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PREPARATION, CHEMICAL CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF PYROLIGNEOUS ACIDS FROM *SALIX PSAMMOPHILA* BRANCHES

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ABSTRACT

In order to improve the additional values of *Salix psammophila* bio-waste, pyroligneous acids (PAs) from *Salix psammophila* branches were extracted by the pyrolysis process at two temperature ranges: 90-380°C and 380-550°C. The chemical constituents and antimicrobial activities of PAs were investigated in detail. The GC-MS results showed that 34 compounds were identified from the two kinds of PAs. The main components were organic acids and ketones for PA at 90-380°C, while they were organic acids and phenols for PA at 380-550°C. The total content of acids and phenols was as high as 67.96% for PA at 380-550°C, which contributed to the strong antimicrobial activity. Two PAs both showed good antimicrobial activities for five pathogenic fungi and two pathogenic bacteria, especially against *Verticillium dahlia*. Compared with the antimicrobial activity of PA at 90-380°C, PA at 380-550°C showed greater antifungal activity but against *Fusarium oxysporum*.

KEYWORDS: *Salix psammophila*, pyroligneous acid, chemical constituents, antifungal activity, anti bacterial activity.

INTRODUCTION

Salix psammophila is a shrub mainly distributed in Northwest China and is endemic to the Kubuqi Desert and the Mu Us Desert. *S. psammophila* has extraordinary adaptation to abiotic stress, thus it is mainly planted to prevent wind erosion and control desertification (Hao et al. 2019). In order to maintain a benign ecological system, the stems of *S. psammophila* are required to flat stubble once every 3-5 years (Li et al. 2013, Zhou et al. 2017). Most of branch residues were discarded as waste or burnt as fuel. The burning of such biomass residue can lead to soil degradation by affecting the soil biota (Grewal et al. 2018). In addition, large number of particulates and trace gases released into the atmosphere can cause many environmental

problems. Therefore, it is of great significance to find an effective way to improve the additional values of this biomass residue. Until now, *S. psammophila* has been used as raw material for wood composite board, pulp and paper (Bao and Zhang 2012, Li et al. 2012, Xu et al. 2006, Ji et al. 2019). It is also a promising biomass feedstock for biofuels and bioenergy (Zhang et al. 2014). In addition, the branch residues can be converted into activated charcoal and pyroligneous acid (PA) by the pyrolysis process. Several researches concerning the activated charcoal from *S. psammophila* have been reported (Gong et al. 2018, Liu and Lang 2020, Liu et al. 2019). However, to the best of our knowledge, taking the *S. psammophila* branch residues as a bio-resource to make PA through pyrolysis, as well as for further development, has not yet been reported.

PA is an acidic, reddish-brown aqueous liquid resulted from the distillation of smoke in the anaerobic charcoal-making process (Mathew et al. 2015). The chemical compounds of PA belong to different classes of organic compounds, such as organic acids, phenols, ketones, aldehydes, alcohols, esters, in which the major ones are organic acids and phenols (Wei et al. 2010b). In China, PA has been used in medicine for more than 400 years. Currently, PA is mainly used as soil fertilizer, antimicrobial agents, sterilizing agent and flavor additive in food industry (Li and Wang 2014, Zhang et al. 2010, Pan et al. 2016). There are lots of studies showing that chemicals, yield, performance and applications of PA vary with the process conditions and raw materials (Ma et al. 2013, Wei et al. 2010a), so it is very necessary to investigate the chemical constituents and antimicrobial activities of PAs from *S. psammophila* branches.

In this paper, *S. psammophila* branches were used as raw material to produce PAs by the pyrolysis process at two temperature ranges: 90-380°C and 380-550°C. Antimicrobial activities of the PAs were evaluated, and the chemical constituents of PAs were analyzed by GC–MS in detail. The purpose of the study is to provide evidence for the development of a green antimicrobial agent from *S. psammophila* pyroligneous acid.

MATERIAL AND METHODS

S. psammophila branches were collected from the sandy land of Ordos city in Inner Mongolia, China. The materials were dried and cut into chips with an average size about 15 mm (diameter) ×100 mm (length).

Preparation and refinement of PAs from *S. psammophila* branches

A pyrolytic retort with dimension of 130 mm (diameter) × 270 mm (height) was used to make PA. About 600 g *S. psammophila* samples were loaded into the retort equipped with a water-cooling condenser. The retort was heated from room temperature to the target temperature with a rate of $1°C \cdot min^{-1}$ and maintained for 30 min to ensure completed pyrolysis. The condensed liquids were collected at two temperature ranges: 90-380°C and 380-550°C based on our preliminary test. We found that the color of pyroligneous acid (PA) at this two temperature ranges was different obviously. PA at 90-380°C was red brown, and PA at 380-550°C was black brown. The color of the former was slightly lighter than the latter, so we decided the two temperature range.

The collected raw PAs were stood for 3 months, and then the crude PA was divided into three distinct layers. The upper layer was thin oil, the middle layer was clear liquid, and the bottom layer was sticky wood tar with other substances. The middle layer was siphoned off and 5 wt% activated charcoal powders were added into. The mixture was stirred for 10 min, and then stood for 72 h. Refined PAs were labeled as E_1 for that collected at 90-380°C and E_2 at 380-550°C.

GC-MS test

About 50 ml refined PA was extracted with 15 ml ether for 6 times to remove the water in PA completely, and obtain the purified and concentrated PA, then the mixed solution was dried and distilled to remove ether. Concentrated PA was obtained and subjected to GC-MS test (TRACEGC-TRACEDSQ, Finnigan). DB-WAX capillary column with a size of 30.00 m × 0.25 mm × 0.25 μm was used to perform separation and N_2 was used as carrier gas with a constant flow rate of 1.0 $ml \cdot min^{-1}$. The injection port temperature was set at 220°C and the injection volume was 1.00 μl. The column temperature was maintained at 40°C for 2 min, and then increased to 150°C at a rate of 4.0°C·min^{-1} for 3 min, finally increased to 240°C at a rate of 6.0°C·min^{-1} for 8 min. Split injection was conducted at a split rate of 80 : 1.

Mass spectroscopic conditions were set as follows, electron energy: 70 eV, ion source temperature: 280°C, mass scanning range: 35-400 $amu \cdot s^{-1}$. Compounds were identified by comparison of the retention time and mass spectra with library data of mass spectra (NIST). The corresponding peak areas were used to determine the relative content of compounds.

Antimicrobial activity

The antifungal effects of PAs from *S. psammophila* branches were investigated on five pathogenic fungi, such as *Verticillium dahlia, Fusarium oxysporum, Fusarium equiseti, Alternaria solani* and *Fusarium graminearum.* The pathogenic fungi were cultured on potato dextrose agar (PDA) medium. The antibacterial effects of PAs were investigated on two pathogenic bacteria, including *Escherichia coli* and *Staphylococcus aureus.* The pathogenic bacteria were cultured on beef extract peptone agar (BPA) medium.

The Oxford cup method was adopted to investigate the antimicrobial activities of PAs from *S. psammophila* branches (Wang et al. 2017). The refined PAs were diluted 10 times and 100 times with sterile water, respectively. About 20 ml sterile PDA or BPA medium was heated until completely melted and then poured into a culture dish. Bacterium or fungus was dissolved in sterile physiological saline to form mixed suspension with the microbial content of 10^6-10^7 CFU·ml^{-1}. Then about 0.1 ml mixed suspension was inoculated on the cured BPA (PDA) medium uniformly. Two Oxford cups were placed within equal distance in each medium culture dish. About 0.25 ml refined PA suspension of different concentration was injected into the Oxford cups. Finally, the culture dishes were placed into a conditioning chamber at a temperature of 28°C for 3 days. The antimicrobial activity was represented by the inhibition zone diameter, which was measured via the cross intersect method with a vernier caliper.

RESULTS AND DISCUSSION

GC-MS analysis

The chemical constituents of PAs from *S. psammophila* branches at different pyrolysis temperature ranges were analyzed by GC-MS and listed in Tab. 1. A total of 34 compounds were identified from the PAs of *S. psammophila* branches. For E_1, 33 compounds were identified, accounting for 97% of the total amount of chemicals, and for E_2, 31 compounds were identified, representing 91% of the total amount. The chemical composition of the two kinds of PAs was very similar to each other with 30 identical compounds, except for different content. Organic acids were the dominant component of the two PAs, accounting for 36.63% and 41.86% for E_1 and E_2, resp. Among three organic acids (acetic acid, propionic acid and butanoic acid), the content of acetic acids was the highest, accounting for 79.12% and 91.76% of the total acids for E_1 and E_2. Ketones were the second highest compound for E_1, representing 29.85% of the total amount, while for E_2, phenols were the second highest constituent, representing 26.1% of the total amount. The different results indicate that the dominant components of PA from *S. psammophila* branches are organic acids and ketones at relatively low temperature stage, but organic acids and phenols at relatively high temperature stage. In addition, three unique compounds could be identified from E_1, including 1,6-dehydrated pyranoglucose, 1,2,3-trimethoxy-5-methyl-benzene and oligogalactose trisaccharide. Only one unique compound could be identified from E_2, which is furfuryl alcohol with the content of 3.37%. All above results suggest that collection of PAs at different temperature ranges is an effective way to pre-fractionate chemicals in PAs. Different products could be achieved based on the different thermal degradation characteristics for chemical constituents (cellulose, hemi-cellulose, and lignin) (Bradbury et al. 1979, Ben et al. 2013, Zhai et al. 2015, Nakamura et al. 2007). As we know, the pyrolysis of hemicelluloses mainly happened at 220-315°C, and that of cellulose mainly happened at 315-400°C. Cellulose and hemicellulose were thermally degraded into ketones, alcohols, furan and pyran derivatives. However, lignin was more difficult to decompose in a wide temperature range (from 160°C to 900°C), and mainly converted into phenol, guaiacol, syringol, pyrocatechol, and their derivatives (Que et al. 2019).

Tab.1: The chemical composition of PAs from S. psammophila branches.

Chemicals	Name	Molecular formula	PA content (%)	
			E_1	E_2
Organic acids	Acetic acid	$C_2H_4O_2$	28.98	38.41
	Propionic acid	$C_3H_6O_2$	5.04	1.80
	Butanoic acid	$C_4H_8O_2$	2.61	1.65
	Total content		36.63	41.86
	1-Hydroxy-2-acetone	$C_3H_6O_2$	18.42	0.92
	2-Hydroxy-3-methyl-2-cyclopenten-1-one	$C_6H_8O_2$	3.84	2.27
	2(5H)-Furanone	$C_4H_4O_2$	3.49	2.93
	1-Acetoxyl-2-acetone	$C_5H_8O_3$	2.22	1.51
Ketones	2-Methyl-2-cyclopenten-1-one	C_6H_8O	0.54	0.74
	1-Hydroxy-2-butanone	$C_4H_8O_2$	0.33	0.83
	2, 4-Dimethyl-1, 3-cyclopentone	$C_7H_{10}O_2$	0.39	0.13

	2-Methyl-3-hydroxy-4-pyranone	$C_6H_6O_3$	0.36	0.28
	2, 3-Pentanedione	$C_5H_8O_2$	0.21	0.55
	2, 5-Hexanedione	$C_6H_{10}O_2$	0.05	0.17
	Total content		29.85	10.33
	Furfural	$C_5H_4O_2$	8.85	6.87
	5-Methyl furfural	$C_6H_6O_2$	1.93	2.95
Aldehydes	Butanedial	$C_4H_6O_2$	0.36	1.00
	Glutaraldehyde	$C_5H_8O_2$	0.20	0.15
	Total content		11.34	10.97
	Phenol	C_6H_6O	5.06	4.74
	1, 2-Benzenediol	$C_6H_6O_2$	3.10	12.09
	3-methoxy-1, 2-Benzenediol	$C_7H_8O_3$	0.73	2.49
	2-Methoxyphenol	$C_7H_8O_2$	0.76	2.81
Phenols	4-Methyl-1, 2-benzenediol	$C_7H_8O_2$	0.45	3.04
	2, 6-Dimethoxy-4-methylphenol	$C_9H_{12}O_3$	0.37	0.61
	2-Methoxy-4-methylphenol	$C_8H_{10}O_2$	0.22	0.32
	Total content		10.69	26.1
	Propionic acid vinyl ester	$C_5H_8O_2$	1.25	0.33
Esters	Butyrolactone	$C_4H_6O_2$	0.87	2.35
	4-Oxo-methyl pentanoic acid	$C_6H_{10}O_3$	0.54	3.37
	Total content		2.66	6.05
	Butyric anhydride	$C_8H_{14}O_3$	2.54	0.33
	Tetrahydro-2-furancarbonyl chloride	$C_5H_7ClO_2$	0.97	0.45
	2, 5- Dimethoxy tetrahydrofuran	$C_6H_{12}O_3$	0.39	0.54
Others	1, 6- Dehydrated pyranoglucose	$C_6H_{10}O_5$	3.55	-
	1, 2, 3-Trimethoxy-5-methyl-benzene	$C_{10}H_{14}O_3$	0.84	-
	Oligogalactose trisaccharide	$C_{18}H_{32}O_{16}$	0.54	-
	Furfuryl alcohol	$C_5H_6O_2$	-	3.37

Antifungal activity

The antifungal activities of PAs extracted at two temperature ranges are shown in Tab. 2 and Fig. 1. Both two kinds of PAs exhibited antifungal activities against five pathogenic fungi when the PA samples were diluted 10 times. The inhibition zone diameters were distributed in the range of 13 mm and 45 mm, indicating that PAs from *S. psammophila* branches possessed a broad antifungal spectrum against different pathogenic fungi. Most notably, PAs had excellent antifungal performance against *Verticillium dahlia* with the inhibition zone diameter of 42.5 mm for E_1, and 44.6 mm for E_2. After the PAs were diluted 100 times, they still had good antifungal activities with the inhibition zone diameter of 12.5 mm for E_1, and 21.5 mm for E_2. It suggested that PA originated from *S. psammophila* branches had the potential to be a low-cost and environmental friendly antifungal agent for preventing attack by *Verticillium dahlia*. As shown in Tab. 2, it could be seen that E_1 had better antifungal performance against *Fusarium oxysporum* and *Fusarium equiseti* as compared to the other two fungi. However, E_2 had better antifungal performance against *F. equiseti* and *F. graminearum* than the other two fungi. In general, E_2 showed higr antifungal effect as compared to E_1 with the exception of against *F. oxysporum*. The reason for high antifungal activity of PA produced at high temperature might be the high total amount of phenols and acids. The total content of phenols and acids for E_2 was 67.96%, while the corresponding data was 47.59% for E_1. Compared to other raw materials in Tab. 3, the total

content of phenols and acids for E_2 extracted from *S. psammophila* branches was much higher than that from some common forestry residues (Lu et al. 2017), such as China fir, pine nut shell, bamboo, and so on. Many papers reported that the components of phenol and acid contributed to the increase in controlling the growth of fungal. This might be due to the inhibition of enzymatic activity by the compounds of phenol and acid in wood vinegar (Oramahi et al. 2018). In addition, from the results, it could be seen the antifungal effect of PA was affected seriously by the process conditions, such as the final temperature and heating rate (Choi et al. 2015, Wu et al. 2015). PAs with complex compositions at different temperature ranges selectively changed the permeability of cell membrane, inhibited the division speed of fungus or the synthesis of protein, which could result in the metabolism disorder, thus showing the inhibitory effect (Walsh et al. 2003).

Tab. 2: The inhibition zone diameter of PAs from S. psammophila branches with different concentration against pathogenic fungi.

Fungi	The inhibition zone diameter (mm)			
	E_1		E_2	
	10 times	100 times	10 times	100 times
Verticillium dahlia	42.5	12.5	44.6	21.5
Fusarium oxysporum	16.2	8.0	13.5	8.0
Fusarium equiseti	15.5	8.0	19.8	8.0
Alternaria solani	13.3	8.0	14.6	8.0
Fusarium graminearum	14.8	8.0	16.1	8.0

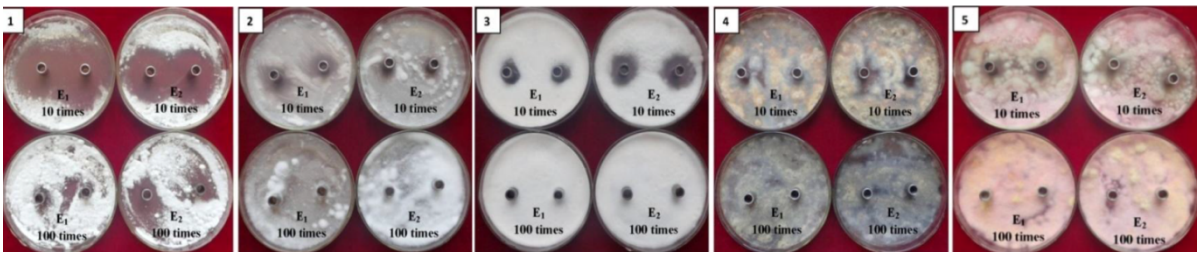

Fig. 1: The antifungal activity of PAs from S. psammophila branches with different concentration against pathogenic fungi: 1) Verticillium dahlia, 2) Fusarium oxysporum, 3) Fusarium equiseti, 4) Alternaria solani, 5) Fusarium graminearum).

Tab. 3: The chemical compositions of pyroligneous acids from different materials (Lu et al. 2017).

Materials	Phenols content (%)	Acids content (%)	Total content of phenols and acids (%)
China fir	17.62	9.97	27.59
Apple wood	13.44	52.73	66.17
Pear wood	13.23	53.09	66.32
Pine nut shell	53.88	3.78	57.66
Walnut shell	43.18	37.52	80.70
Apricot shell	42.83	23.00	65.83
Bamboo	23.29	33.04	56.33
Rice straw	9.37	38.02	47.39
Corn stalk	6.09	35.71	41.80

Antibacterial activity

The antibacterial activities of PAs produced at different temperature ranges on two kinds of bacteria including *Escherichia coli* and *Staphylococcus aureus* are shown in Tab. 3 and Fig. 2. PAs extracted at low and high temperature ranges both showed antibacterial activities for the two bacteria when they were diluted 10 times. However, the antibacterial activities were relatively weak when the PAs were diluted 100 times. Furthermore, E_2 had better antibacterial effect than E_1 against *E. coli.* While for *S. aureus,* E_1 had better antibacterial effect than E_2. It indicated that the antibacterial activity was not only determined by the chemical constituents of PAs, but also by the characteristic of the tested bacteria. Therefore, the optimum pyrolysis temperature range of PAs should be selected based on the characteristic of bacteria (Hou et al. 2018). Furthermore, it could be found that the antibacterial activity (the inhibition zone diameter range: 20-22 mm) was greater than the antifungal activity (the inhibition zone diameter range: 13-19 mm) but against the fungus *Verticillium dahlia.* The antibacterial mechanism of PAs was unclear until now. Some research reported that the pH value of PAs (2.20-3.01) was much lower than the optimal value of the bacteria growth (7.0), leading to the inhibition of bacterial growth (Oramahi and Yoshimura 2013). But some insisted that the phenolic compounds, such as phenol, cresols, were directly responsible for the antibacterial activity of PAs (Loo et al. 2007, Baimark and Niamsa 2009).

Tab. 4: The inhibition zone diameter of PAs from S. psammophila branches with different concentration against pathogenic bacteria.

Bacteria	**The inhibition zone diameter (mm)**			
	E_1		**E_2**	
	10 times	100 times	10 times	100 times
Escherichia coli	20.8	8.0	22.0	8.0
Staphylococcus aureus	21.6	8.0	20.5	8.0

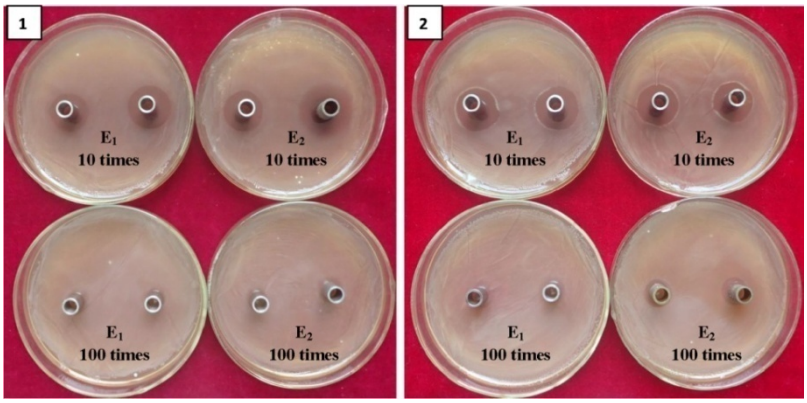

Fig. 2: The antibacterial activity of PAs from S. psammophila branches with different concentration against pathogenic bacteria: 1) E. coli, 2) S. aureus.

CONCLUSIONS

Pyroligneous acids were prepared from *S. psammophila* branches by the pyrolysis process at two temperature ranges, and the chemical constituents and antimicrobial activities were investigated in detail: *1)* 34 compounds were identified from the pyroligneous acids of *S. psammophila* branches. The main components were organic acids and ketones for E_1, and organic acids and phenols for E_2. The total content of acids and phenols for E_2 was as high as 67.96%, which was much higher than that of PAs from some common biomass waste. *2)* PAs extracted at two temperature ranges both showed good antimicrobial activities for the five pathogenic fungi and two pathogenic bacteria, indicating PAs from *S. psammophila* had a broad antimicrobial spectrum. It was worth noting that PAs had excellent antifungal performance against *Verticillium dahlia* with the inhibition zone diameter of 42.5 mm for E_1, and 44.6 mm for E_2. Except for the *Verticillium dahlia,* the antibacterial activity was superior to the antifungal activity. *3)* In general, E_2 showed better antifungal activity compared to E_1 but against *Fusarium oxysporum.* Therefore, PA at high temperature range had a high potential to be an antifungal agent. However, for bacteria, E_2 had better antibacterial activity against *Escherichia coli* than E_1, and E_1 had better antibacterial activity against *Staphylococcus aureus* than E_2.

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