ANTIMICROBIAL ACTIVITY OF *RUBUS ROSIFOLIUS* J.E. SM. (ROSACEAE) FRUIT CRUDE EXTRACT

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Abstract: Berries have a variety of beneficial biological properties which play an important role in maintenance of human health. *Rubus rosifolius*, locally known as "sapinit" or Philippine wild raspberry, was tested against wide variety of microorganisms to evaluate its antimicrobial property. Fruit crude ethanolic extract yield was 3.19%. It exhibited growth inhibition by disc-diffusion assay on *Staphylococcus aureus, S. epidermidis, Salmonella typhi, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Candida albicans* and *Aspergillus oryzae*. Only *Aspergillus niger* showed total resistance to the extract. Regression analysis showed strong positive linear correlation between concentration of extract and size of inhibition zone. Phenolic compounds, anthraquinones, alkaloids, steroids and higher alcohols were the secondary metabolites detected in the extract by thin-layer chromatography. The minimum inhibitory concentration of the fruit crude extract to *S. aureus* is 78.13 µg/mL, 156.25 µg/mL for *S. epidermidis* and *S. typhi*, and *S. cerevisiae, C. albicans* and *A. oryzae*, 312.50 µg/mL. Bioautography suggests that the phenolic compounds, anthraquinones, alkaloids and higher alcohols are responsible for observed antimicrobial activity of the extract.

Keywords*: Rubus rosifolius, antimicrobial activity, secondary metabolites, minimum inhibitory concentration, bioautography*

1. INTRODUCTION

The emergence of microbial resistance and the decrease in effectiveness of currently available antimicrobial agents have spurred an increased effort to search for new and alternative antimicrobial substances with novel inhibitory mechanism (Esterhuizen, 2006). Many scientists exploit our natural resources especially plants to find cure to infectious diseases caused by microorganisms. Plant extracts are screened for their secondary metabolites with relevant biological activities. However, extensive research could not be done because of the huge plant kingdom's diversity.

Rubus rosifolius, locally known as "sapinit," or Philippine Wild Raspberry (Figure 1) is one of the medicinal plants currently endorsed by the Department of Agriculture. It thrives in the forest of Laguna and Quezon Provinces in Luzon. Today, it is being cultivated to be processed as jam, wine, vinegar and other commercial products which generate additional income for the people.

Figure 1. Fruits of *Rubus rosifolius* (left) and herbarium specimen (right).

Sapinit belongs to the family of Rosaceae in which strawberry, blueberry and other berries are included. Berries are known for their variety of phenolic compounds in various plant parts as a protection against environmental stress and pathogens (Puupponen-Pimia, 2001). The presence of such compounds led the researcher to become interested with the antimicrobial activity of sapinit.

The present paper studied the antimicrobial activity of *Rubus rosifolius* fruit extract. Specifically, the fruit extract was examined for the presence of secondary metabolites by thin layer chromatography (TLC); tested for antimicrobial activity against gram positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*), gram negative (*Salmonella typhi*, *Pseudomonas aeruginosa*), yeasts (*Saccharomyces cerevisiae*, *Candida albicans*) and molds (*Aspergillus oryzae*, *Aspergillus niger*); and subjected to bioautography in determining which active constituents induced microbial inhibition.

2. METHODOLOGY

2.1 Plant Material and Extraction

Sapinit fruit was locally obtained from San Pablo City Wet Market. Authentication was done at Philippine National Museum and herbarium specimens were deposited at Philippine National Museum Botany Section and Polytechnic University of the Philippines Herbarium.

The fruit was oven-dried at 40^{o}C for 72 hours, subjected to blender and further pounded by mortar and pestle. Two successive extractions were done to the fruit paste using 95% ethanol, with maceration for 48 hours for each extraction. The ethanolic extract was concentrated into syrup over water bath at reduced temperature.

2.2 Phytochemical Analysis

Detection of secondary metabolites present in the fruit crude extract was done using thin-layer chromatography. Crude extract was spotted on TLC plates (silica gel G60 F265) and developed in two solvent systems MeOH: $CHCl_3$ 7:3 (v/v) and $CHCl_3$: MeOH 5:1 (v/v). Dry and spray reagents were applied to TLC plates forming specific color reaction: antimony III chloride for steroids (glow at UV365nm); potassium ferricyanide-ferric chloride for phenols (blue); Dragendorff's reagent for alkaloids (brown-orange); magnesium acetate for anthraquinones (blue); Borntrager's reagent for coumarins (blue) and; vanillin-sulfuric acid for higher alcohols and essential oils (blue-violet for higher alcohols and variety of colors for essential oils).

2.3 Antimicrobial Assay

The antimicrobial activity of the extract was tested by disc-diffusion assay. Gram positive bacteria *Staphylococcus aureus* UST and *S. epidermidis* ITDI-24; Gram negative bacteria *Salmonella typhi* ITDI-7 and *Pseudomonas aeruginosa* UST; yeasts *Saccharomyces cerevisiae* UST and *Candida albicans* ITDI-2049; and molds *Aspergillus oryzae* UST and *Aspergillus niger* ITDI-3008 were used for the antimicrobial assay. All microorganisms were inoculated in nutrient broth and one-day old cultures were used in all assays.

Disc-Diffusion Assay Ten µL aliquot of each microbial culture was pour-plated with 10 mL of Muller Hinton Agar. Sterilized 6mm diameter discs from #1 Whatman filter paper was placed on the surface of inoculated MHA. A total of 6 discs were used, each permeated with 5 µl of the following: 100%, 75%, 50%, and 25% crude extract; positive control 1% ampicillin (for Gram positive bacteria and *S. typhi*), 0.05% ampicillin for *P. aeruginosa*, 100 000 units/mL of nystatin (for yeasts and molds); and negative control 95% ethanol. All plates were incubated for 24 hours at room temperature before diameter of zones of inhibition were measured using a ruler. Three agar plates were prepared for each microorganism.

2.3.1 Minimum Inhibitory Concentration

Ten test tubes with 5mL sterile nutrient broth were arranged in row using a test tube rack. Five mL of crude extract mother stock solution with 20,000 µg/mL was added to the first test tube and obtained 10,000 µg/mL concentrations. This procedure was repeated until 39.07 µg/mL of crude extract was obtained. Five µL of one day-old cultures was added to all test tubes except the 10th tube (pure water). The test tubes were incubated at room temperature for 24 hours. Minimum inhibitory concentration (MIC) was expressed as the lowest dilution which inhibited microbial growth.

2.4 Direct-Overlay Bioautography Assay

Chromatograms were overlaid in MH agar plates with test microorganisms, and then incubated for 24 hours at room temperature. The chromatograms were removed from the agar and incubated further for 48 hours. The areas of growth inhibition of bacterial lawn were equated to spot on the reference TLC chromatograms.

3. RESULTS AND DISCUSSION

3.1 Yield of Fruit Crude Extract

A total of 28.48 g (3.19% yields) of crude extract was obtained. The extract was deep red in color, sticky gum-like with sweet scent.

3.2 Secondary Metabolites in Fruit Crude Extract

Table 1 shows the summarized categories of secondary metabolites detected in thin layer chromatography with corresponding spray reagent, their positive color reaction and HR_f values. Both solvent systems, $CHCl_3$: MeOH, 5:1 v/v and MeOH: $CHCl_3$, 7:3 v/v, gave almost identical results.

Phenolic compounds, with $10HR_f$ values, are the abundant secondary metabolites present in the fruit crude extract. Other secondary metabolites detected are anthraquinones, steroids, alkaloids, and higher alcohols.

3.3 Antimicrobial activity of Fruit Crude Extract

Staphylococcus aureus, S. epidermidis, Salmonella typhi, P. aeruginosa, S. cerevisiae, C. albicans and *A. oryzae* are all susceptible and *A. niger* is resistant to the extract. Table 2 shows the zones of inhibition of the positive control and different concentrations of the fruit crude extract. Compared to the corresponding positive controls, inhibitions by the extract were significantly greater ($p<0.05$) at 25% for *S. cerevisiae and C. albicans*, at 50% for *A. oryzae*, at 75% for Gram positive *S. aureus* and S. *epidermidis*. The opposite was the case for the Gram negative *S. typhi* and *P. aeruginosa*.

Figure 3 shows the bar graph of the mean zones of inhibition at different concentrations of fruit crude extract to test microorganisms. *Candida albicans* showed the largest R value of 0.972 and *S. typhi* the least at 0.823. Nevertheless, linear correlations were high for all test microorganisms, indicating that inhibition increased with extract concentration.

Table 1. Secondary metabolites detected from *Rubus rosifolius* fruit crude extract by TLC with corresponding HRf in the MeOH: CHCl3 and CHCl3: MeOH solvent systems.

Secondary Metabolites	MeOH: $CHCl_3$ (7:3) HR_f	$CHCl_3$: MeOH (5:1) HR_f
Steroids (antimony III chloride, glows under UV365)	99	99
Phenols (potassium ferricyanide-ferric chloride sol., blue)	15 49 58 65 69 81	 14 22 35 95
Antraquinones (Magnesium acetate, blue)	11 43 88	2 31 38
Alkaloids (Dragendorff reagent, brown orange)	73	91
Coumarins (Borntrager reagent, blue)	not detected	not detected
Higher Alcohols and Essential oils (blue violet for sterols and triterpines and variety of color for essential oils)	35 80	50 69 88

3.4 Minimum Inhibitory Concentration of Crude Extract

Figure 2 shows MICs of extract to test microorganisms. It was 78.13µg/mL for S. *aureus*, 156.25µg/mL for *S. epidermidis* and *S. typhi* and 312.50µg/mL for *P. aeruginosa*, *S. cerevisae*, *C. albicans* and *A. oryzae*.

3.5 Determination of Secondary Metabolites of Fruit Crude Extract that Induced Antimicrobial Activity

The areas that did not develop growth in bacterial lawn after 48 hours of incubation matched roughly with the HR_f values 65, 69, 73, 80, 81, and 88 for MeOH:$CHCl_3$ (7:3) and HR_f 2,14, 22, 91, and 95 for $CHCl_3$:MeOH(5:1) shown in Table 3. These HR_f values were those for phenols, alkaloids, anthraquinones and higher alcohols.

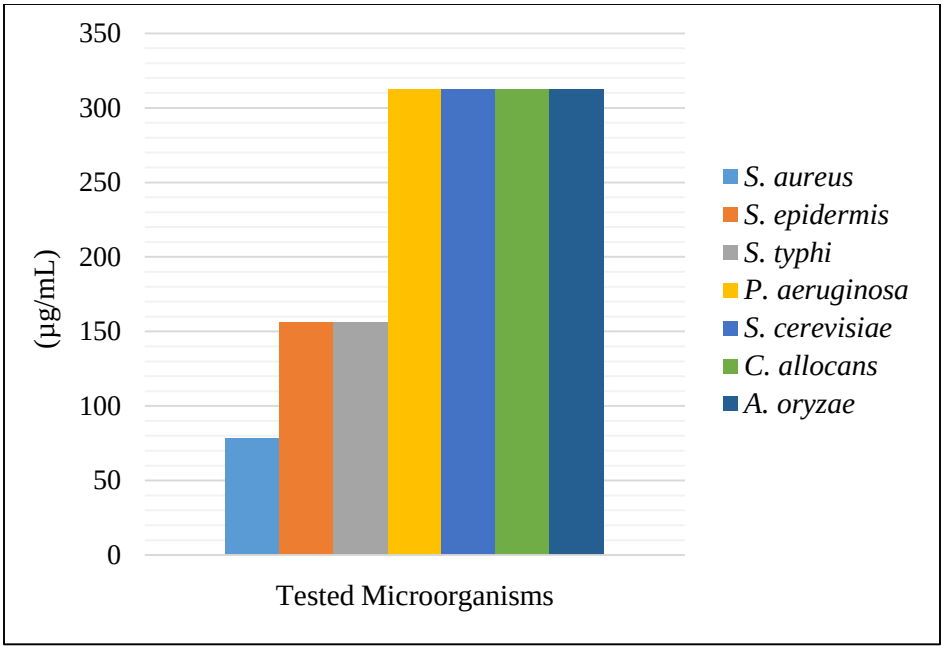

Figure 2. Minimum inhibitory concentrations extract to test microorganisms.

The array of secondary metabolites produced by plants constitutes the source of bioactive substances that gain much interest in scientific field. Berries are rich in phenolic compounds as reported by (Bowen-Forbes, 2010). This is parallel to the result of this study through TLC having phenolic compounds with $10HR_f$ values reported.

The red coloration of the crude extract can be attributed to the phenolic compounds (Puupponen-Pimia, 2001). Specifically, anthocyanins, one of the subgroups of flavonoids, are responsible for the red coloration of the fruit. Antimicrobial activity of *Rubus rosifolius* fruit crude extract has been remarkable against almost all test microorganisms except for *A. niger*. Puupponen-Pimia (2001) reported that berries *R. ideaus* and *R. chamaemorus* also showed antimicrobial activity to *S. aureus*, *S. epidermidis*, *S. typhi* and *C. albicans*.

For the minimum inhibitory concentration, *S. aureus* was least inhibited after 24 hours of incubation at 78.13 ug/ mL concentration. Twice as much was needed to inhibit *S. epidermidis*, *S. aureus* and *S. epidermidis* are the least means of zone of inhibition at disc diffusion assay contrary to the MIC value results.

Table 2. Mean inhibition of *Rubus rosifolius* fruit crude extract to the test organisms at different concentrations.

	S. aureus	*S. epidermis*	*S. typhi*	*P. aeruginosa*	*S. cerevisiae*	*C. albicans*	*A. oryzae*	*A. niger**
Positive	11.00±	9.33±	15.33±	17.76±	8.00±	6.00±	8.00±	-
	0.00^{a}	0.33^{a}	2.07^{abc}	1.20^{a}	0.58^{a}	0.00^{a}	0.58^{a}	-
100%	16.33±	17.33±	18.67±	18.33±	18.33±	19.33±	17.67±	-
	0.33^{b}	0.33^{b}	1.45^{b}	0.33^{a}	0.33^{b}	0.33^{b}	0.88^{b}	-
75%	15.33±	16.00±	17.00±	16.33±	17.00±	17.00±	16.67±	-
	0.33^{b}	0.00^{a}	1.53^{a}	0.88^{a}	0.58^{bc}	0.58^{b}	0.88^{b}	-
50%	11.00±	11.33±	14.67±	11.00±	15.33±	14.33±	11.33±	-
	0.58^{a}	0.33^{a}	0.67^{a}	0.58^{b}	0.67^{cd}	0.67^{c}	0.33^{c}	-
25%	9.33±	10.67±	12.33±	8.00±	13.67±	11.33±	10.00±	-
	0.67^{a}	0.88^{a}	0.67^{c}	0.58^{b}	0.98^{d}	0.67^{d}	0.00a^{c}	-
Negative	0	0	0	0	0	0	0	-

**No Inhibition*
Same letter in same column are not significant $\alpha_{0.05}$

The low MIC value can be attributed to the cell wall morphology of the gram positive bacteria (Parekh, 2007). Surface adhesion is vital prerequisite for colonization and infection for Staphylococci species (Elliapola, 2001; Nohynek, 2006; and Karou, 2005). *Salmonella typhi* and *P. aeruginosa* inhibited at 156.25 µg/mL and 312.50 µg/mL, respectively. *Salmonella typhi* retains its median status on both disc diffusion and MIC while *P. aeruginosa* growth and mean zone of inhibition agreed on both assays. Fungi and mold, *Saccharomyces cerevisae, Candida albicans* and *Aspergillus oryzae* were all inhibited at 312.50 µg/mL

Most of the studies about berries were only concerned on phenolic compounds and their bioactivity. For this research, the alkaloids, antraqiunones and higher alcohols found in the fruit extract showed antimicrobial activity to the tested microorganisms. Indoquinoline alkaloids isolated and purified from *Sida acuta* (Malvaceae) exhibited antimicrobial activity in the study of (Omulkoli, 1997). Diterpenoids alkaloids commonly isolated from species of Ranunculaceae also exhibit growth inhibition to pathogenic microorganisms (Suleiman, 2010). Polyhidric alcohols, a group of higher alcohols, are inhibitory to gram positive bacteria (Conley, 1973). Antraquinones, extensively studied because of their anticancer effect, can likewise inhibit gram negative and gram positive bacteria.

Phenolic compounds are the major secondary metabolite that were detected in the crude extract based on phytochemical analysis and the major growth inhibitors on the tested microorganisms based on direct-overlay bioautography. Berry phenolics exhibit strong antimicrobial property to variety of microorganisms. *R. idaeus* and *R. chamaemorus*, the temperate cousins of *R. rosifolius*, are rich in ellagitannins the main phenolic compounds that inhibit the growth of *C. albicans*. Ellagtannins may be present also in its congener *R. rosifolius*. *S. cerevisiae* and *A. oryzae* may also been inhibited by ellagitannins, although it was also not inhibited based on the study.

The antimicrobial activity of *R. idaeus* and *R. chamaemorus* were previously tested to *S. typhi*, *S. aureus* and *S. epidermidis*. The fruit extract disintegrates the outer membrane of *S. typhi* as indicated by the uptake of permeability assay. Part of the activity may occur by chelation of divalent cations from the outer membrane or intercalation into the outer membrane with the replacement of the stabilizing cations. Phenolic materials adhere on the cell membrane of staphylococci thereby inhibiting its adhesion on surface which is vital prerequisite for successful microbial colonization and infection.

Table 4. Retention factor and secondary metabolites that induce antimicrobial activity of *Rubus rosifolius* fruit crude extract by bioautograph.

Test Microorganism	HR_F at 7:3 MeOH: $CHCl_3$	Secondary Metabolities	HR_F at 5:1 $CHCl_3$: MeOH	Secondary Metabolities
S. aureus	65, 69, 81	Phenols	14, 22, 95	Phenols
	88	Antraquinones		
	80	Higher Alchohols		
	73	Alkaloids		
S. epidermis	81	Phenols	14	Phenols
	80	Higher Alchohols		
	73	Alkaloids		
Salmonella typhi	91	Phenols	91	Alkaloids
P. auruginosa	None	None	95	Phenols
S. cerevisiae	88	Antraquinones	95	Phenols
C. albicans	73	Alkaloids	91	Alkaloids
	80	Higher Alkaloids	95	Phenols
	81	Phenols	91	Alkaloids
	88	Antraquinones	95	Phenols
A. oryzae	80	Higher Alchohols	95	Phenols
	81	Phenols		
	88	Antraquinones		

4. CONCLUSIONS AND RECOMMENDATIONS

The present research shows that the fruit of *Rubus rosifolius* has antimicrobial activity that can inhibit growth of wide range of pathogens. Phenolic compounds are the major inhibitors found in this study, although anthraquinones, alkaloids, and higher alcohols detected in the extract also exhibited same activity.

For future researchers, the complete profiling of secondary metabolites and their other bioactivity should be tested. The antimicrobial activity of the fruit extract should also be tested on other groups of microorganisms. Physiological mechanisms of antimicrobial activity of this plant should also be investigated in preparation for the industry-based researches like drug development, natural preservative and other commercial products.

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