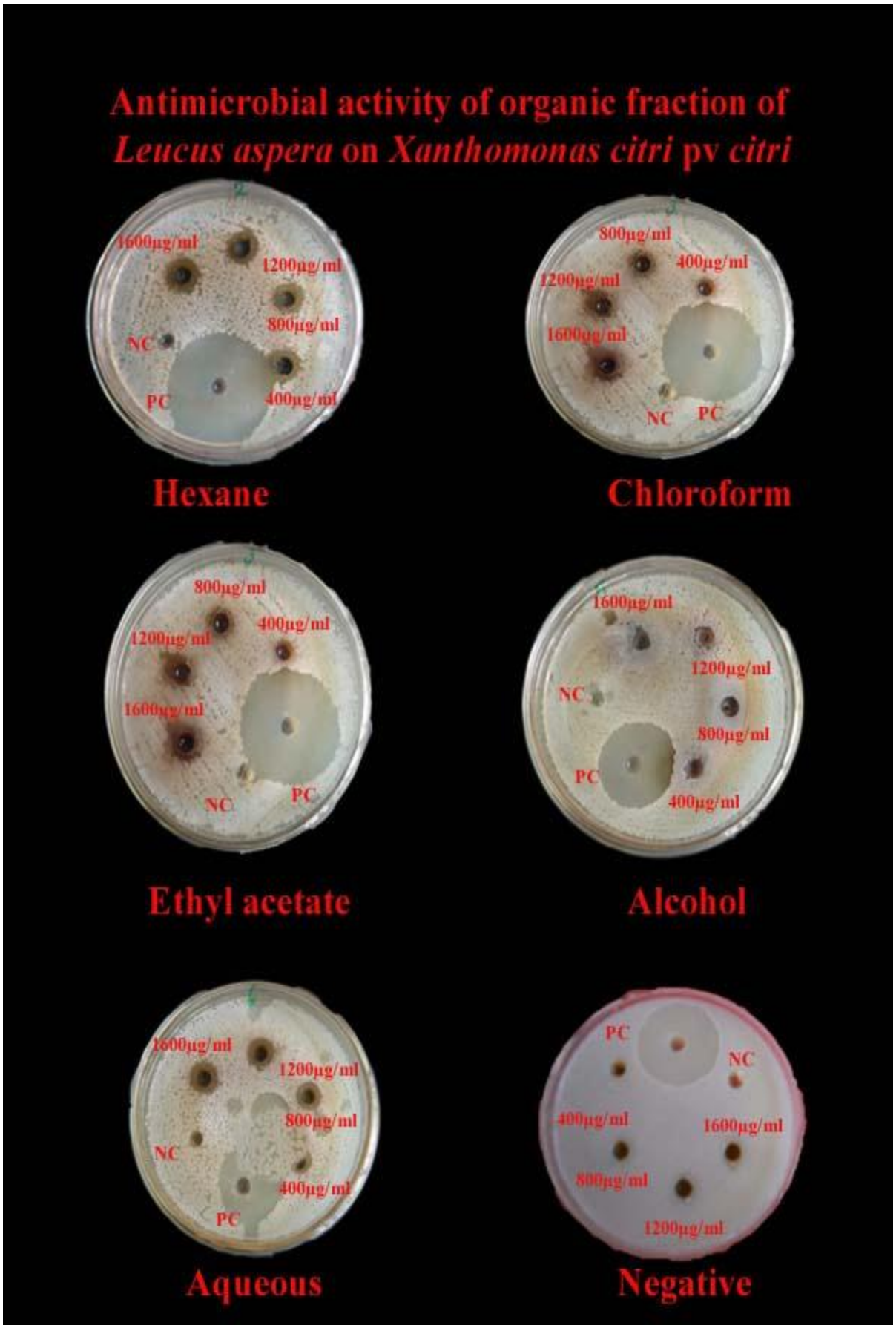

Figure 2. *Antimicrobial activity of cow urine extract of Leucus aspera on Xanthomonas citri pv citri.*

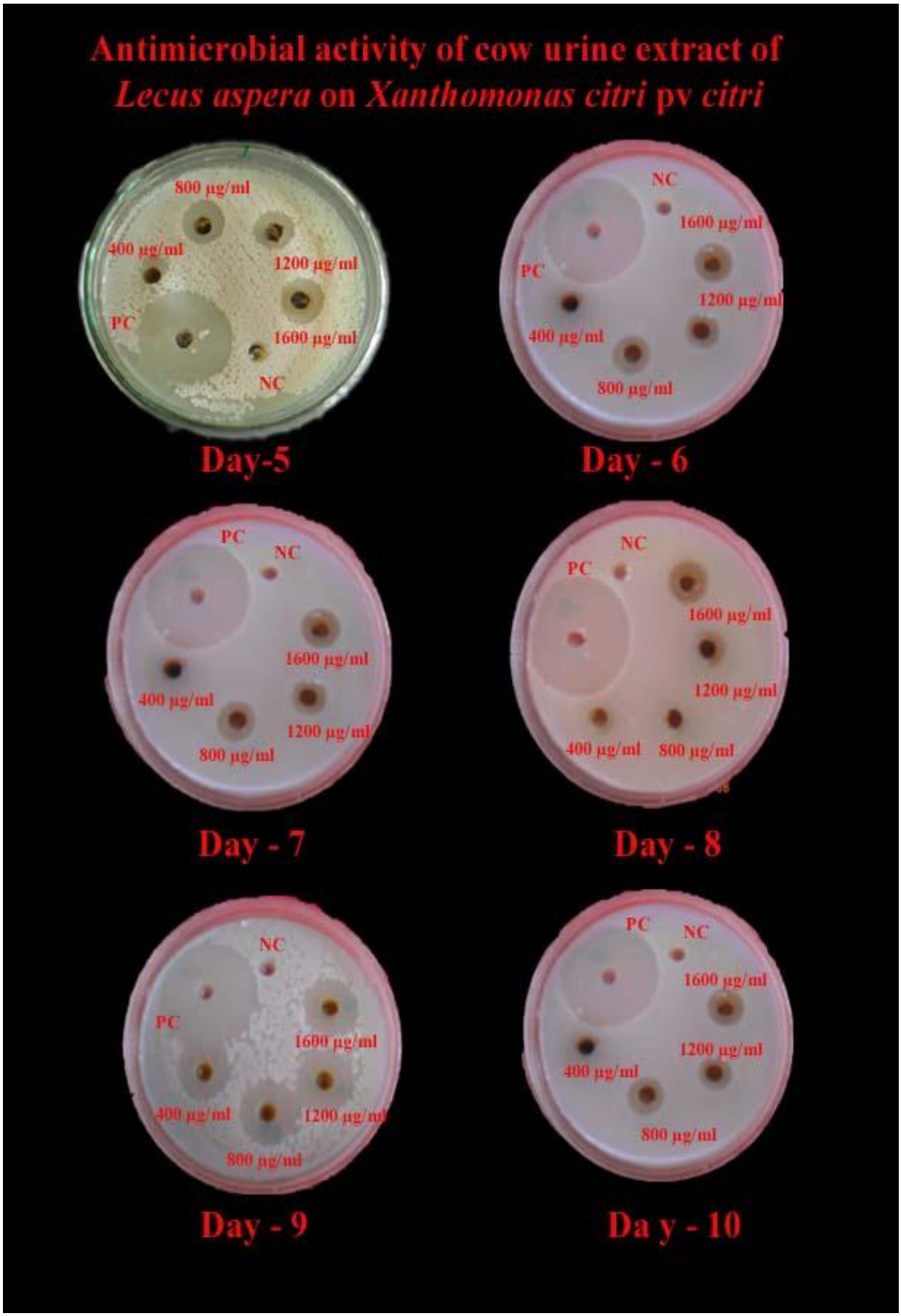

Figure 3. *Minimum inhibitory concentration of cow urine extract of vitex negundo and Leucus aspera on Xanthomonas citri pv citri.*