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ANTIMICROBIAL POTENTIAL OF DIFFERENT SOLVENT EXTRACTS OF TOBACCO (*NICOTIANA RUSTICA*) AGAINST GRAM NEGATIVE AND POSITIVE BACTERIA

JEHAN BAKHT[1*], AZRA[1] AND MOHAMMAD SHAFI[2]

[1]*Institute of Biotechnology and Genetic Engineering, University of Agriculture Peshawar, KPK Pakistan*
[2]*Department of Agronomy, University of Agriculture Peshawar, KPK Pakistan*
** Corresponding author email: jehanbakht@yahoo.co.uk*

Abstract

The present study was aimed to evaluate the antimicrobial activity of tobacco extracts from *Nicotiana rustica* at different concentration in different polar solvents. For this purpose 6 different extracts were prepared, using 5 different polar solvents viz., ethanol, ethyl acetate, n-hexane, acetone, butanol and water. Four different concentrations (6, 12, 18 and 24 mg $Disc^{-1}$) of each extract were subjected for preliminary antibacterial screening against 7 pathogenic bacteria by Kirby-Bauer Disk Diffusion method. The result of *In vitro* antibacterial screening showed that 6 extracts from *Nicotiana rustica* had different ranges of antibacterial activities. The ethyl acetate extracts showed more potent effects followed by butanol, very little in the ethanol extracts while no significant inhibitory effects were observed in acetone or hexane extracts. When tobacco extracts were studied for their antibacterial potential against gram-positive bacteria and gram-negative bacteria, ethyl acetate extracted samples were more effective against *Bacillus cereus, Staphylococcus aureus,* and *Erwinia carotovora* at highest concentrations. Hexane and acetone extracted samples did not inhibit the growth of both gram +ive and –ve bacteria.

Introduction

Plants have been used as a valuable source of natural products for maintaining human health since ancient times in all parts of the world. During the last decade more intensive studies have been devoted to natural therapies (Rahman *et al.,* 2004; Agra *et al.,* 2007a; Ushimaru *et al.,* 2007). Researchers are employing extracts for their antibacterial, antifungal and antiviral activities. It is reported that more than 400, 000 plant species of tropical origin have medicinal properties (Lopez *et al.,* 2001; Odugbemi, 2006). As a result of microbial resistance to available antibiotic and increasing popularity of traditional medicine, researchers around the globe are investigating the antibacterial compounds in different plants species (Yildirim *et al.,* 2000 and 2001; Naz *et al.,* 2010; Bakht *et al*., 2011a, b, c, d; 2012; 2013).

Solanaceae family includes different crop species such as tomato, potato pepper and tobacco etc., however, tobacco is the major member of this family. The principal specie producing commercial tobacco is *Nicotiana tabacum*. Commercial tobacco can also be obtained from its other sister species such as *Nicotiana rustica*, which is smaller in height with fewer leaves than *N. tabacum*. Nicotine inhibits the growth of pathogens in dose dependent manner (Maria *et al.,* 2007; Wang *et al.,* 2008; Suresh *et al*., 2008). It is equally effective against gram-positive and gram-negative bacteria along with the acid-fast *Mycobacterium phlei* and the opportunist fungi *Candida albicans* and *Cryptococcus neoformans*. Levels of inhibition (≥50%) occurred when most of the affected organisms were cultured with nicotine at 100-250 µg ml^{-1}. The above mentioned concentrations of nicotine can be found *In vivo* (Russel *et al.,* 1981), especially in the oral cavity of smokeless tobacco users, making these findings physiologically relevant. With these considerations, the present study was initiated to investigate the effect of different solvents extracts of *N. rustica* on microbial activity of gram +ive and –ve bacteria.

Materials and Methods

Plant material: The present study was conducted at the Institute of Biotechnology and Genetic Engineering, University of Agriculture Peshawar KPK Pakistan. The tobacco plant specie *Nicotiana rustica* used in the present research work was collected from the farmer's tobacco fields at Jamal Garhi Mardan Khyber Pukhtun Khwa Province of Pakistan. Plants were washed with distilled water to remove dirt and soil particles. The plants were cut into small pieces and dried in shaded area at room temperature for a period of seven days. The dried plants leaves were grinded and sieved through sever.

Procedure for plant extracts: Six hundred grams of dried powdered plant material of tobacco (*Nicotiana rustica*) were taken into separate round bottom flasks and filled with 95% ethanol until dipped and fixed with the condenser. The material was boiled at 50ºC for 24 hrs and filtered with the help of vacuum pump using Buchner funnel. Ethanol was isolated from the mixture of the extract through rotary evaporator at 60ºC under reduce pressure. Ethanol extract was collected from the flask and dried through water bath at 60ºC. After drying, the extract was weighed and stored into a vile. Extract from the same plant material was collected exhaustively and this procedure was repeated thrice for the same plant material.

Part of the crude extract was used for further fractionation. The extract for fractionation was suspended into 100 ml distilled water having methanol (water: methanol at the ratio of 8:2) and made the total volume up to 200 ml and poured into a separating funnel, defatted it with 200 ml n-hexane. The compounds soluble in n-hexane separated in the upper phase were collected and the lower aqueous phase was extracted thrice with n-hexane for maximum recovery. Extract was dried through water bath and weighed and stored into a vile. The same procedure was adopted for ethyl acetate, acetone, butanol and water.

Antibacterial activity bioassay: Antibacterial activities of the different extracts against various microorganisms were determined by Kirby Baur Disc Diffusion method (Table 1). For gram +ve organisms, Azithromycin (30 µg $disc^{-1}$) was used as positive control while solvent media as negative control. In case of gram –ive organisms, Ciprofloxacin (30 µg $disc^{-1}$) was used as positive and solvent media as negative controls.

Result and Discussion

Analysis of the data revealed that ethanol extracted samples from *Nicotiana rustica* did not inhibit the activity of *Bacillus cereus* when compared with their respective controls (Table 2). Zero percent inhibition of *Bacillus cereus* was recorded by ethanol extracted sample from *Nicotiana rustica* (Table 2). In case of *Staphylococcus aureus*, ethanol extracted samples were more effective to control the growth of *Staphylococcus aureus* at highest concentration (i.e., 37% at 24 mg of sample $disc^{-1}$) when compared with other concentrations. Similar results were also reported by Wang *et al.,* (2008). It is clear from the result that ethyl acetate extracted samples had a profound inhibition effects against *B. cereus* and *S. aureus* and was more effective to control the growth of *B. cereus* and *S. aureus* (Table 2). On the average, ethyl acetate extracted samples were more effective against *B. cereus* than *S. aureus*. Our results also showed that acetone extracted samples had no inhibiting effects on *B. cereus* and recorded zero percent inhibition. In case of *S. aureus*, highest inhibition was achieved at higher concentration when compared with other concentrations (Table 3). Butanol extracted samples also inhibited the growth of *B. cereus* and *S. aureus*. However, butanol extracted samples inhibited the growth of *S. aureus* more than *B. cereus*, maximum control being noted at highest concentration of 24 mg of sample $disc^{-1}$ (Table 3). Water extracted samples were effective to control both *B. cereus* and *S. aureus* at higher concentration only (Table 4). These results are similar to those reported by Bakht *et al.,* (2012).

Table 1. Microbial strains tested for susceptibility to *Nicotiana rustica* extracts.

Microbial species	Gram strain type	Details of the microbial strains used
Bacillus cereus	Positive	Clinical isolate obtained from Microbiology Laboratory, Quaid-e-Azam University Islamabad Pakistan
Erwinia carotovora	Negative	Department of Plant Pathology, University of Agriculture Peshawar KPK Pakistan
Escherichia coli	Negative	ATCC # 25922
Agrobacterium tumefacian	Negative	Recombinant DNA Technology of IBGE, University of Agriculture Peshawar KPK Pakistan
Pseudomonas aeruginosa	Negative	ATCC # 9721
Salmonella typhi	Negative	Clinical isolate obtained from Microbiology Laboratory, Quaid-e-Azam University Islamabad Pakistan
Staphylococcus aureus	Positive	ATCC # 6538

Table 2. Antibacterial activity of ethanol and ethyl acetate extracted samples from *Nicotiana rustica* (NR) against *B. cereus* and *S. aureus* (gram +ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Bacillus cereus						*Staphylococcus aureus*					
		ZI during replicates			% ZI	+VE control 30 µg $disc^{-1}$	-ve control 6µl/disc	ZI during replicates			% ZI	+VE control 30 µg $disc^{-1}$	-ve control 6µl $disc^{-1}$
Ethanol	06	-	-	-	0	27	–	-	-	-	0	27	–
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			08	08	07	30		
	24	-	-	-	0			10	8	11	37		
Ethyl acetate	06	14	12	15	52			07	08	08	30		
	12	20	18	17	67			07	09	11	37		
	18	21	22	22	81			16	13	15	56		
	24	26	25	25	93			22	18	20	74		

Table 3. Antibacterial activity of acetone and butanol extracted samples from *Nicotiana rustica* (NR) against *B. cereus* and *S. aureus* (gram +ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Bacillus cereus						*Staphylococcus aureus*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Acetone	06	-	-	-	0	27	–	-	-	-	0	27	–
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		
Butanol	06	8	6	8	26			10	8	7	30		
	12	8	10	11	44			10	8	11	44		
	18	14	11	12	44			14	11	13	48		
	24	12	16	14	52			16	15	15	56		

Table 4. Antibacterial activity of water extracted samples from *Nicotiana rustica* (NR) against *B. cereus* and *S. aureus* (gram +ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Bacillus cereus						*Staphylococcus aureus*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Water	06	-	-	-	0	27	–	-	-	-	0	27	–
	12	-	-	-	0			-	-	-	0		
	18	07	10	08	30			-	-	-	0		
	24	10	08	11	37			11	10	08	37		

Our results also indicated that ethanol extracted samples were effective to control *E. carotovora* and *E. coli* at higher concentration when compared with other concentrations (Table 5). These results suggested that n-hexane extracted samples showed zero percent inhibition against *E. carotovora* and *E. coli* at any concentration (Table 5). Similar results are also reported by Zaidi *et al.,* (2005), Suresh *et al.,* (2008) and Bakht *et al.,* (2012). The data further revealed that ethyl acetate extracted samples were more effective to control *E. carotovora* (100% Zone of Inhibition (ZI) than *E. coli* (74% ZI). Acetone extracted samples recorded zero percent inhibition against *E. coli* at any concentration when compared with positive control. The results further showed maximum control of both the micro-organisms at higher concentration when compared with other treatments (Table 6). The data also indicated that ethyl acetate and acetone reduced the growth of *E. carotovora* than *E. coli*. Maximum inhibition of *E. carotovora* was achieved at higher concentrations of ethyle acetate and acetone extracted samples from tobacco (Table 6). Similarly, maximum control (59% ZI) against *E. carotovora* was recorded in butanol extracted samples when compared with *E. coli* (Table 7). In case of water extracted samples, maximum (34% ZI) inhibition was observed against *E. coli* compared with *E. carotovora* at 24 mg sample disc^{-1} concentration (Table 7). These results agree with those reported by Bakht *et al.,* (2012).

Table 5. Antibacterial activity of ethanol and n-hexane extracted samples from *Nicotiana rustica* (NR) against *E. carotovora* and *E. coli* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Erwinia carotovora						*E. coli*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Ethanol	06	-	-	-	0	27	–	-	-	-	0	35	–
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			05	05	06	14		
	24	07	05	05	22			09	08	08	23		
n-Hexane	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		

Table 6. Antibacterial activity of ethyl acetate and acetone extracted samples from *Nicotiana rustica* (NR) against *E. carotovora* and *E. coli* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Erwinia carotovora						*E. coli*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Ethyl acetate	06	18	20	21	74	27	–	12	14	16	40	35	–
	12	25	22	25	89			20	22	23	63		
	18	28	27	25	100			22	24	25	69		
	24	29	27	30	100			25	26	27	74		
Acetone	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	05	06	07	26			-	-	-	0		
	24	06	07	07	26			-	-	-	0		

Analysis of the data also revealed that ethanol and n-hexane extracted samples were ineffective to control the growth of *Agrobacterium tumefacien* and *Pseudomonas aeruginosa* (0% ZI) at any concentration (Table 8). These results agree with those reported by David & Obuotor (2000) when they studied the effect of methanolic extract of *N. tabacum* leaves against *Pseudomonas aeruginosa.* Similar results were also reported by Bakht *et al.,* (2012). Ethyl acetate extracted samples were more effective in controlling *Agrobacterium tumefacien* (86% ZI) than *Pseudomonas aeruginosa* (73% ZI) at higher concentration (Table 9). Acetone extracted samples were ineffective against *Agrobacterium tumefacien* and *Pseudomonas aeruginosa* causing zero percent inhibition (Table 9). Butanol extracted samples were more effective to control *Pseudomonas aeruginosa* (71% ZI) than *Agrobacterium tumefacien* (57% ZI) at higher concentration (Table 10). Our results also suggested that water extracted samples were ineffective to control *Agrobacterium tumefacien* and *Pseudomonas aeruginosa* at any concentration (Table 9). These results agree with those reported by Stojanovic *et al.,* (2000). Our data also suggested that ethanol, acetone, n-hexane and water extracted samples did not control *S. typhae* at any concentration (0% ZI). The data further suggested that ethyl acetate extracted samples inhibited the growth of *S. typhae* by 63% compared with other solvents (Table 11). Similar results are also reported by Zaidi *et al.,* (2005) and Bakht *et al.,* (2012).

Table 7. Antibacterial activity of butanol and water extracted samples from *Nicotiana rustica* (NR) against *E. carotovora* and *E. coli* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Erwinia carotovora						*E. coli*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Butanol	06	07	08	10	30	27	–	08	10	12	29	35	–
	12	09	10	12	37			14	15	17	43		
	18	11	12	14	44			17	19	19	51		
	24	15	16	16	59			18	21	21	57		
Water	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	08	08	10	30			06	06	07	17		
	24	10	10	12	37			10	13	13	34		

Table 8. Antibacterial activity of ethanol and n-hexane extracted samples from *Nicotiana rustica* (NR) against *A. tumefacien* and *P. aeruginosa* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Agrobacterium tumefacien						*Pseudomonas aeruginosa*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Ethanol	06	-	-	-	0	30	–	-	-	-	0	35	–
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		
n-hexane	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		

Table 9. Antibacterial activity of ethyl acetate and acetone extracted samples from *Nicotiana rustica* (NR) against *A. tumefacien* and *P. aeruginosa* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Agrobacterium tumefacien						*Pseudomonas aeruginosa*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Ethyl acetate	06	10	10	11	33	30	–	12	13	13	37	35	–
	12	10	12	14	40			20	20	21	57		
	18	19	20	22	66			26	27	27	77		
	24	20	22	23	73			29	29	32	86		
Acetone	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		

Table 10. Antibacterial activity of butanol and water extracted samples from *Nicotiana rustica* (NR) against *A. tumefacien* and *P. aeruginosa* (gram -ive).

Plant extract	Conc. mg/disc	Zone of inhibition in mm											
		Agrobacterium tumefacien						*Pseudomonas aeruginosa*					
		ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}	ZI during replicates			% ZI	+VE control 30 µg disc^{-1}	-ve control 6µl disc^{-1}
Butanol	06	07	08	10	27	30	–	08	10	12	29	35	–
	12	12	12	13	40			14	15	17	43		
	18	14	15	17	50			18	21	21	57		
	24	16	16	18	57			23	26	26	71		
Water	06	-	-	-	0			-	-	-	0		
	12	-	-	-	0			-	-	-	0		
	18	-	-	-	0			-	-	-	0		
	24	-	-	-	0			-	-	-	0		

Table 11. Antibacterial activity of ethanol, ethyl acetate, acetone, butanol, n-hexane and water extracted samples from *Nicotiana rustica* (NR) against *S. typhe*.

Plant extract	Conc. mg/disc	Zone of inhibition in mm					
		S . typhae (Gram negative)					
		ZI during replicates			% ZI	+ve Ctrl 30 µg disc^{-1}	-ve Ctrl 6µl disc^{-1}
Ethanol	06	-	-	-	0	40	-
	12	-	-	-	0		-
	18	-	-	-	0		-
	24	-	-	-	0		-
Ethyl acetate	06	07	08	08	18		-
	12	09	10	12	25		-
	18	15	15	16	38		-
	24	24	26	26	63		-
Acetone	06	-	-	-	0		-
	12	-	-	-	0		-
	18	-	-	-	0		-
	24	-	-	-	0		-
Butanol	06	07	09	09	20		-
	12	09	11	11	25		-
	18	13	12	12	30		-
	24	14	15	15	38		-
n-Hexane	06	-	-	-	0		-
	12	-	-	-	0		-
	18	-	-	-	0		-
	24	-	-	-	0		-
Water	06	-	-	-	0		-
	12	-	-	-	0		-
	18	-	-	-	0		-
	24	-	-	-	0		-

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