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ANTIMICROBIAL ACTIVITY OF PESTICIDE RESIDUE AND IMPLICATIONS OF INTERNATIONAL MRLS IN INDIAN AND IMPORTED WINES

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ABSTRACT

Objectives: International food safety standards having a great interest in public health and setting legislative Maximum Residue Limits (MRLs) of the host country with respect to applied agrochemicals. India is the leading country known for its highest tonnage per hector grape yield due to the favorable microclimatic conditions. Present investigation aimed to determine pesticidal residues in International and Indian wine samples using various sophisticated biophysical instrumentation. Further, antimicrobial effect of these samples was to be assessed against various commensal pathogens.

Methods: Determination of pesticide residue in Indian and Imported wine samples and its comparison with respect to European Union (EU) MRLs was done. Pesticide residues in grapewine were detected by GC, GC/MS & LC-MS/MS.

Results: Majority of wine samples were found to be contaminated with different pesticides like Thiophanate Methyl Carbendazime, Metalaxyl, Trideminol Fenpyroximate, Endrin, Propargite and Chlorpyriphos-Methyl. Antimicrobial activity of pesticides was tested against *E. coli, P. mirabilis, S. aureus* and *K. aerogenes*. The result indicates that majority of the pesticides were more effective against *P. mirabilis* and *S. aureus*.

Conclusion: The result revealed that majority of the wine samples were contaminated by pesticides in the range 0.01 to 0.04 ppm, which is within the permissible limit as per EU norms. However, if the concentration of pesticide exceeds the permissible limit, there is a high risk of human health hazard. The present research will be helpful for the wine stack holders, policymakers and research community for planning the effective dissemination of technology and sensible use of pesticides in vineyards so as to prepare healthy and quality wines.

INTRODUCTION

India is the leading country known for its highest tonnage per hector grape yield due to its favorable microclimatic condition and grapes are considered as one of the important commercial fruit crop, earning highest foreign exchange of the country and having highest potential for its value added health enhancing products like juice, resins, drakshasawa, wine and nutraceutical products etc. Pesticides can have short term toxic effects on directly exposed organisms or long-term effects by causing changes in habitat and the food chain. It is found that their residues are persisting into grape and their value added products, deteriorating their quality and adversely affecting not only on human health in the form of different non communicable diseases but on the viticulture and consequently Indian economy also. Appearance of pesticide residues in wine have impelled government to set up monitoring programmes. Maximum Residual Limits (MRLs) are the allowed concentration of pesticide residue in the food products. Various wine producing countries have introduced legislation for protection of the consumer's health from hazards of pesticides in food. Unfortunately, no such regulations have been enforced in India, hence protection and awareness is required in this sector. Considering this background, current investigation was undertaken to understand the pesticide levels in various types of Indian and International wines using the most sophisticated biophysical techniques. These levels are reported in terms of ppm and compared with the EU standards. To understand the effect of the detected pesticides on microorganisms, the study was undertaken wherein; individual predominantly occurring

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pesticides were assessed for the antibacterial effect at various concentrations.

MATERIALS AND METHODS

Bacterial Strains

Antimicrobial activity of wine, solid pesticides (Thiophanate Methyl, Carbendazim and Metalaxyal) and liquid pesticides (Propargite, Chlorpyriphos) was assessed. Standard were obtained from the National Collection of Industrial Microorganisms (NCIM, National Chemical Laboratory, and Pune, India). Amongst four microorganisms investigated, one Gram-positive bacterium was *Staphylococcus aureus* NCIM-2079, while, three Gram-negative bacteria were *Proteus mirabilis* NCIM-2241, *Escherichia coli* NCIM-2065 and *Klebsiella aerogenes* NCIM-2239. All the microorganisms were maintained on nutrient agar slants at 4ºC.

Chemicals used

Pesticide certified reference standards obtained from M/s Ehrenstorfer GmSH, Germany. All the solvents used like Ethyl Acetate, Methanol, water were of HPLC grade.

Sample collection

Wine samples of Indian and imported origin were collected from the market. Among the collected wine samples, 50% belonging to red and 50% to the white wine category. All samples were sealed properly and stored in refrigerator until analyzed. They were labeled as S1 to S12 before submitting to NABL accredited National Horticultural Research and Development Foundation. (NHRDF) laboratory at Chittegaon for analysis of pesticide residue commercially. Extraction and analysis of samples was done by following standard United States of America Environmental Protection Agency (US EPA) Methodology.

Pesticide extraction from wine sample

Octadecyl C-18 solid-phase cartridges as the sorbent and ethyl acetate as the extraction solvent were effective for the extraction of pesticides in wines. For this research, Pesticides from the wine sample were extracted using Caliper's Auto trace solid phase extraction workstation. C18 columns were used for extraction of pesticides. The columns were initially soaked with methanol and 400 ml of wine samples loaded on to the column, the columns were dried with nitrogen gas and finally ethyl acetate were eluted through the column and the elute collected (Holland *et al.,1994;* Kaufmann *et al.,* 1997).

Pesticide Residue Analysis

The reduced solvent extracts were subsequently screened and analyzed by GC, GC/MS and LC/MS/MS. All wine samples were analyzed for Organophosphorus pesticide (OPP), Organochlorine pesticide (OCP), synthetic pyrethroids, triazines, carbamate, triazoles, benzimidazole, nicotinoids, natural product derivative, substituted thiourea (Handore *et al.,* 2011; Beltran *et al.,* 2000)

Instrumentation and operating conditions:

Gas Chromatography

GC – Chemito (Mumbai) ceres 800 plus gas chromatograph fitted with Ni 63 –ECD was used for analysis of organchlorine pesticides viz. alpha HCH, beta HCH, gamma HCH, heptachlor, aldrin, alpha endosulphan, dieldrin, beta endosulfan.

LC-MS/MS

LC/MS-MS analysis was done using API 2000 having triple quadrupole LCMS-MS system of AB Sciex Instruments for analysis of mixture of standards and sample. Each compound were tuned for reclustering potential, focusing potential, entrance potential, collision energy and collision cell exit potential for it Q1 and Q3 ions. MS/MS method. LC- 200 quaternary pump used with column Euro sphere 100-5 (C-18) 250 mm x 4.6mm, PE 200 Auto sample was used for injection & the sample. Injection volume was 20 µl. All the calculations were done as per Normalization method.

Recovery experiments were done in the lentvol sample at 0.5ppm level spiking of pesticides amenable to GC, GC-MS, and LC-MS/MS. The recovery was found to be within 70-120%.

Antimicrobial activity tests

Standard cultures were individually inoculated in Nutrient broth (Hi-Media) to achieve a specified inoculums size. O.D. was adjusted at 0.7 to 0.8 of overnight grown cultures using UV- Visible Spectrophotometer (Chemito, Mumbai.). Different dilutions of wine pesticides were prepared (0.01ppm to 0.04ppm) viz., Solid pesticides (Thiophanate Methyl, Carbendazim, and Metalaxyl): 0.1 ppm, 0.04 ppm, 0.02 ppm for and for Liquid pesticides Propargite, Chlorpyriphos): 1:1, 1:9 (Chrissanthy *et al.,* 2004). The antimicrobial effect of the wine extracts was tested using the agar well diffusion method. Approximately 15 mL of Nutrient agar was seeded with 0.1 ml of overnight grown bacterial cultures organism. Each agar well was loaded with 10µl samples of wine and pesticides individually. After pre-diffusion, plates were incubated at37^{0}C for24 hours. Plates were observed for zone of inhibition (Bauer, *et al*, 1996).

RESULTS AND DISCUSSION

Pesticide Residue Analysis

Liquid Chromatography Tandem -Mass Spectrometry (LC-MS/MS)

Under the appropriate operation conditions, LC-MS/MS analysis showed the contamination of wine samples with carbendazim (0.01ppm), fenpyroximate (0.01ppm), metalaxyl (0.04ppm), propargite (0.01ppm), thiophanate-methyl (0.01ppm), and tridemenol (0.01ppm). Thiophanate methyl was detected in sample S5, S6, S10, while carbendazime was detected in S6, S10, S11 whereas metalaxyl was detected in S11. Propargite was found in S10 and S12, fenpyroximate was detected in S10 and S12 samples and sample S2 and S12 exhibited the presence of tridemenol. Altogether, 12 brands of wines were tested for pesticides LC/MS-MS. 6 out of 12 samples were detected for pesticides between 0.01 ppm to 0.04ppm (Table 1). Chromatogram of sample S6 exhibited the presence of Thiophanate methyl at concentration of 0.01 ppm and Carbendazim at Concentration of 0.01ppm and as per EU recommendation, this range is within the permissible limit (Figure 2). In 1991, Zironi and his team has observed that due to the presence of pesticide thiophanate-methyl and fenarimol delay in the wine fermentation occurred. In 1999, Wong and Halverson has carried out multiresidue analysis of some common pesticides in wines including chloropyrifos and propagite using C-18 Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry (GC-MS) and confirmed the method as one of the fastest and effective method. In 2004, Ruediger and his team carried out study on the fate of pesticides during the winemaking process in relation to malolactic fermentation and detected seven fungicides (carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone, and tridemenol) and three insecticides (carbaryl, chlorpyrifos, and dicofol) in the red wine.

GC and Gas Chromatography -Mass Spectrometry (GC-MS) Under the operation condition described in materials and methods, 5 samples viz. S1, S2, S3, S9 and S12 were analyzed by GC-ECD and found to be contaminated with pesticide endrin at 0.01ppm concentration (Figure 1) (Table 2) (Garcia-Repetto, 1996). It is reported that this pesticide is banned since more than two decades. The reason for presence of pesticide may be due to its persistence in water and also uptake of endrin by plants was noted from soils treated as long as 16 years after planting. The presence of endrin indicates the hazardous effect on the health of consumer (Nash and Harris 1973) (Margni *et.al*. 2002). GC-MS analysis of sample S11 shows presence of chlorpyriphos-methyl at 0.01ppm concentration (Table 1) for which the EU MRL value is 0.2 ppm. Thus the concentration found is within the permissible limit (Table 2) (George Soleas *et.al.* 2000). It was investigated that, out of the total wine samples analyzed, majority of them were contaminated with endrin (41.66%), carbendazime (25%), thio-phanatemethyl (25%), fenpyroximate (16.66%), propargite (16.66%), tridimenol (16.66%), chlorpyriphos-methyl (8.33%) and metalaxyl (8.33%).

Table 1. Pesticide Residue in Wine Sample

S.N	Details of Compounds	Q1/Q3 Mass	LCMS/MS											
			S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12
1	Acetamiprid	223.0 / 126.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
2	Atrazine	216.2 / 174.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
3	azoxystrobin	404.2 / 372.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
4	Buprofezin	306.1 / 201.3	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
5	Carbaryl	202.0 / 145.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
6	Carbendazim	192.0 / 160.0	BDL	BDL	BDL	BDL	BDL	**0.01**	BDL	BDL	BDL	**0.01**	**0.01**	BDL
7	Difenconazole	406.3 / 250.9	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
8	Difenthiuron	385.4 / 329.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
9	dimethomorph	388.4 / 301.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
10	Etrimphos	293.0 / 125.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
11	fenpyroximate	422.3 / 366.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**0.01**	BDL	**0.01**
12	Flusilazole	316.2 / 247.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
13	Iprobenphos	289.0 / 91.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
14	Metalaxyl	280.0 / 220.3	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**0.04**	BDL
15	Myclobutanil	289.4 / 70.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
16	Penconazole	284.0 / 70.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
17	Phorate	261.0 / 75.2	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
18	Phosalone	368.0 / 182.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
19	Phosphamidon	300.0 / 174.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
20	Propargite	368.3 / 231.4	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.01	BDL	**0.01**
21	Propiconazole	342.2 / 158.9	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
22	Simazine	202.0 / 124.0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
23	Thiocloprid	253.1 / 126.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
24	Thiophanate methyl	343.1 / 151.1	BDL	BDL	BDL	BDL	**0.01**	**0.01**	BDL	BDL	BDL	**0.01**	BDL	BDL
25	Tridemenol	296.0 / 70.0	BDL	**0.01**	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**0.01**
26	Emmamectin benzoate	886.0 / 158.1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
			GCMS											
27	Diazinon		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
28	Chlorpyriphos methyl		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**0.01**	BDL
29	Metyl parathion		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
30	Malathion		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
31	Parathion ethyl		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
32	Chlorfenvinphos		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
33	Fipronil		BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL

Table 2. Detected Pesticides and Their MRL Values

Pesticides	Sample No.	Detected level (ppm)	MRL values (mg/kg)				
			EU	UK	Netherlands	Germany	Codex
Thiophanate Methyl	S_5,S_6, S_{10}	0.01	0.10	0.10	0.10	0.10	---
Carbendazim	S_6,S_{10},S_{11}	0.01	0.30	0.30	0.30	0.30	10.00
Metalaxyl	S_{11}	0.04	2.00	2.00	2.00	2.00	1.00
Chlorpyriphos Methyl	S_{11}	0.01	0.20	0.20	0.20	0.20	0.20
Propargite	S_{10}, S12,	0.01	#	#	10.00	3.00	7.00
Tridimenol	S_2, S_{12}	0.01	2.00	2.00	2.00	2.00	2.00
Fenpyroximate	S_{10}, S12	0.01	#	#	#	0.50	----
Endrin	S_1, S_2 ,S_3, S_9, S12	0.01	0.01	0.01	0.01	0.01	------

No MRL exists

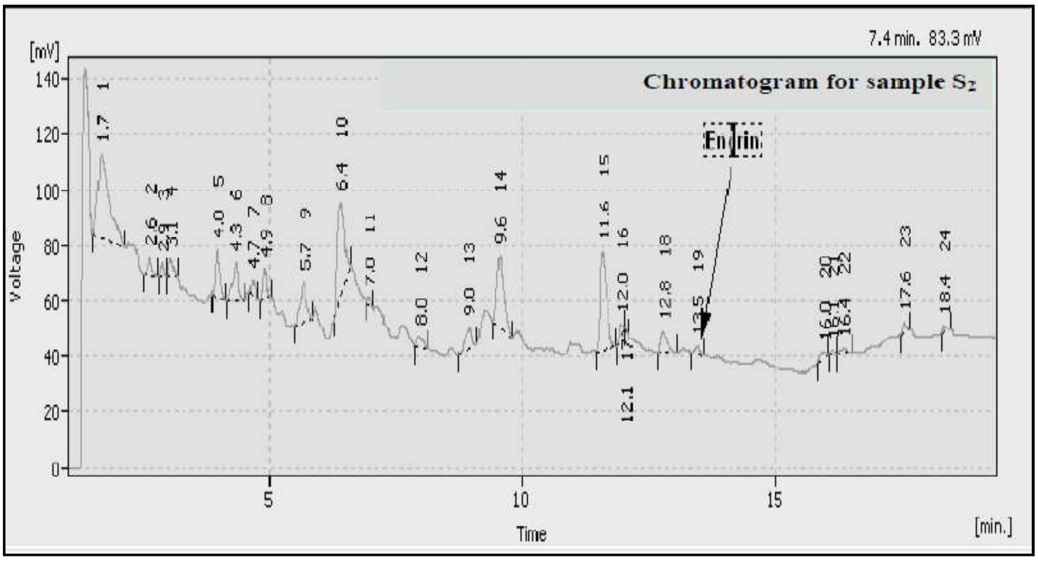


Figure 1. GC analysis of S2 sample

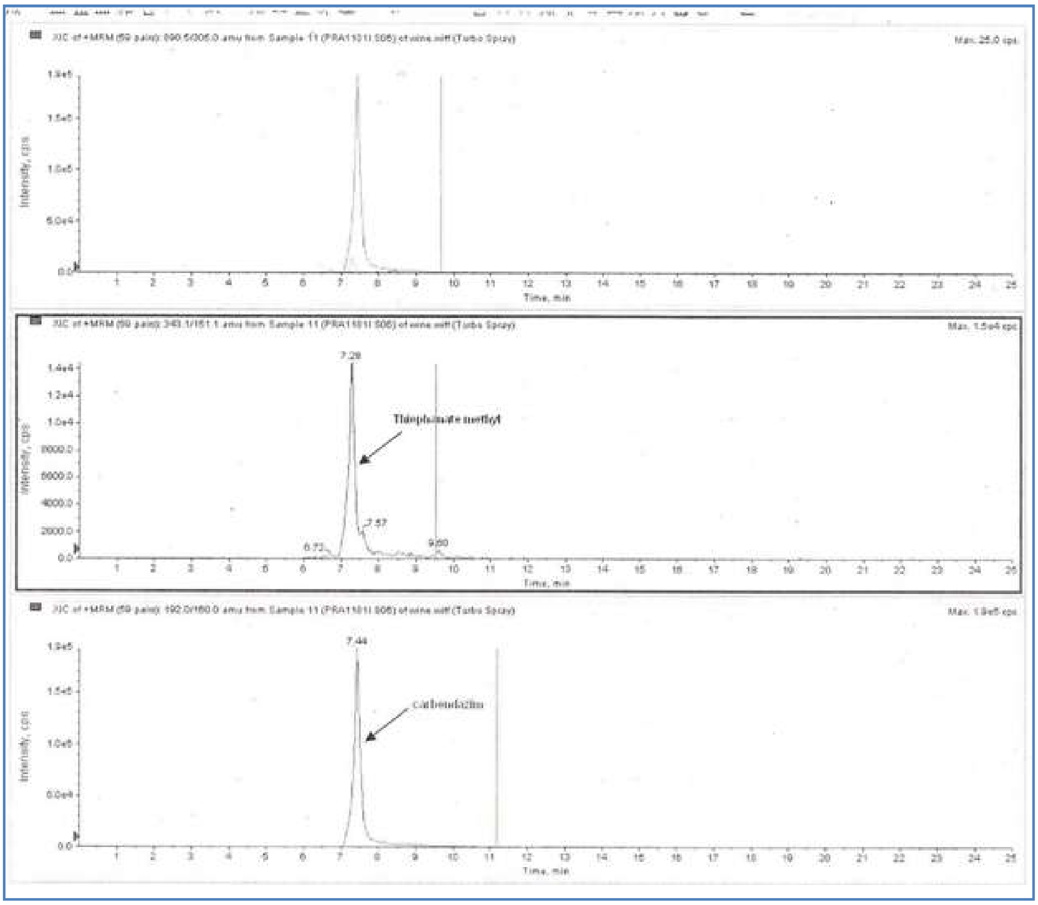
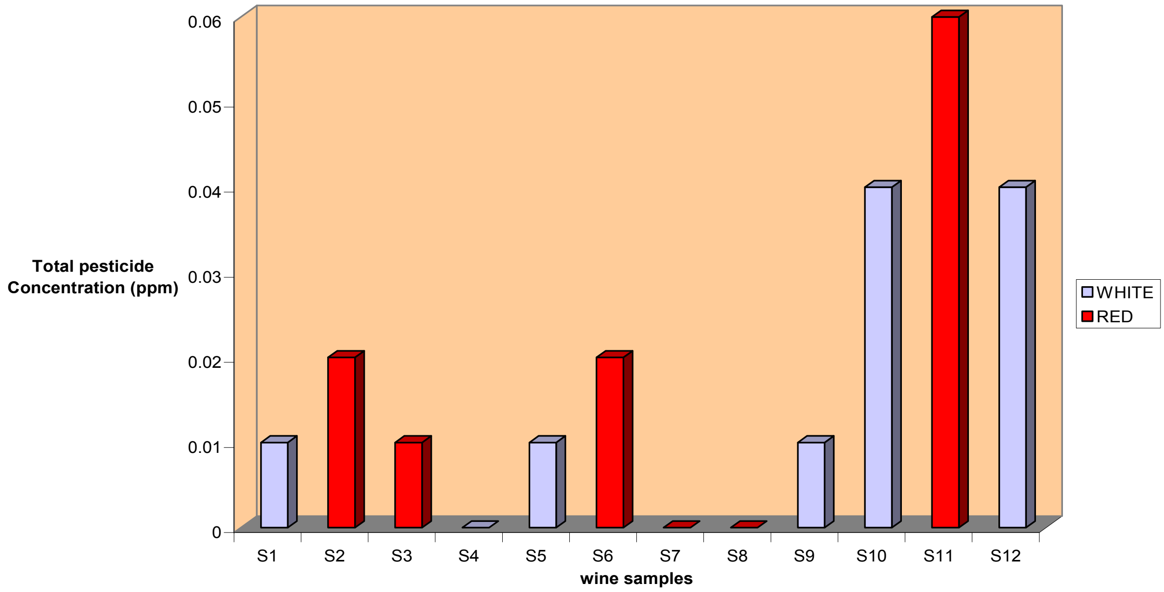


Figure 2. Chromatogram of sample S6

Table 3. Sample Category and Total Pesticide Residue

Wine Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Indian/Imported Red/White	Indian White	Indian Red	Imported Red	Imported White	Indian White	Indian Red	Imported Red	Indian Red	Imported White	Indian White	Indian Red	Indian White
Total Pesticide concentration (ppm)	0.01	0.02	0.01	0	0.01	0.02	0	0	0.01	0.04	0.06	0.04



Graph 1. Total Pesticide Residue in Red and White Wine Samples

All the samples were found to contain one or more pesticides but at concentrations (0.01-0.04 ppm) below the MRLs set by EU. Chromatograms of samples S1, S2, S3, S9, and S12 analysed by GC, exhibited the presence of endrin at concentration of 0.01ppm. It is observed that sample S11 shows highest concentration of total pesticides which is 0.06 ppm and sample S10 and S12 exhibited concentration level as 0.04ppm. Sample S2 and S6 exhibited total concentration at 0.02 ppm whereas sample S1, S3, S5, S9 showed 0.01ppm. Presence of pesticide residues in the wine samples are due to lack of regular monitoring scheme of MRLs on pesticides and also Good Agricultural Practice (GAP) Good Manufacturing Practices (GMP), Good Laboratorial Practice (GLP) and Total Quality Management (TQM) are not followed as per recommendations. On the contrary, remaining samples such as S4, S7, and S8 do not show the presence of any pesticide which probably indicates Good Manufacturing Practice and Good Laboratorial Practice (Wong *et.al*., 1999; Jimenez *et.al*., 2001). Graph.1 shows presence of pesticides in red and white wine samples.

Amongst the red wine samples, it is observed that in sample S2 and S6 total pesticides present are at 0.02ppm level. Sample S11 shows highest concentration of 0.06ppm whereas none of the pesticides were detected in S7 and S8 samples. Similarly in white wine samples, it is observed that, Sample S1,S5,S9 have pesticide concentration at 0.01ppm; sample S10 and S12 shows total pesticide concentration as 0.04ppm, while S4 doesn't show presence of any pesticide. This clearly indicates that red wine samples exhibited higher concentration of pesticides as compared to the white wine samples (Graph 1). One of the parameters supporting these findings is the difference in the manufacturing process of the red and white wine i.e. Fermentation with or without maceration (Cabras and Alberto 2000). Comparison of pesticide contamination in Indian and imported wine samples shows that 75% of Indian red wines are found contaminated with pesticides whereas 50% imported red wine samples are contaminated with pesticides. In addition, 100% Indian white wine sample are contaminated whereas 50% imported white wine samples are observed to be contaminated with pesticides (Table 3). On the basis of the previous investigation, Chromatogram of sample S6 shows presence of Thiophanate methyl at concentration of 0.01 ppm and Carbendazim at Concentration of 0.01ppm and as per EU recommendation, this range is within permissible limit (Figure 2). Graph.1 exhibited presence of pesticides in red and white wine samples. Amongst the red wine samples, it is observed that in sample S2 and S6 total pesticides present are at 0.02ppm level. Sample S11 shows highest concentration of 0.06 ppm while, sample S7 and S8 do not show presence of any pesticides. Similarly in white wine samples, it is observed that, Sample S1,S5,S9 have pesticide concentration at 0.01ppm; sample S10 and S12 shows pesticide concentration at 0.04ppm, while S4 doesn't show presence of any pesticide. This clearly indicates that red wine samples exhibited higher concentration of pesticides as compared to the white wine samples.

Antibacterial activity

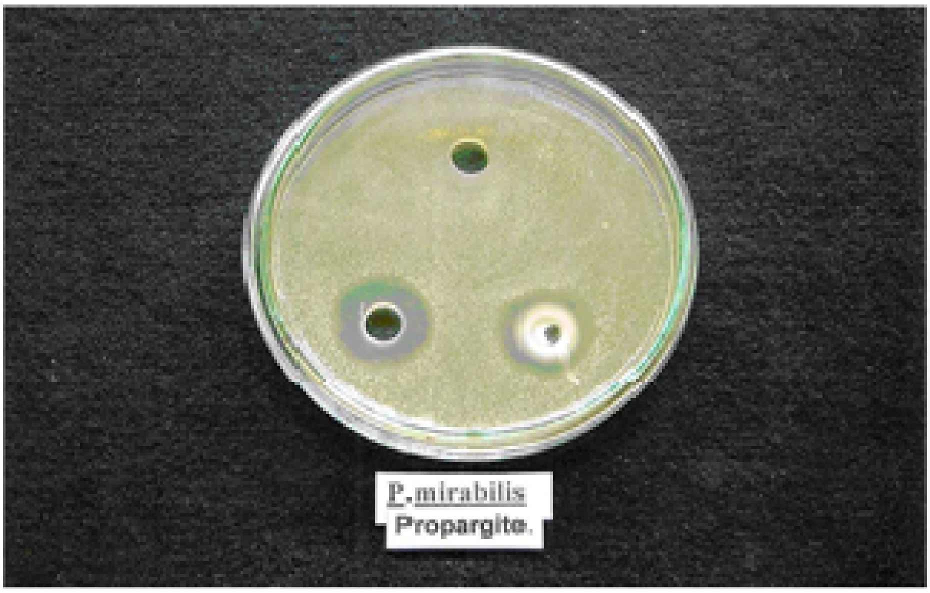
Due to the detection of considerable levels of pesticides in majority of wine samples, they were further subjected to antibacterial activity against commensal pathogenic bacteria. Photoplate.1 indicates larger zone of 2.4 mm using 1:1 dilution

Table 4. Inhibition of microorganisms by pesticides

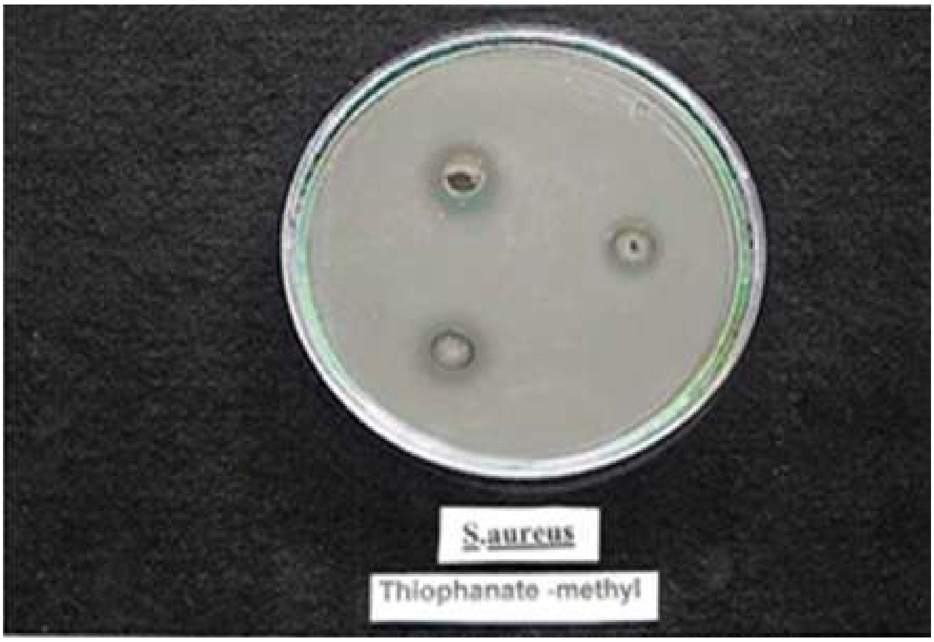
Sr.No.	Test Organisms	Zone of Inhibition in mm												
		Solid Pesticides Concentration (ppm)									Liquid Pesticides proportion			
		Thiophanate Methyl			Carbendazim			Metalaxyl			Propargite		Chlorpyriphos	
		0.1	0.04	0.02	0.1	0.04	0.02	0.1	0.04	0.02	1:1	1:9	1:1	1:9
1	*E. coli*	----	---	--	--	--	--	1.3	--	1.1	1.9	1.5	1.7	1.2
2	*P. mirabilis*	1.6	--	1.1	--	--	--	1.6	--	--	2.4	2.0	2.4	2.0
3	*S. aureus*	2.1	1.3	1.6	1.7	1.1	1.4	1.7	--	1.2	2.1	1.4	1.8	1.2
4	*K. aerogenes.*	--	--	--	--	--	--	1.2	--	--	1.7	1.3	1.6	1.1

Table 5. Antimicrobial effect of Indian red wine (S11)

Particulars		Detected Pesticide (ppm)	Zone of Inhibition in 'mm'			
			E.coli	*P.mirabilis*	*S.aureus*	*K.aerogenes*
Sample S11 (Indian Red wine) Total pesticide		0.06	2.9	2.4	3.1	1.9
Detected Pesticides	Metalaxyl	0.04	0	0	0	0
	Carbendazim	0.01	0	0	1.4	0
	Chlorpyriphos Methyl	0.01	1.2	2.0	1.2	1.1



Photoplate 1. Zone of inhibition of pesticide propargite on *P. mirabilis*



Photoplate 2. Zone of inhibition of pesticide Thiophanate on *S. aureus*

of pesticide propargite against *P.mirabilis.* It could be concluded that the presence of propargite pesticide in S10 and S12 sample may be inhibitory to the test organisms. Photoplate. 2 indicates larger zone of 2.1 mm using 0.1 ppm of pesticide Thiophanate methyl with *S.aureus*. Concentration of 0.02 ppm of Thiophanate methyl exhibited 1.6 mm zone of inhibition and 0.04 ppm exhibited 1.3 mm zone of inhibition. It could be concluded that the presence of Thiophanate methyl pesticide in S5, S6, and S10 sample is inhibitory to the test organisms Table 4. Summarizes the values of zones of inhibition (control was 11.1% alcohol which was subtracted from the test reading) obtained using pesticide metalaxyl. It is observed that 0.1 ppm concentration gives higher zone inhibition with all test organisms as compared to all 0.02 ppm and 0.04 ppm concentration (Maria Rosa Alberto 2006). It can be concluded that metalaxyl is inhibitory to *E.coli, P. mirabilis, S.aureus and K. aerogenes.* Wine has been previously explored for its effective antioxidant and antibacterial properties against aerobic mesophilic bacteria, lactic acid bacteria and Enterobacteriaceae. The product derived from the whole wine pomace exhibited a bacteriostatic effect on these bacteria (García-Lomillo *et al.,* 2014).

The analytical observations in the present work will be helpful to streamline the infrastructure of vineyard growers and winemakers. It will also prove to be a vital footstep ahead for entrepreneur, policy makers and research community in planning the effective dissemination of technology, judicious use of pesticides in vineyards so as to prepare quality wines. From the present investigation, it can be concluded that, Indian wines are more contaminated with pesticides than imported wines (Zironi, *et al.,* 1991). According to the current investigation, International wines had lesser pesticides as compared to Indian wines whereas; Red wine samples exhibited relatively more amounts of pesticides than the white wine samples. Predominantly present pesticides in wines were observed to be inhibitory against the test microorganisms except *E. coli*; hence consumption of these wine types will not prove unsafe to the human health limiting the values of Acceptable Daily Intake (ADI). Majority of the wine samples were observed to be contaminated by pesticides in the range 0.01 to 0.04 ppm, which lies in the permissible limit. However, if the concentration of pesticide exceeds the permissible limit then there is a high risk of human health hazard.

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