Anti-Phytopathogenic Evaluation of Synergistically Formulated Cow Urine and Aqueous Extract of some Selected Plants

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Abstract – Plants extracts have been known to possess antimicrobial components that are effective to inhibit the pathogens.The different phytochemical present in plant extracts are effective in eliminating phytopathogens and could be better option for using them as biopesticides. The aqueous extracts of neem (Azadirachta indica) leaves, garlic (Allium sativum) tuber and chilly (Capsicum annum) fruits and cow urine extracts were prepared and tested for presence of phytochemicals. The antimicrobial activity of plant extracts against Xanthomonas axonopodis pv citri, Xanthomonas oryzae, Fusarium oxysporum f.sp. cubense and Bipolaris oryzae which were the potent phytopathogens was analyzed by agar well diffusion method and minimum inhibitory concentration (MIC). All the plant extracts reported significant zones of inhibition against phytopathogens. The cow urine extracts of plants extracts showed effective inhibitory activity against selected phytopathogens. The inhibitory activity of A. indica and A. sativum combination was found be effective against X. axonopodis pv citri and F. oxysporum f.sp cubense with lowest MIC of 312.5µg/mL. The antimicrobial activity of different plant aqueous and cow urine extracts were statistically significant ($p<0.05$).This study concluded that botanical extracts in combination with cow urine can be an effective Biopesticides for the farmers. The rational use of plant extracts could be more beneficial to replace the use of chemical pesticides.

Keywords: Plant extracts, Cow urine, Synergistic effect, Phytopathogens, Antimicrobial activity

INTRODUCTION

Plant pathogens such as fungi, bacteria, nematodes and viruses cause various diseases in plants enough to damages the plants (Montesinos, 2003). Irrational use of synthetic agents to control bacterial disease of crop plants has caused health hazard in animals and humans due to their residual toxicity (Raghavendra et al., 2006). Plant extracts exhibiting antibacterial compounds can assist plant disease management because of their eco-friendly nature (Bolkan and Renert, 1994). Neem has been proven to possess many medicinal properties (Packiaet al., 2012). Garlic has been used to prevent wound infection and food spoilage (Arora and Kaur, 2007) as well as chilli peppers are used worldwide in foods for their pungent flavor, aroma, and to prolong food spoilage. It has been found that cow urine is effective in agricultural operations in form of biofertilizer and biopesticide as it exhibit antimicrobial activity against pesticide and herbicide resistant bacteria, viruses, fungi (Dhama et al., 2005). Cow urine with plant extracts antimicrobial agents which are biodegradable with eco-friendly nature (Mandavgane et al., 2005). The antifungal and antibacterial compounds present in plants have long been identified to resist various plant diseases (Mahadevan, 1982). Hence, the main objective of this work is to evaluate the effect of plant extract as natural pesticide for the control of different plant disease causing pathogens reducing the indiscriminate conventional pesticide application among farmers.

MATERIAL AND METHODS

The study was carried out in central campus of Technology, Dharan, Nepal from November 2018 to April 2019. The plants materials were collected from the different areas of Dharan. The selected plants were firstly identified from herbarium collection of Postgraduate Campus Biratnahar, Nepal. The selected plant materials were washed with water to remove soil and unwanted particles and were chopped into small pieces to reduce time for drying

and to grind easily. The plant materials were kept under shade at room temperature for 2 weeks to dry and grinded with grinder to obtain fine powder of plant material.

Preparation of plant extract

Plant extract were prepared by following the method described by Ndip *et al.*, (2007) and Rajpandiyan *et al.*, (2011). Sterile distilled water and cow urine were used as a solvent to prepare the crude plant extract as to mimic the traditional style and they are easily available. These plant parts were administered as either infusions or decoctions. Hundred grams of each powdered plant material were macerated in 1000 ml of each solvent in extraction pots such that the level of the solvent was above that of the plant material. The macerated mixtures were then left on the waterbath shaker for 72 hours at room temperature for continuous shaking. The mixtures were then allowed to settle for 24 hour and solvent containing water was decanted. The decanted extracts were then filtered by two fold muslin cloth followed by Whattman filter paper No.1 (pore size 45 µm). After filtration solvent was evaporated in water bath at 40°C to dryness to obtain solid mass of the extract and were stored at 4°C until use. 400mg of crude extract was dissolved in 10ml DMSO to make concentration of 40000µg/mL stock solution.

Calculation of percentage yield of extract

After the complete dryness of plant material, the percentage yield of plant extract was calculated. The percentage yield of the plant extract was calculated as below:

$$\text{Percentage yield (\%)} = \frac{\text{Dry wt.of Extract}}{\text{Dry wt.of plant material}} \times 100$$

Phytochemical screening of plant extract

The crude extracts (water and cow urine) were subjected to qualitative phytochemical screening to detect the constituents (tannins, phlobatannins, saponins, alkaloids, flavonoids, terpenoids) using standard procedures as described by Sofowora, (1993), Trease and Evans, (1989) and Harborne, (1973), Tiwari*et al.*, (2011).

Isolation of Phytopathogen

The plant samples for the isolation of these bacteria were obtained from agricultural fields of Tarahara, Sunsari, Nepal. *Xanthomonas oryzea* pv *oryzea* was isolated as described by Kala *et al.*, (2015), characterized and pathogenicity test was performed as described by Patil and Devanna, (2016). Similarly, isolation of *Xanthomonas axonopodis* pv *citri* was isolated, characterized and pathogenicity test was performed as described by Gadhe *et al.*, (2016). *Fusarium oxysporum* f.sp *cubense* was isolated, identified and pathogenicity test was performed as described by Udompongsuk and Soytong, (2016). Similarly, *Bipolaris oryzea* was isolated, identified and pathogenicity test was performed as described by Sobanbabu *et al.*, (2018).

Antimicrobial screening via Agar Well Diffusion technique and Poison food technique

The antimicrobial activity of plant extract was evaluated against the phytopathogens by agar well diffusion method for bacteria as described by Ginovyan *et al.*, (2017) and Poison food technique for fungal plant pathogens as described by Rao and Srivastava, (1994). For antibacterial bioassay, the fresh bacterial inoculum with standard turbidity i.e., 0.5 Mc Farland was seeded over the MHA (Himedia, Mumbai, India) plates using sterile cotton swab. With the help of corkborer no. 6, wells of 6mm in diameter were made in the inoculated plates and where 100µl of the extract with concentrations; 25mg/mL and 50mg/mL was pipette onto separate wells. DMSO itself was tested as a control in a separate well. The plates were then incubated at 37°C for 24 hours. After the incubation, the plates were observed for the halo zone around the well and the zone of inhibition was measured and recorded.

For antifungal bioassay volume of 0.5ml of each concentration was aseptically poured into the petriplate followed by the addition of 9.5ml of melted PDA (HiMedia, Mumbai, India) and was swirled gently to achieve thorough mixing of the contents. In the control set, no extract was used. After the solidification of the media, one inoculum disc of the test fungus was aseptically inoculated upside down at the center of the petriplate and incubated at

25°C. The average diameters of the fungal colonies were measured on the 7th day of incubation and percentage of mycelial growth inhibition was calculated.

Mycelial growth inhibition (%) $=\frac{gc-gt}{gc} X\ 100$

Where,

gc = growth of mycelial colony in control set after incubation period subtracting the diameter of inoculum disc.

gt= growth of mycelial colony in treatment set after incubation period subtracting the diameter of inoculum disc.

Broth microdilution assay for minimum inhibitory concentration (MIC) of bacteria

MIC values of the plant extracts against bacterial strains were determined based on a micro well dilution method as described by Swanson *et al.*, (1992).The 96-well plates were prepared from plant crude extracts initially prepared at the concentration of 40mg/mL in DMSO (2.5%). Serial dilutions were transferred into different consecutive wells to achieve concentrations from 40000µg/mL to 0.625µg/mL. The negative and positive control was maintained. The microtitre plates were covered with sterile lid and incubated at 37°C for 24 hrs. The lowest concentration of the test samples, which did not show any growth of tested organism, was determined as MICs, which were expressed in µg/mL.

Determination of minimum inhibitory concentration (MIC) of fungus

The in-vitro fungicidal activity of plant extracts were performed according to Dellavalle *et al.*, (2011). The crude extracts of plants were dissolved in 2.5% DMSO solution to get the initial concentration of 40mg/mL. The growth assay was performed in microtitre wells incorporated with PDB and fungus inoculums. Serial dilution was performed to get concentration ranging from 40000µg/mL to 0.625µg/mL. The plates were incubated at 27°C for 48 hours. The lowest concentration of the test samples, which did not show any growth of tested organism, was determined as MICs, which were expressed in µg/mL.

Data analysis

The information collected was documented and tabulated. The data were statistically analyzed at 5% level of significance by SPSS version 16.The p value less than equal to 0.05 was known to be statistically significant.

RESULTS AND DISCUSSION

Phytochemical Screening of Samples

In our study the Phytochemical screening of aqueous neem extract had shown the presence of phytochemicals such as flavonoids, alkaloids and carbohydrates etc. whose result was consistent to study performed byRamadas and Subramanan, (2018). Similarly,in our study the aqueous chili extract showed the presence of tannins, saponins and alkaloids whereas the study performed by Hemalatha, (2013) had shown the presence of terpenoids along with these phytochemicals. This vary in result might be due the ratio of solvent to plant sample while extraction, reagents used etc. The result of aqueous garlic extract phytochemicals was similar to the study of Huzaifa*et al.*, (2014). Our results have shown similarity with the study of Rajapandiyan*et al.*, (2011), where cow urine neem extract also showed the presence of similar phytochemicals except phenol. The difference in antimicrobial properties of different plant extract might be due to the difference in the type and amount of phytochemicals present in them.

Test	*A. indica*		*A.sativum*		*C. annum*	
	Aqueous extract	Cow urine extract	Aqueous extract	Cow urine extract	Aqueous extract	Cow urine extract
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+

Phlobatanins	-	-	-	-	-	-
Flavonoids	+	+	+	+	-	-
Terpenoids	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+
Carbohydrates	+	+	+	+	-	-
Proteins	-	-	-	-	-	-
Phenols	-	-	-	-	-	-
Amino Acids	+	+	+	+	+	+

Physical characteristics of plants extracts

In this work, different parts of plants were selected on the basis of reported use for its antimicrobial properties. *A. indica, A. sativum* and *C. annum*had been used by local farmers as they were easily available and possessed antimicrobial properties for the control of disease causing plant pathogens. Water and cow urine had been used as a solvent to obtain the plant extract as they are easily available and cheap for the farmers or local people to use.There was a difference in the percentage yield of the solvent extracts from different plant samples. The differences ranges from 12.53% to 31.55% with water extract whereas 14.52% to 40.28% with cow urine extract. The differences in yield might be due different type and part plant materials, particle size of the plant sample, maturity of plant during sampling and extent of dryness etc.

SN	Plant	Solvent	Characteristics of extract		Dry weight (gm)	Weight of extract (gm)	% Yield
			Color	Consistency			
1.	*A. indica*	Water	Dark green	Solid	100	12.53	12.53
		Cow urine	Dark green	Solid and sticky	100	14.52	14.52
2.	*A. sativum*	Water	Yellow	Solid	100	31.55	31.55
		Cow urine	Yellow	Semi-solid	100	40.28	40.28
3.	*C. annum*	Water	Dark red	Solid	100	19.87	19.87
		Cow urine	Dark-red	Semi-solid and sticky	100	25.42	25.42

Zone of Inhibition (ZOI) of plant extract (aqueous and cow urine) against bacterial plant pathogen

In fact ZOI and MIC are two different attributes for the evaluation of antibacterial effect and MIC for antifungal effect of obtained plant extract. The MIC value is important to evaluate the dose response relationship of plant extract with bacteria/fungi. Jabeen, (2011) reported the significant activity of extract of *Azadirachta indica* on isolates of *Xanthomonas oryzae*. Naqvi *et al*., (2019) showed significant efficacy of Neem extract and chilly extract on *Xanthomonas oryzae* pv *oryzea*. Similar findings were even observed on our study where Neem extract exhibited greatest antimicrobial property against selected phytopathogens. Rakesh *et al*., (2013) reported antifungal activity being displayed by the cow urine extract of certain plants against *Fusarium* sp. Even in our study the cow urine extracts of all the selected plants exhibited antifungal activity against *F. oxysporum* f.sp *cubense* and *B. oryzea*.

SN	Plant extract	Bacteria	Zone of Inhibition (mm) Aqueous			Zone of Inhibition (mm)Cow urine	
			DMSO	25mg/mL (mm)	50mg/mL (mm)	25mg/mL (mm)	50mg/mL (mm)
1.	*A. indica*	*X. oryzea* pv *oryzea*	-	9	12.3	11	14
		X. axonopodis pv *citri*	-	10	14	12	14.7
2.	*A. sativum*	*X. oryzea* pv *oryzea*	-	8	11	10	12
		X. axonopodis pv *citri*	-	8.5	11.8	10.4	12.6

3.	*C. annum*	*X. oryzea* pv *oryzea*	-	7.8	10	9.2	12.8
		X. axonopodi spv *citri*	-	8	10.6	10	13

Mycelial growth inhibition by the crude aqueous and cow urine extract of selected plants against fungal plant pathogens

In our study, cow urine extract of *A. indica* showed the highest mycelia growth inhibition followed by *A. sativum* and *C. annum* with 92%, 75% and 65% mycelial growth inhibition of *Fusarium oxysporum* f.sp *cubense* at the concentration of 50 mg/mL respectively. The result was similar with the research of Shrestha and Tiwari, (2009) where the extract of *A. sativum* inhibited the mycelia growth of *F. solani* at the concentration of 40%. Similarly, in our study, the cow urine extracts of *A. indica* showed maximum mycelia growth inhibition followed by *A. sativum* and *C. annum* with 50%, 40% and 35% mycelial growth inhibition of *Bipolaris oryzea* which was similar with the research of Bisht and Khulbe, (1995) and Ganguly (1994) where *A. indica* extract had shown best inhibitory effect against *Bipolaris oryzea*.

SN	Plant extract	Fungi	Mycelial growth inhibition (%) Aqueous			p-value	Mycelial growth inhibition (%) Cow urine		p-value
			DMSO	25mg/mL (mm)	50mg/mL (mm)		25mg/mL (mm)	50mg/mL (mm)	
1.	*A. indica*	*F.oxysporum* f.sp. *cubense*	-	42	78	0.05	56	92	0.039
		B. oryzea	-	35	72		50	86	
.	*A. sativum*	*F. oxysporum* f.sp. *cubense*	-	36	68		42	75	
		B. oryzea	-	30	64		35	62	
3.	*C. annum*	*F.oxysporum* f.sp. *cubense*	-	30	58		35	65	
		B. oryzea	-	25	52		40	60	

Minimum Inhibitory concentration of crude aqueous and cow urine extracts of plant extract against *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri*

The aqueous extract of *A. indica* and in combination with *A. sativum* showed the best inhibitory effect against *X. axonopodis* pv *citri and* with lowest MIC 1250 µg/mL and 625 µg/mL respectively. Similarly, combined extract of *A. indica* and *C. annum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250 µg/mL and the plant extract of *A.indica*, *A. indica*+ *A.sativum*, *A.sativum* + *C. annum* showed the similar MIC of 5000µg/mL against *X. oryzea* pv *oryzea*. Chowdappa *et al.*, (2018) studied the effect of neem, chilli and garlic against *Xanthomonas* sp. and found that the aqueous extract also possessed the good antimicrobial property which increased with increase in the concentration of aqueous formulation. Shanti *et al.*, (2015) reported cow urine extract of *A. indica* most effective than the other extracts exhibiting maximum antibacterial activity against *X. oryzae* pv *oryzae* with MIC at 1600 µg/mL concentration. Similarly, cow urine and extract of *A. indica* and *A. indica* + *A. sativum* showed maximum antibacterial activity against *X. axonopodis* pv *citri* with lowest MIC of 625 µg/mL and 312.5µg/mL respectively. Similarly, combined extract of *A. indica* + *A. sativum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250 µg/mL.

SN	Plant extract	MIC (µg/mL) aqueous		p-value	MIC (µg/mL) cow urine		p-value
		X. oryzea pv *oryzea*	*X. axonopodis* pv *citri*		*X. oryzea* pv *oryzea*	*X. axonopodis*pv *citri*	
1	*A. indica*	5000	1250		2500	625	
2	*A. sativum*	10000	2500		5000	2500	
3	*C. annum*	20000	5000		10000	1250	
4	*A. indica* + *A. sativum*	5000	625	0.00	1250	312.5	0.04
5	*A. sativum* + *C. annum*	5000	2500		5000	1250	
6	*C. annum* + *A. indica*	1250	2500		5000	625	

Minimum Inhibitory concentration of crude aqueous and cow urine extracts of plant extract against *Fusarium oxysporum* f.sp *cubense* and *Bipolaris oryzea*

Moslem et al., (2009) found that neem leaves and seed extracts were effective against all tested *Fusarium oxysporium* with a complete inhibition (100%) of growth of Fusarium oxysporium at 40% level of ethanolic and methanolic extracts. Even in our study, the inhibitory effect of aqueous extract of *A. indica* alone and in combination with *A. sativum* and *C. annum* showed the best inhibitory effect against *F. oxysporum* f.sp *cubense* with similar MIC 1250 µg/mL. In our study the combined aqueous extract of *A. indica* in combination with *A. sativum* showed the best inhibitory effect against *B. oryzea* with lowest MIC of 2500µg/mL. Similarly, Manimegalai *et al.*, (2012) also obtained good inhibitory activity of aqueous extract of Neem against *Bipolaris oryzae*. Rakesh *et al.*, (2013) found antifungal activity being displayed by cow urine extract of certain plants against *Fusarium* sp. causing rhizome rot disease in ginger. In our study, the cow urine extract of *A. indica*, *A. indica* + *A. sativum, C.annum* + *A. indica* showed the best inhibitory effect against *B. oryzea* and *F. orysporum* f.sp *cubense* with MIC of 2500 µg/mL and 312.5 µg/mL respectively. Mehta *et al.*, (2014) also observed formulations

SN	Plant extract	MIC (µg/mL)Aqueous		p-value	MIC (µg/mL) Cow urine		p-value
		*F. oxysporum*f.sp *cubense*	*B. oryzea*		*F. oxysporum*f.sp*cubense*	*B. oryzea*	
1	*A. indica*	1250	5000		625	2500	
2	*A. sativum*	5000	10000		2500	5000	
3	*C. annum*	5000	20000		5000	10000	
4	*A. indica* + *A. sativum*	1250	2500	0.027	312.5	2500	0.047
5	*A. sativum* + *C. annum*	5000	10000		1250	5000	
6	*C. annum* + *A. indica*	1250	5000		312.5	2500	

containing crude extracts from four plants with cow urine were shown to exhibit good antimycotic activity.

In our study all the plant extract had shown the significant effect on the control of both fungal and bacterial plant pathogens. The cow urine extract showed greater inhibition than the aqueous extract which might be due to the presence of some antimicrobial substances in cow urine. The extract of *A. indica* had shown pronounced effect than other plant extracts alone and in combination with *A. sativum* and *C. annum*. The differences in antimicrobial properties might be due to differences in phytochemical composition

(Owuor*et al.*, 1986; Toda *et al.*, 1989). The antimicrobial activity of different plant aqueous and cow urine extracts against phytopathogens were statistically significant ($p<0.05$).

CONCLUSIONS

This study revealed that selected plants contained antimicrobial properties against the plant pathogens. In comparative study plant extract using cow urine as a solvent showed significantly better result as compared to aqueous plant extract. From this work, it can be concluded that the botanical extract in combination with cow urine could be a safe method for the control of plant pathogen and might be helpful to replace the harmful chemical pesticide in the field too.

Abbreviations: MHA-Mullen-Hinton Agar, PDA-Potato Dextrose Agar, DMSO-Dimethyl Sulfoxide.

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Authors' contributions

Bidhya Dhungana participated in study design, sample collection, processing, organism identification, data analysis and preparing the manuscript. Bijay Kumar Shrestha participated in sample collection, sample processing, organism identification, data analysis and interpretation. Jenish Shakya participated in data analysis and interpretation. Hemanta Khanal supervised the whole work. All the authors assisted in preparing and approving the manuscript.

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