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PHENOLIC ACIDS FROM SUGARCANE BAGASSE LIGNIN: QUALITATIVE AND QUANTITATIVE DETERMINATION, ISOLATION, DERIVATIZATION, AND BIOLOGICAL ACTIVITY EVALUATION

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Lignin has a high service potential and its practical applications turn it into a research material of great interest. There are several processes that can be used to release and purify lignin components from biomass. Various analytical procedures for qualitative and quantitative determination of phenolic compounds by high-performance liquid chromatography have been reported in the literature [1, 2]. The monomers degraded from lignin, such as ferulic acid, coumaric acid, and vanillin and their derivatives, afford good antioxidant, anti-inflammatory, antihypertensive, choleretic, and antispasmodic properties [3]. Among the components, *p*-coumaric acid is recognized as an important chemical for the pharmaceutical industry as well as for human health as a drug against stomach cancer [1]. Literature reports reveal that *p*-coumaric acid and its derivatives possess a wide spectrum of biological activities like antimicrobial [4], antioxidant [5], antiplatelet [6], anti-infertility [7], anti-tyrosinase [8], and antiviral [9]. Ferulic acid has been identified in Chinese medicine herbs such as *Angelica sinensis* (female ginseng), *Lignsticum chuangxiong*, and *Cimicifuga heracleifolia* [10]. Ferulic acid may be useful as a precursor in the synthesis of important aromatic compounds such as vanillin, a synthetic flavoring agent often used in place of natural vanilla extract.

These phenolic acids have been much less exploited for their derivatization into amides and their biological activities. Therefore, in the present study, phenolic constituents of sugarcane bagasse lignin were determined using high-performance liquid chromatography followed by isolation, derivatization, and biological activity evaluation of the major phenolic acids i.e., *p*-coumaric acid and ferulic acid.

The lignin black liquor extracted from sugarcane bagasse was analyzed for its constituent phenolic acids. Phenolic acids, along with being components of lignins, are also attached to lignin and hemicelluloses mainly *via* ester bonds as bridges between them, forming lignin/phenolics-carbohydrate complexes [11]; their qualitative and quantitative determination was carried out in alkaline extracts as ester linkages are completely hydrolyzed with NaOH treatment during extraction of lignin [12]. Table 1 depicts the results of HPLC analysis of lignin black liquors obtained by alkaline extraction from bagasse with 1, 5, and 10% NaOH as explained in our previous study [13]. The major components observed were phenolic acids, viz. *p*-coumaric, ferulic, vanillic, and syringic acids, whereas aldehydes like vanillin and *p*-hydroxybenzaldehyde were found in very small amounts. Syringaldehyde was not detected in any of the lignin extracts. The amounts of *p*-coumaric, ferulic, vanillic, and syringic acids in the lignin liquor obtained with 10% NaOH were found to be 1.47, 1.01, 0.64, and 0.30% w/w bagasse, respectively, taking the lignin content of sugarcane bagasse as 19.20% [13]. The concentrations of all components appeared to increase with increase in concentration of NaOH solution, with *p*-coumaric acid always the major product followed by ferulic acid. The results were found to be in agreement with those reported by [11] for the same raw material. The phenolic acids *p*-coumaric acid (**1**) and ferulic acid (**2**), present in major amounts in bagasse lignin, were isolated in pure form from the methanolic extract of lignin liquor and identified by their physical properties, spectral data, and elemental analyses.

Derivatization of isolated Phenolic Acids to Amides (1a–c, 2a–c). Amide synthesis was carried out in the presence of HOBt (1-hydroxybenzotriazole) additive. The main advantage of using the amide coupling reagent EDC-HCl (*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride) is in the ease of handling of the reagent, the enhanced solubility of EDC, and particularly the easily removable water-soluble urea by-product formed during the reaction; therefore it is superior to conventional coupling reagents like DCC (*N*,*N′*-dicyclohexylcarbodiimide).

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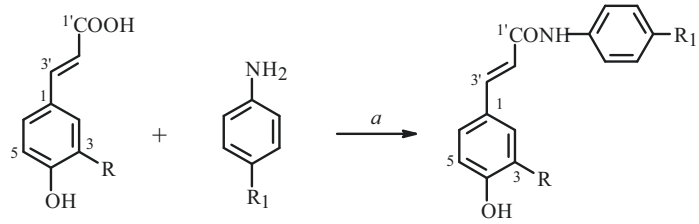
TABLE 1. Results of Qualitative and Quantitative Analysis of Major Components of Bagasse Lignin Extracted with Alkaline Solutions of Different Concentrations (mg g^{-1} bagasse*)

Components of lignin**	NaOH		
	1%	5%	10%
p-Coumaric acid	3.20 ± 1.4	7.10 ± 0.9	14.76 ± 1.8
Ferulic acid	2.01 ± 1.9	5.22 ± 0.8	10.09 ± 1.5
Vanillic acid	1.70 ± 0.6	3.30 ± 0.4	6.43 ± 0.7
Syringic acid	0.51 ± 0.4	1.20 ± 0.01	2.99 ± 0.6
Vanillin	0.08 ± 0.07	0.18 ± 0.08	0.38 ± 0.1
p-Hydroxybenzaldehyde	0.05 ± 0.03	0.08 ± 0.03	0.20 ± 0.08

*Values depicted are mean ± SD of quadruplicate determinations; **syringaldehyde was found in traces (results not shown).

TABLE 2. EC_{50} of DPPH Radical Scavenging Activity and RSI Values of Phenolic Acids and Their Derivatives

Compound	EC_{50}, mg/mL	RSI	Compound	EC_{50}, mg/mL	RSI
1	0.30	3.33	**2a**	0.14	7.14
2	0.18	5.55	**2b**	0.16	6.25
1a	0.16	6.25	**2c**	0.18	5.55
1b	0.20	5.00	BHT	5.60	0.17
1c	0.30	3.33	BHA	0.11	9.09



1a-c, 2a-c

1a-c: R = H; **2a-c:** R = OCH_3; **1a,2a:** R_1 = OCH_3
1b,2b: R_1 = H; **1c,2c:** R_1 = NO_2

a. EDC-HCl/HOBt, DCM, 23°C, 24 h

The physical properties, spectral data, and elemental analysis results of the synthesized derivatives are presented. The isolated phenolic acids and their derivatives were evaluated for their antioxidant activities in terms of DPPH radical scavenging activities in comparison with BHT and BHA (Fig. 1 and Table 2).

DPPH radical scavenging by phenolics is attributed to their hydrogen donating ability [14] and stability of the phenoxyl radical; substituents contributing to stability of the phenoxyl radical further increase the antioxidant activity of the compounds. Ferulic acid showed higher antioxidant activity than *p*-coumaric acid as methoxy groups at the *ortho* position in ferulic acid stabilize phenoxyl radicals by resonance. Amide derivatives of phenolic acids were found to be stronger antioxidants than their corresponding phenolic acids. The presence of methoxyl groups at the *para* position in compounds **1d** and **2d** may be responsible for the increased conjugation and hence stabilization of phenoxyl radicals, leading to higher antioxidant activity, whereas the presence of nitro groups at the *para* position in compounds **1c** and **2c** may be responsible for destabilization of phenoxyl radicals and hence decreased antioxidant activity. Moreover, phenolic acids and amides were found to have higher radical scavenging activity than BHT and lower than that of BHA.

The antibacterial activities of phenolic acids and their derivatives (Table 3) were found to be concentration dependent. The MIC values for all the compounds were highest against *Klebsiella* sp. and lowest against *Bacillus* sp., suggesting that all the compounds were more effective for the control of *Bacillus* sp. and less effective against *Klebsiella* sp. Ferulic acid showed a larger zone of growth inhibition and hence lower MIC values than *p*-coumaric acid. The amide linkage and different aromatic moieties, especially the nitro group, were found to be responsible for the increased activity of phenolic acid amides.

TABLE 3. Antibacterial Activity of Phenolic Acids and Their Derivatives against *Bacillus aryabhattai* and *Klebsiella* sp. at Different Concentrations (Diameter of growth inhibition zone, mm)

Compound	Concentration, μg/disc					
	200	150	100	50	25	10
			Bacillus aryabhattai			
1	16.5 ± 0.5	15.0 ± 0.2	12.0 ± 0.5	9.5 ± 1.0	8.0 ± 0.5	6.5 ± 0.2
2	18.5 ± 1.0	17.0 ± 0.5	15.0 ± 1.0	12.5 ± 1.0	9.0 ± 0.2	7.0 ± 0.5
1a	16.5 ± 0.4	15.5 ± 0.5	13.0 ± 0.5	10.5 ± 0.2	8.5 ± 0.4	6.5 ± 0.5
1b	17.5 ± 0.6	16.5 ± 0.1	13.0 ± 0.2	10.5 ± 0.5	9.0 ± 0.4	7.0 ± 0.6
1c	19.0 ± 0.5	17.5 ± 1.0	15.5 ± 0.8	12.0 ± 0.2	10.0 ± 0.5	7.5 ± 0.5
2a	19.5 ± 1.0	17.5 ± 0.2	15.5 ± 0.1	13.0 ± 1.0	9.5 ± 0.6	7.0 ± 0.7
2b	20.5 ± 0.5	18.0 ± 0.7	16.0 ± 0.5	13.5 ± 1.0	10.5 ± 0.8	7.0 ± 0.2
2c	21.0 ± 1.0	18.5 ± 1.0	17.0 ± 0.6	14.5 ± 0.3	11.0 ± 0.6	7.5 ± 0.3
Tetracycline	25.0 ± 0.3	23.5 ± 0.5	20.0 ± 0.8	19.0 ± 1.0	14.5 ± 0.5	13.0 ± 0.8
			Klebsiella sp.			
1	10.0 ± 0.2	9.5 ± 0.4	8.5 ± 0.6	7.5 ± 0.2	6.5 ± 0.5	6.0 ± 0.2
2	11.5 ± 0.5	11.0 ± 0.3	9.0 ± 0.8	8.5 ± 0.1	7.5 ± 0.1	6.5 ± 0.3
1a	10.0 ± 0.4	10.0 ± 0.4	9.0 ± 0.5	7.5 ± 0.2	7.0 ± 0.5	6.0 ± 0.6
1b	11.0 ± 0.3	10.5 ± 0.3	9.0 ± 0.1	8.0 ± 0.4	7.0 ± 0.2	6.0 ± 0.2
1c	11.5 ± 0.2	10.5 ± 0.4	9.5 ± 0.7	8.5 ± 0.5	8.0 ± 0.4	6.0 ± 0.1
2a	12.5 ± 0.5	11.5 ± 0.5	9.5 ± 0.5	9.0 ± 0.3	7.5 ± 0.2	6.5 ± 0.3
2b	13.0 ± 0.2	12.0 ± 0.7	10.0 ± 0.5	9.5 ± 0.4	8.0 ± 0.2	6.5 ± 0.2
2c	13.5 ± 0.5	13.0 ± 0.1	11.5 ± 0.2	10.0 ± 0.7	8.5 ± 0.7	6.5 ± 0.2
Tetracycline	17.0 ± 0.5	15.5 ± 0.2	14.5 ± 1.0	13.5 ± 0.5	12.0 ± 0.2	11.0 ± 0.3

Values depicted are mean (± SD) of triplicate determinations.

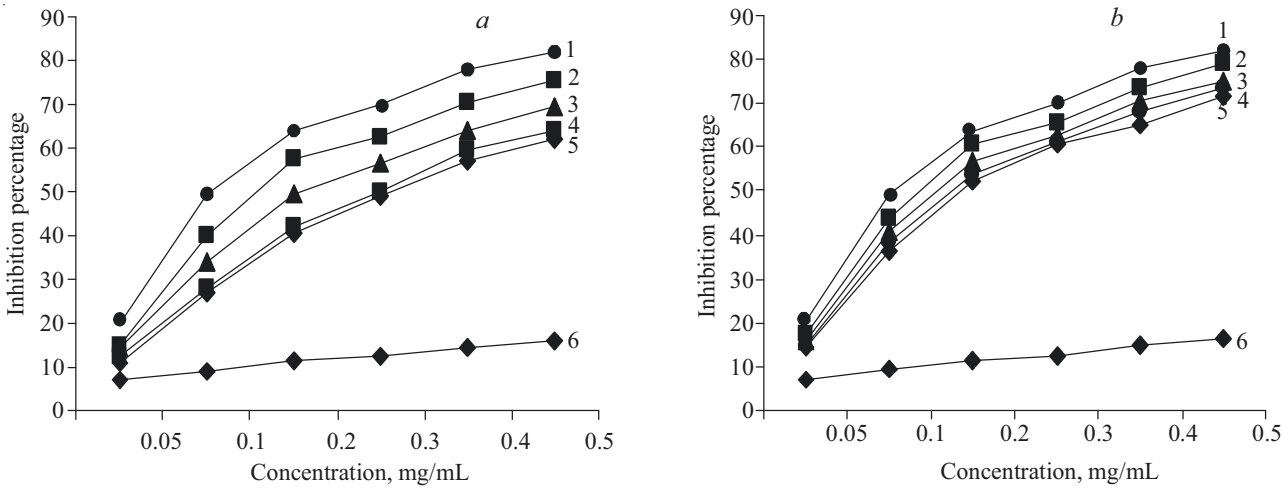


Fig. 1. DPPH radical scavenging activity of (*a*) *p*-coumaric acid and its derivatives and (*b*) ferulic acid and its derivatives in comparison to BHT and BHA. *1* – BHA; *2* – **1a**; *3* – **1b**; *4* – **1c**; *5* – **1**; *6* – BHT.

In general, variations in antibacterial activities among bacteria may reflect differences in cell surface structures between gram-negative and gram-positive species. Also, the number and position of substituent, in the benzene ring of phenolic acids influence the antibacterial potential of the phenolic acids against different microorganisms, but in different ways. The antibacterial activities of phenolic acids and amides were found to be less than that of the standard tetracycline.

In conclusion, *p*-coumaric acid and ferulic acid are major phenolic acids present in alkali lignin liquor of sugarcane bagasse as determined by HPLC analysis. These phenolic acids can be conveniently isolated and derivatized into amides. *p*-Coumaric acid, ferulic acid, and their derivatives can serve as natural, safer, and cheaper potential antioxidants as compared to synthetic antioxidants like BHT. Also, these phenolic acids extracted from sugarcane bagasse and their synthesized derivatives can be used as natural antibacterials.

Materials and Chemicals. Sugarcane bagasse (obtained from a local sugar mill), dried at 60°C and ground to pass through 45-mesh sieve, was put into plastic bags and stored in a desiccator at room temperature until used. The reference standards, namely *p*-coumaric, *t*-ferulic, syringic, vanillic acids, vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), and 3-*tert*-butyl-4-hydroxyanisole (BHA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents like acetonitrile (HPLC grade) and water (HPLC grade) were obtained from Merck, Darmstadt, Germany. HOBt (1-hydroxybenzotriazole), EDC-HCl (*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide), and dry dichloromethane were purchased from Spectrochem Pvt. Ltd., Mumbai, India. All other chemicals used in the study were of analytical grade and obtained from SD Fine-Chem Limited, Mumbai, India. All the filtrations performed during the study were carried out using Whatman Grade 91 wet strengthened qualitative filter papers (Whatman International Ltd. Maidstone, England) or mentioned otherwise.

The cultures of bacteria, i.e., *Bacillus aryabhattai* (KF 853102) and *Klebsiella* sp. (KF 424316), were procured from Pulses Microbiology Laboratory, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The standard tetracycline and HiMedia sterile susceptibility discs were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Instrumentation and Characterization. HPLC analysis was carried out on a Waters HPLC 2489 system equipped with UV/visible detector and a reverse-phase symmetry C18 column (5.0 μm) with dimensions of 4.6 mm × 250 mm. FT-IR spectra were recorded using KBr pellets on a PerkinElmer RX-1 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 400 NMR spectrometer using tetramethylsilane as internal standard. Elemental analyses of compounds were performed on a CHNS-O Elemental Analyzer (Thermo Scientific FLASH 2000). Mass spectra of compounds were recorded on a Micromass/Waters Quattro Micro LC-MS/MS spectrometer. Absorbance values were recorded on a UV-VIS spectrophotometer (Shimadzu UV-1800).

Analysis of the major constituents of bagasse lignin was performed using reverse-phase high-performance liquid chromatography (HPLC).

Lignin was extracted from sugarcane bagasse using NaOH solutions (1, 5 and 10%) as reported in our previous work [13]. The black liquors obtained in the extraction process were filtered through 0.45 μm pore filters (Millipore membrane filter paper-HVLP04700), and the filtrates were acidified with H_3PO_4 to pH 3.0. These solutions were shaken with activated charcoal for 2 h, filtered, and concentrated under reduced pressure, and the final volume of the solutions (3 mL) was made up in acetonitrile. The samples were filtered through 0.20 μm pore filters and analyzed by RP-HPLC for determination of the major components of bagasse lignin using methodology standardized by [2] with some modifications. Acetonitrile:1% acetic acid in HPLC grade water was used as mobile phase under isocratic conditions at a flow rate of 0.5 mL/min. The injection volume was 20 μL with detection wavelengths of 274 and 313 nm. Each run was performed for 15 min. Reference standard solutions were prepared (10–100 μg/mL) from corresponding stock solutions of 1.0 mg/mL. A linear calibration ($R^2 > 0.999$) was obtained for all the standards. Quantification of major components was accomplished by comparing the peak area response for samples with the peak area of standards.

Isolation of *p*-Coumaric Acid and Ferulic Acid from Lignin. Phenolic acids were extracted from lignin black liquor with methanol and isolated by preparative thin-layer chromatography on chromatoplates (20 × 20 cm) of thickness 1500 μm using ethyl acetate–hexane (8:2) as mobile phase. The strips corresponding to spots (visualized under UV lamp) of the standards *p*-coumaric acid (R_f 0.58) and ferulic acid (R_f 0.49) were scratched off separately, eluted with methanol, and centrifuged at 12000 rpm for 15 min in order to remove silica, and the supernatants were allowed to evaporate to yield pure compounds which were identified by melting points, FT-IR, ^{1}H, ^{13}C NMR spectra, and elemental analyses.

Synthesis of Derivatives 1a–c, 2a–c. To a solution of 0.65 mmol of amine and 0.90 mmol of phenolic acid in 5 mL of dry dichloromethane was added 0.30 mmol of HOBt (1-hydroxybenzotriazole) and 3.0 mmol of EDC-HCl (*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide). After stirring for 24 h at room temperature, the reaction was quenched with 1 N HCl, extracted with ether, washed with brine, dried over Na_2SO_4, and concentrated to get pure amides which were characterized by melting point, spectral studies, and elemental analysis. Elemental analyses of all compounds agreed with those calculated.

Compound 1a, mp 220–223°C; yield 78.5%. FT-IR (ν, cm^{-1}): 3205 (N-H stretching), 1661 (C=O, amide), 1549 (N-H bending). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.89 (1H, s, OH), 9.25 (1H, s, NH), 7.58–7.54 (1H, d, J = 15.9, H-3′), 7.20–6.50 (8H, m, Ar-H), 6.30–6.26 (1H, d, J = 15.9, H-2′), 3.83 (3H, s, OCH_3). Elemental anal. calcd for $C_{15}H_{13}O_2N$, 239.

Compound 1b, mp 237–240°C; yield 74.6%. FT-IR (ν, cm^{-1}): 3270 (N-H), 1657 (C=O), 1544. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.90 (1H, s, OH), 9.17 (1H, s, NH), 7.66–7.63 (2H, m, Ar-H), 7.60–7.56 (2H, d, J = 8.6, Ar-H),

7.54–7.52 (3H, m, Ar-H), 6.87–6.85 (1H, d, J = 15.9, H-3′), 6.34–6.30 (1H, d, J = 15.9, H-2′), 6.28–6.24 (2H, d, J = 8.6, Ar-H). Elemental anal. calcd for $C_{16}H_{15}O_3N$, 269.

Compound 1c, mp 216–220°C; yield 79.8%. FT-IR (ν, cm^{-1}): 3263, 1653, 1550. ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.85 (1H, s, OH), 9.20 (1H, s, NH), 8.24–8.20 (2H, d, J = 16.5, Ar-H), 7.82–7.80 (2H, d, J = 16.5, Ar-H), 7.56–7.52 (2H, d, J = 8.6, Ar-H), 7.40–7.36 (1H, d, J = 15.9, H-3′), 6.88–6.86 (1H, d, J = 15.9, H-2′), 6.60–6.56 (2H, d, J = 8.6, Ar-H). Elemental anal. calcd for $C_{15}H_{12}O_4N_2$, 284.

Compound 2a, mp 231–234°C; yield 75.5%. FT-IR (ν, cm^{-1}): 3270, 1657, 1544. ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.91 (1H, s, OH), 9.76 (1H, s, NH), 7.47–7.43 (1H, d, J = 16.0, H-3′), 7.32–7.30 (2H, d, J = 8.0, Ar-H), 7.11–7.10 (1H, d, J = 1.8, H-2), 7.03–7.00 (1H, dd, J = 1.8, 8.1, H-6), 6.81–6.79 (1H, d, J = 8.1, H-5), 6.37–6.35 (2H, d, J = 8.0, Ar-H), 6.31–6.27 (1H, d, J = 16.0, H-2′), 3.77 (6H, s, OCH_3). Elemental anal. calcd for $C_{17}H_{17}O_4N$, 299.

Compound 2b, mp 205–208°C; yield 68.5%. FT-IR (ν, cm^{-1}): 3268, 1646, 1545. ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.92 (1H, s, OH), 9.28 (1H, s, NH), 7.50–7.48 (2H, m, Ar-H), 7.46–7.42 (1H, d, J = 15.8, H-3′), 7.15–7.13 (3H, m, Ar-H), 7.08–7.06 (1H, d, J = 1.8, H-2), 6.98–6.96 (1H, dd, J = 1.8, 8.1, H-6), 6.80–6.78 (1H, d, J = 8.1, H-5), 6.52–6.50 (1H, d, J = 15.8, H-2′), 3.82 (3H, s, OCH_3). Elemental anal. calcd for $C_{16}H_{15}O_3N$, 269.

Compound 2c, mp 258–261°C; yield 70.0%. FT-IR (ν, cm^{-1}): 3250, 1655, 1540. ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.88 (1H, s, OH), 9.12 (1H, s, NH), 8.22–8.18 (2H, d, J = 16.6, Ar-H), 7.80–7.78 (2H, d, J = 16.6, Ar-H), 7.40–7.36 (1H, d, J = 15.8, H-3′), 7.10–7.08 (1H, d, J = 1.9, H-2), 7.04–7.02 (1H, dd, J = 1.9, 8.2, H-6), 6.90–6.92 (1H, d, J = 8.2, H-5), 6.86–6.84 (1H, d, J = 15.8, H-2′), 3.84 (3H, s, OCH_3). Elemental anal. calcd for $C_{16}H_{14}O_5N_2$, 314.

Evaluation of Antioxidant Activity. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the phenolic acids and their derivatives was evaluated [13, 15] using BHT and BHA as standards. The inhibition percentage (IP) of the DPPH" was plotted as a function of concentration. From the graphs, EC_{50} and radical scavenging index (RSI) were calculated.

Evaluation of Antibacterial Activity. The antibacterial activities of the phenolic acids and their derivatives against *Bacillus aryabhattai* and *Klebsiella* sp. were assessed by the bacterial sensitivity-filter paper disc method [16]. Sterilized filter paper discs dipped in dimethyl sulfoxide served as control. Plates were incubated at 28°C ± 2°C, and the diameters of growth inhibition zones (mm) were measured after 24 h.

The phenolic acids and amides showing varying inhibition at different concentrations were further evaluated for their minimum inhibitory concentrations (MIC) for *Bacillus aryabhattai* and *Klebsiella* sp.

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