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Phenotype microarray profiling of the antibacterial activity of red cabbage

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ABSTRACT

Background: Functional food can be a potent source of wide array of biocomonents with antimicrobial activity. We investigated the antibacterial activity of red cabbage (RC) extract on Gram negative and positive ATCC strains. Most intersting, we, for the first time, explored and analysed the complete phenotypic profile of RC-treated bacteria using Omnilog Phenotype Microarray.

Results: This study revealed that the phenotype microarray (PM) screen was a valuable tool in the search for compounds and their antibacterial mechanisms that can inhibit bacterial growth by affecting certain metabolic pathways. It was shown that RC exerted remarkable antibacterial effect on S. aureus and E. coli bacteria, and PM showed a wide range phenotypic profile of the exerted RC antibacterial activity. RC targeted the peptide, carbon, nutriontional assembly, and sulfur metbolic pathways altogether. The peptidoglycan synthesis pathway was inferred to be targeted by RC extract at a metabolic point different from other available cell wall-targeting drugs; these could be hot targets for the discovery of new therapy for many problematic microbes.

Conclusions: Taken together, the phenotype microarray for functional food and medicinal plants can be a very useful tool for profiling their antimicrobial activity. Moreover, extracts of functional food can exert antibacterial activity by hitting a wide range of metabolic pathways, at the same time leading to very difficult condition for bacteria to rapidly develop resistance. Therefore, using functional foods or medicinal plants as such, or as extracts, can be superior on mono-targeting antibiotics if the optimal concentrations and conditions of these functional foods were sought.

Key words: red cabbage, bacteria, antibacterial, phenotype microarray, Omnilog, Biolog

BACKGROUND:

The continuous escalation of resistant bacteria and fungi against a wide range of antibiotics, and the indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, have caused many microorganisms to develop resistance to several antibiotics; this necessitates the discovery of novel and unconventional sources of antibiotics [1]. Therefore, there is a critical need to move quickly and develop alternative antimicrobial drugs from natural products and functional foods. One approach is to screen local medicinal plants, or functional foods, which represent a rich source of novel antimicrobial agents.

In the past few decades, plant phenolic metabolites have gained interest due to their potential role in the prevention and treatment of human diseases. In addition, the use of phytochemicals as natural antimicrobial agents, commonly called 'biocides', is gaining popularity [2]. Interestingly, natural phenolic agents from plants may have a major advantage since they might contain a spectrum of phenolic antimicrobials, instead of a single antimicrobial substance, directed toward a certain spectrum of microbes which potentially be ineffective against the 'antibiotic resistance' phenomenon commonly seen with long-term use of synthetic antibiotics [3]. It is noteworthy to mention that flavonoids have been associated with many of the biological effects such as antibacterial, antiviral, anti-inflammatory, antiplatelet, antioxidant, free radical scavenging, vasodilatory actions, and the ability to lower the risk of coronary heart diseases [4]. Accordingly, some plant phenolics are being developed as potential antimicrobial agents and used in the defense against human pathogens [5, 6]. Besides, synthetic chemicals, which have long been used as active agents in reducing the incidence of diseases in plants, animals and humans, are costly and loaded with side effects [1]. Therefore, the need for more research on new sources, such as medicinal plants and functional food, has become necessary to provide the society with cheap and effective alternative medical drugs. Such medicinal plant products are believed to be highly crucial to improve the society health level and prevent the development of serious diseases [7].

Red cabbage (*Brassica oleracea* L. var. *capitata f. rubra* DC), which belongs to the family Brassicaceae, is one of the most important vegetables grown worldwide. Recently, red cabbage has attracted much attention because of its physiological functions and applications. The dye of red cabbage has been used as a pH indicator in pharmaceutical formulations, [8] and as a food colorant in food systems [9]. There were some studies conducted on red cabbage extract proving its ability to suppress the oxidative stress *in vivo* [10, 11], anticancer [12], anti-inflammatory [13], and anti-diabetic effects [14]. However, only two studies on the antibacterial effects of red cabbage extract and juice were found worldwide [12, 15].

Among the substances that seem to be responsible for the biological activities of red cabbage, are polyphenols [16]. Red cabbage is a rich source of phenolic compounds, with the anthocyanins being predominant over other flavonoids [16, 17]. The highest stability and strongest antioxidative properties in red cabbage extract were assigned to anthocyanins [18]. To date, about 36 anthocyanins have been identified in red cabbage [19]. The amount of total anthocyanins in red cabbage was found to be positively correlated with total antioxidant power, implicating the potential of health benefit of red cabbage to human health. The content of phenolic compounds and the antioxidant activity are partly dependent on the color of the variety of the vegetables. The very high values of antioxidant activity were found in intensely colored

vegetables like red cabbage, red onion, etc. [20, 21]. Besides flavonoids, the antioxidant activity in cruciferous vegetables, such as red cabbage, has also been associated with glucosinolates which present in high quantities [16].

METHODS:

Preparation of the Stock Extract: The stock extract of RC was prepared by redissolving the extract in methanol for the preparation of disc diffusion assay [22]. On the other hand, extracts were dissolved in dimethyl sulfoxide (DMSO) 10%, (BIO BASIC INC., NY, USA). At this concentration, DMSO was shown to be non toxic to microorgansims. It was used for the preparation of microdilution assay, electron microscope observations, and Biolog microarray assay [23]. The dissolved suspension was centrifuged at 134 g for 10min and filtrated by 0.45 μm Millipore filters (Nalgene, UK). The stock was stored at -20°C until further use. The concentration of the stock extract was determined as required in each section.

Disc Diffusion Assay: Briefly, the stock extracts were prepared at 200 and 500 mg/ml for antibacterial tests. The primary screening antimicrobial test was carried out by using the disc diffusion assay [24]. The bacteria were cultured in Nutrient Broth (Merck, Darmstadt, Germany) for 24h at 37°C. One hundred microliter of suspension containing 10^8 CFU/ml were spread evenly on the surface of the nutrient agar. Sterile Whatman No. 1 filter paper (MACHEREY-NAGEL, MN 615, Germany) was used to prepare 6 mm in diameter discs. Ten microliters of the samples with the concentrations of 200, 500, and 700 mg/ml of the plant extracts were loaded into the discs in triplicate in order to give final concentrations of 2, 5, and 7 mg/disc, respectively. These discs were then impregnated in the inoculated agar. The inoculated plates were incubated for 24h at 37°C. Clear inhibition zones around discs indicated the presence of antimicrobial activity. More details are described in another report from our team [25]

Microdilution Assay: The minimum inhibitory concentration (MIC) values were assessed for the microorganisms which were shown to be sensitive to the extract by the disc diffusion assay. The microdilution method was used according to the methodology referred by Zgoda and Porter (2001) [26] with some modifications [25]. The inocula of the bacteria were prepared from 12h broth cultures and standardized to 10^8 CFU/ml. To confirm MIC and to establish minimum bactericidal concentrations (MBC), 10 μl of each culture medium for bacteria with no visible growth were removed from each well and inoculated, in triplicates, on nutrient agar. After incubation for 24h at 37°C, the number of surviving organisms was determined [25].

Transmission Electron Microscope (TEM): Transmission electron microscope observations were carried out on *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29247) as an example for the extract effect on the bacterial cells of Gram negative and Gram positive bacteria. The inocula of the bacteria were prepared from 12h nutrient broth cultures and standardized to 10^8 CFU/ml. The stock extract was to a concentration of 200 mg/ml. One milliliter of 10^8 CFU/ml of *S. aureus* and *E. coli* bacteria suspensions were incubated with 1 ml extract solution at final concentration 100 mg/ml for 24h at 37°C. The detailed procedure of TEM is described by our team in another report [25].

Biolog Phenotype Microarray Technology: The phenotype analysis was carried out by using a new tool, Phenotype MicroArrays (PMs). Biolog's Phenotype MicroArray technology (Biolog, USA) offers a unique way to identify natural product hits and to infer a mode of action by which the novel inhibitor (the extract) prevents microbial growth [27]. The assays are pre-filled and dried in 96-well microplates that can monitor chemical sensitivities. Cell response in each assay well is determined by the amount of color development produced by the reduction of a tetrazolium compound (a redox indicator) during cell respiration [28]. To identify the method of action of RC extract on Gram negative and Gram positive bacteria, PMs were employed to screen various sources of carbon, nitrogen, sulfur and phosphorous. PMs 1-8 were used in which PM1 and 2 measures carbon utilization phenoytpe while PM3 to 8 measures nitrogen, sulfur and phosphorous utilization phenotypes. The methods were done according to the PM procedure for *E. coli* and other Gram negative bacteria and the PM procedure for *B. subtilis* and other Gram positive bacteria provided by Biolog Inc., USA. All the fluids, PMs, and instruments were purchased from Biolog Inc., USA. Kinetic data were analyzed with OmniLog PM software (Biolog, USA). The results were expressed by the differences of the treated bacterial cells from untreated bacterial cells (control group).

PMs of RC Extract with *E. Coli*: *E. coli* (ATCC 25922) was grown overnight at 37°C on nutrient agar plates. In order to prepare the control group bacteria (untreated bacteria), colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 ml of Inoculating Fluid-0a (IF-0a), in a 20 ml sterile capped glass tube. The cell density must be equaled to 42% transmittance (T) on a Biolog turbidimeter. The IF-0a with Biolog Redox Dye Mix A was prepared by adding 0.9 ml of Dye Mix A to 62.5 ml of IF-0a and completed the volume to 75 ml by sterile distilled water. To prepare the bacterial cell suspension, 15 ml of IF-0a with bacteria was added to 75 ml of IF-0a with Dye Mix A; the cell density must be 85% T. Later, 22 ml of this 85% T suspension were transferred to a sterile reservoir and 100 µl/well using Biolog multichannel pipette were added to PM1 and 2 plates. D-glucose (Sigma, Steinheim, Germany) with concentration of 20 mM was added as a carbon source to the rest of 85% cell suspension in order to inoculate PM3 to 8 plates with 100 µl/well. To prepare the treated group bacteria (bacteria treated by RC extract), the same previous steps were followed except the addition of RC extract (100 mg/ml) instead of distilled water in IF-0a plus Dye Mix A. All the plates were incubated at 37°C in the OmniLog plate incubator and reader and were monitored for any color change of the wells. Readings were recorded for 48h for all PM plates.

PMs of RC Extract with *S. Aureus*: *S. aureus* (ATCC 29247) was grown on nutrient agar plates overnight at 37°C. In order to prepare the control group bacteria (untreated bacteria), colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 ml of Inoculating Fluid-0a (IF-0a), in a 20 ml sterile capped glass tube. The cell density must be equaled to 81% transmittance (T) on a Biolog turbidimeter. To enhance the Gram positive bacterial growth some additives were added to PMs inocula. Each PM plate needs its own PM additive (see below) which was prepared as 120x stock solution final volume 100ml, sterilized by filtration using 0.22 µm Millipore filters (Nalgene, UK) and stored at 4°C till further use. All the chemicals used in PM additives were purchased from Sigma, Steinheim, Germany.

To prepare the inocula for PM plates, four plastic tubes (50ml) were used. The first tube was for PM1 and 2. The second one was for PM3, 6, 7 and 8. The third and the fourth tubes were for PM4 and 5, respectively. To prepare the inocula for PM1 and 2, the following additions were done. Twenty milliliter of IF-0a (1.2x) were added and mixed with 0.24 ml Dye Mix F (100x). Two milliliter of PM1 and 2 additives (120x) were also added to give final concentration of 12x. PM1 and 2 additives (120x) were composed of the following ingredients: 10 ml of (240 mM of $MgCl_2$. $6H_2O$ with 120 mM of $CaCl_2.2H_2O$), 10 ml of (3 mM of L-arginine.HCl with 6 mM of L-glutamic acid), 30 ml of (1 mM of L-cystine pH8.5 with 1 mM of Uridine 5`-monophosphat.2Na), 10 ml of 0.6% yeast extract, 10 ml of 0.6% Tween-80 and 30 ml sterile distilled water. Later, the bacterial cell suspension was added as 1.76 ml to PM1 and 2 tube, and the inoculum was ready to inoculate PM1 and 2 plates with 100 µl/well. For the preparation of PM3,6,7 and 8 inocula, 40 ml of IF-0a (1.2x) were added to the second tube and mixed with 0.48 ml Dye Mix F (100x). Four milliliter of PM additives specific for these panels were added from 120x stock to give final concentration of 12x. PM3, 6, 7, and 8 additive stock solution was composed of the following: 30 ml of (800 mM of tricarballylic acid, pH 7.1), 10 ml of (240 mM of $MgCl_2.6H_2O$ with 120 mM of $CaCl_2.2H_2O$), 30 ml of (1 mM of L-cystine pH8.5 with 1 mM of Uridine 5`-monophosphat.2Na), 10 ml of 0.6% yeast extract, 10 ml of 0.6% Tween-80, and 10 ml of (300 mM of D-glucose with 600 mM of sodium pyruvate). A cell suspension of 3.52 ml was added to this tube (tube for PM3,6,7, and 8 inocula) and the inoculum was ready to be applied to PM3, 6, 7, and 8 plates as 100 µl/well. Finally, to prepare PM4 and 5 inocula, 10 ml of IF-0a (1.2x) were added to each specified tube then mixed with 0.12 ml Dye Mix F (100x). One milliliter of PM4 and 5 additives was added from 120x stock solution to give final concentration of 12x. PM4 additive solution was composed of the following: 30 ml of (800 mM of tricarballylic acid, pH 7.1), 10 ml of (240 mM of $MgCl_2.6H_2O$ with 120 mM of $CaCl_2.2H_2O$), 10 ml of (3 mM of L-arginine.HCL with 6 mM of L-glutamic acid), 10 ml of 0.6% yeast extract, 10 ml of 0.6% Tween-80 and 10 ml of (300 mM of D-glucose with 600 mM of sodium pyruvate) and 20 ml of sterile distilled water. While PM5 additive solution was composed of: 30 ml of (800 mM of tricarballylic acid, pH 7.1), 10 ml of (240 mM of $MgCl_2.6H_2O$ with 120 mM of $CaCl_2.2H_2O$), 10 ml of (300 mM of D-glucose with 600 mM of sodium pyruvate) and 50 ml of sterile distilled water. Then 0.88 ml of bacterial cell suspension was added to each tube of PM4 and 5 and the inocula were ready to inoculate PM4 and 5 plates as 100 µl/well. Regarding the *S.aureus* treated with RC extract, the extract solution was prepared as 100 mg/ml final concentration. All the steps were the same as for the control group except the addition of the extract solution to each PM inoculum. One milliliter of RC extract was added to the prepared PM1,2,4 and 5 inocula. While 2 ml of RC extract were added to the prepared PM3,6,7 and 8 inocula. All the plates were inoculated as 100 µl/well. The plates were incubated at 37°C in the OmniLog plate incubator and reader and were monitored for color changes in the wells. Readings were recorded for 48h for all PM plates.

RESULTS:

Antibacterial Activity of Red Cabbage: The extract of red cabbage showed remarkable antibacterial activity toward both Gram negative, namely *E. coli* ATCC strain, and Gram

positive, namely *S. aureus* ATCC strain. The antibacterial level of RC shown through disc diffusion and microdilution assay is shown in tables 1 and 2, respectively.

Table 1: Antibacterial activity of RC against *S. aureus* and *E. coli* ATCC strains via disc diffusion assay

Microorganisms		Inhibition zone in diameter (mm)			
Bacterial species	Number of strains	*B.oleracea* methanol extract (mg/ml)		Negative control (MeOH*)	Positive control (streptomycin 10 µg/disc)
		200	500		
S. aureus	1 (ATCC 29247 stain)	-	11 ± 1.3	-	17 ± 0.8
E. coli	1 (ATCC 25922 strain)	15 ± 1.1	20 ± 1.0	-	11 ± 0.3

Table 2. The MIC and MBC values of RC extract against *S. aureus* and *E. coli* ATCC strains by microdilution method

Bacterial species	Number of strains	MIC* (mg/ml)	MBC† (mg/ml)	MBC: MIC ratio	Antibacterial Mode	Positive control streptomycin (µg/ml)	
						MIC	MBC
S. aureus	1 (ATCC 29247 stain)	100	400	4: 1	Bacteristatic	0.06	0.1
E. coli	1 (ATCC 25922 strain)	100	400	4: 1	Bacteristatic	0.045	0.09

Transmission Electron Microscopy: The antibacterial effect observed by TEM was demonstrated as clear morphological changes in the treated cells along with detachment of the cell membrane (Figure 1).

Phenotype Microarray for *E. coli* and *S. aureus* Bacteria Treated with RC Extract: In the field of drug discovery, the Phenotype MicroArray (PM) allows researchers to obtain a comprehensive picture of a drug's effect on a specific cell [27]. With PM technology, the drug, or the extract of a functional food, can be incubated with bacterial cells; then, information-rich fingerprint for the phenotypic profile of the treated bacterial cells is created showing the effect of that drug on the metabolic pathways and functionality of the treated bacterial cells [28]. Due to the large number of substrates used in the PM technology, the phenotypic profiling for the effect of drugs or extracts used on certain bacterial cells is unique and highly informative for the possible modes of actions of the tested antibacterial agents [27, 28].

The OmniLog PM software detected many phenotype differences between PMs of treated and the untreated bacterial cells of *E. coli* and *S. aureus* with RC extract (Tables 3 and 4).

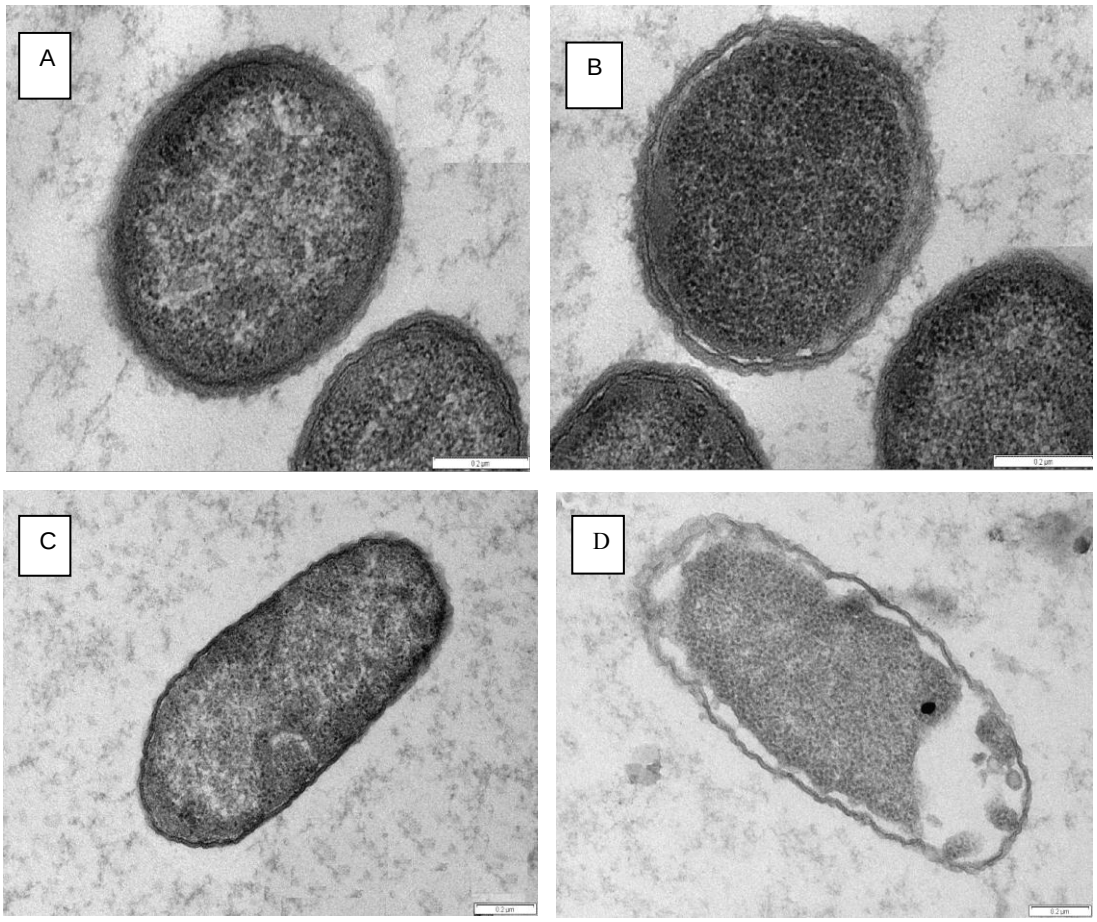

Figure 1. Photos by transmission electron microscope study of *S. aureus* and *E. coli* treated with RC methanol extract showing the cytological changes in *S. aureus* cells: (A) control cells: magnification 100.000k (B) treated cells: magnification 100.000k and in *E. coli* cells: (C) control cells: magnification 60.000k (D) treated cells: magnification 60.000k

Bacteria were exposed to extracts at minimal inhibitory concentrations and phenotype monitoring by OmniLog PM was then conducted. PM tests were performed in 96-well microplates containing different nutrients necessary for the growth of Gram negative and Gram positve bacteria. The differences were calculated when OmniLog PM software system detected growth in the wells of untreated cells and no growth in the corresponding extract-treated wells. These differences were due to the effect of the extract by inhibiting a particular metabolic pathway which needs and uses the coated nutrient on the well. The differences were detected colorimetrically. The results which were expressed as negative values indicate that the control wells had greater rate of respiration than the treated wells. No positive values were detected; positive values indicate that the treated wells had greater rate of respiration than the control wells. The inhibition rate of RC extract on both *E. coli* and *S. aureus* growth was highly manifested in the PM plates with peptide metabolites as nitrogen source, then followed by PM plates with metabolites as carbon source. The level of inhibition in the RC-treated plates was shown to be as follows: nitrogen source (peptide group) > carbon source > nutritional supplement group > other nitrogen source group > phosphorus group > sulfur group.

Table 3: The profile of suppressed growth of RC-treated *E. coli* with the target substrate and the implicated metabolic mode of action.

Plate type	Well number	*Biolog reading	Substrate
Mode of action: C-Source, carbohydrate group:			
PM1	A02	-78	L-Arabinose
PM1	A03	-45	N-Acetyl-D Glucosamine
PM1	B02	-134	D-Sorbitol
PM1	B03	-46	Glycerol
PM1	B04	-103	L-Fucose
PM1	B11	-95	D-Mannitol
PM1	C01	-111	D-Glucose-6-Phosphate
PM1	C07	-75	D-Fructose
PM1	E03	-101	D-Glucose-1-Phosphate
PM1	E04	-42	D-Fructose-6-Phosphate
PM1	H05	-77	D-Psicose
PM2A	B01	-45	N-Acetyl-D Galactosamine
PM2A	B03	-87	β-D-Allose
PM2A	D01	-64	D-Raffinose
PM2A	D04	-78	L-Sorbose
PM2A	D05	-97	Stachyose
PM2A	E05	-96	D-Glucosamine
PM2A	F03	-69	Melibionic Acid
Mode of action: C-Source, carboxylic acid group:			
PM1	C02	-108	D-Galactonic Acid-γ Lactone
PM1	C03	-84	D,L-Malic Acid
PM1	D03	-49	D-Glucosaminic Acid
PM1	F05	-63	Fumaric Acid
PM1	F06	-68	Bromo Succinic Acid
PM1	G07	-67	Acetoacetic Acid
PM1	G12	-72	L-Malic Acid
PM1	H02	-87	p-Hydroxy Phenyl Acetic Acid
PM1	H03	-94	m-Hydroxy Phenyl Acetic Acid
Mode of action: C-Source, amino acid group:			
PM1	B01	-89	D-Serine
PM1	D01	-55	L-Asparagine
PM1	E01	-94	L-Glutamine
PM1	F01	-65	Glycyl-L-Aspartic Acid
PM1	F04	-87	D-Theonine
PM1	G03	-88	L-Serine
PM1	H01	-59	Glycyl-L-Proline
PM2A	G03	-116	N-Acetyl-L glutamic Acid
PM3B	A12	-76	L-Glutamic Acid
PM3B	C03	-248	D-Alanine
PM6	A02	-281	L-Glutamine
Mode of action: C-Source, alcohol group:			
PM1	D04	-76	1,2-Propanediol
Mode of action: C-Source, polymer group:			
PM2A	A06	-87	Dextrin
Mode of action: N-Source, inorganic group:			
PM3B	A02	-152	Ammonia
PM3B	A03	-53	Nitrite
Mode of action: Other N-Source group:			
PM3B	A06	-70	Biuret
PM3B	F01	-70	N-Acetyl-D Mannosamine
PM3B	F02	-113	Adenine
PM3B	F03	-261	Adenosine

PM3B	F04	-104	Cytidine
PM3B	G02	-94	Xanthosine
Mode of action: P-Source, inorganic group:			
PM4A	A02	-155	Phosphate
Mode of action: P-Source, organic group:			
PM4A	B02	-167	Dithiophosphate
PM4A	B03	-231	D,L-α-Glycerol Phosphate
PM4A	C01	-195	Phosphoenol Pyruvate
PM4A	C03	-118	D-Glucose-1-Phosphate
PM4A	D01	-161	D-Mannose-1-Phosphate
PM4A	D02	-141	D-Mannose-6-Phosphate
PM4A	D03	-184	Cysteamine-S Phosphate
PM4A	D04	-193	Phospho-L Arginine
Mode of action: S-Source, inorganic group:			
PM4A	F02	-170	Sulfate
PM4A	F03	-201	Thiosulfate
Mode of action: S-Source, organic group:			
PM4A	G02	-64	S-Methyl-L Cysteine
PM4A	G03	-145	Cystathionine
PM4A	H01	-131	L-Djenkolic Acid
PM4A	H02	-50	Thiourea
Mode of action: Nutritional supplement group:			
PM5	A09	-90	Adenosine-3’,5’-cyclic Monophosphate
PM5	B05	-156	L-Leucine
PM5	B09	-159	Guanosine-3’,5’- cyclic Monophosphate
PM5	B10	-196	Guanine
PM5	B11	-195	Guanosine
PM5	D10	-56	Cytosine
PM5	D11	-169	Cytidine
PM5	E08	-174	D-Pantothenic Acid
PM5	E09	-153	Orotic Acid
PM5	E11	-155	Uridine
Mode of action: N-Source, peptide group:			
PM3B	H01	-119	Ala-Asp
PM3B	H02	-109	Ala-Gln
PM3B	H03	-64	Ala-Glu
PM3B	H04	-87	Ala-Gly
PM6	B01	-242	Ala-Ser
PM6	B02	-270	Ala-Th
PM6	C02	-251	Arg-Phe
PM6	C03	-263	Arg-Ser
PM6	C04	-137	Arg-Trp
PM6	C05	-96	Arg-Tyr
PM6	D01	-248	Asp-Phe
PM6	D03	-293	Asp-Val
PM6	D04	-320	Cys-Gly
PM6	E01	-252	Glu-Val
PM6	E02	-268	Gly-Ala
PM6	E03	-292	Gly-Arg
PM6	E04	-320	Gly-Cys
PM6	F02	-254	Gly-Trp
PM6	F03	-234	Gly-Tyr
PM6	G03	-260	Ile-Ala
PM6	G04	-283	Ile-Arg
PM7	C04	-138	Met-Met
PM7	C05	-222	Met-Phe
PM7	C06	-290	Met-Pro

PM7	C07	-276	Met-Trp
PM7	C08	-174	Met-Val
PM7	D07	-288	Pro-Gly
PM7	E06	-327	Ser-Phe
PM7	E07	-335	Ser-Pro
PM7	E08	-318	Ser-Ser
PM7	F01	-289	Th-Glu
PM7	F02	-257	Th-Gly
PM7	G06	-322	Tyr-Gln
PM7	G07	-313	Tyr-Glu
PM7	G08	-265	Tyr-Gly
PM8	A03	-89	Ala-Asp
PM8	A06	-238	Ala-Met
PM8	B01	-276	Gly-Asp
PM8	B04	-305	His-Glu
PM8	C04	-233	Met-Tyr
PM8	C06	-321	Phe-Glu
PM8	E06	-293	Val-Ala
PM8	E07	-338	Val-Gln
PM8	F01	-240	Val-Ser
PM8	H01	-227	Gly-Gly-Ala
PM8	H03	-152	Gly-Gly-Gly
PM8	H05	-138	Gly-Gly-Leu
PM8	H06	-205	Gly-Gly-Phe
PM8	H08	-141	Gly-Phe-Phe
PM8	H11	-170	Phe-Gly-Gly
PM8	H12	-147	Tyr-Gly-Gly

Note: *Biolog readings: represents the differences between the control bacteria and the treated bacteria. Negative values indicate that the control bacteria had greater respiration rate than the treated bacteria. C: carbon, N: nitrogen, P: phosphorus and S: sulfur.

Table 4: The profile of suppressed growth of RC-treated *S. aureus* with the target substrate and the implicated metabolic mode of action

Plate type	Well number	*Biolog reading	Substrate
Mode of action: C-Source, carbohydrate group:			
PM1	A03	-68	N-Acetyl-D Glucosamine
PM1	B02	-93	D-Sorbitol
PM1	B07	-123	D,L-α-Glycerol-Phosphate
PM1	B11	-58	D-Mannitol
PM1	C01	-158	D-Glucose-6-Phosphate
PM1	E03	-135	D-Glucose-1-Phosphate
PM1	E04	-73	D-Fructose-6-Phosphate
PM1	H05	-91	D-Psicose
PM2A	B01	-89	N-Acetyl-D Galactosamine
PM2A	B03	-185	β-D-Allose
PM2A	B06	-104	D-Arabitol
PM2A	D05	-61	Stachyose
PM2A	E05	-58	D-Glucosamine
Mode of action: C-Source, carboxylic acid group:			
PM1	B10	-111	Formic Acid
PM1	D06	-184	α-Keto-Glutaric Acid
PM1	D03	-85	D-Glucosaminic Acid
PM1	F05	-93	Fumaric Acid
PM1	F06	-88	Bromo Succinic Acid

PM1	G07	-81	Acetoacetic Acid
PM1	G09	-96	Mono Methyl Succinate
PM1	H02	-127	p-Hydroxy Phenyl Acetic Acid
PM1	H03	-102	m-Hydroxy Phenyl Acetic Acid
PM1	H09	-116	L-Galactonic Acid-γ-Lactone
PM2A	C09	-95	β-Methyl-D Glucuronic Acid
Mode of action: C-Source, amino acid group:			
PM1	B12	-173	L-Glutamic Acid
PM1	E01	-108	L-Glutamine
PM1	F01	-53	Glycyl-L-Aspartic Acid
PM1	G04	-48	L-Theonine
PM1	G05	-101	L-Alanine
PM1	G06	-84	L-Alanyl-Glycine
PM1	H01	-105	Glycyl-L-Proline
PM2A	G03	-118	N-Acetyl-Lglutamic Acid
PM3B	A12	-153	L-Glutamic Acid
PM3B	B01	-196	L-Glutamine
PM3B	C03	-204	D-Alanine
PM6	A02	-211	L-Glutamine
Mode of action: N-Source, inorganic group:			
PM3B	A02	-84	Ammonia
PM3B	A03	-69	Nitrite
Mode of action: Other N-Source group:			
PM3B	E08	-63	D-Glucosamine
PM3B	E09	-48	D-Galactosamine
PM3B	F01	-103	N-Acetyl-D Mannosamine
PM3B	F04	-99	Cytidine
PM3B	G02	-96	Xanthosine
Mode of action: P-Source, inorganic group:			
PM4A	A02	-138	Phosphate
Mode of action: P-Source, organic group:			
PM4A	B02	-146	Dithiophosphate
PM4A	C01	-93	Phosphoenol Pyruvate
PM4A	C03	-164	D-Glucose-1-Phosphate
PM4A	C05	-139	2-Deoxy-D-Glucose 6-Phosphate
PM4A	C06	-122	D-Glucosamine-6-Phosphate
PM4A	C07	-94	6-Phospho-Gluconic Acid
PM4A	D04	-173	Phospho-L Arginine
PM4A	E01	-144	O-Phospho-D Tyrosine
PM4A	E02	-89	O-Phospho-Ltyrosine
Mode of action: S-Source, inorganic group:			
PM4A	B01	-57	Thiophosphate
PM4A	B02	-96	Dithiophosphate
PM4A	F02	-168	Sulfate
PM4A	F03	-182	Thiosulfate
Mode of action: S-Source, organic group:			
PM4A	G02	-81	S-Methyl-L Cysteine
PM4A	G03	-89	Cystathionine
PM4A	G07	-117	L-Methionine
PM4A	G08	-74	D-Methionine
Mode of action: Nutritional supplement group:			
PM5	B05	-128	L-Leucine
PM5	B09	-109	Guanosine-3',5'- cyclic Monophosphate
PM5	A03	-117	L-Alanine
PM5	A04	-91	L-Arginine
PM5	C08	-76	Trans-4-Hydroxy L-Proline

PM5	C10	-92	Hypoxanthine
PM5	D10	-66	Cytosine
PM5	D11	-152	Cytidine
PM5	E03	-126	Spermine
PM5	E06	-216	Pyridoxamine
PM5	E08	-203	D-Pantothenic Acid
PM5	E10	-158	Uracil
PM5	E11	-119	Uridine
Mode of action: N-Source, peptide group:			
PM3B	H02	-95	Ala-Gln
PM3B	H03	-74	Ala-Glu
PM3B	H04	-114	Ala-Gly
PM6	B05	-179	Arg-Ala
PM6	B06	-238	Arg-Arg
PM6	C02	-216	Arg-Phe
PM6	C03	-219	Arg-Ser
PM6	C04	-203	Arg-Trp
PM6	C05	-142	Arg-Tyr
PM6	C11	-95	Asp-Leu
PM6	C12	-78	Asp-Lys
PM6	E01	-177	Glu-Val
PM6	E02	-164	Gly-Ala
PM6	E03	-199	Gly-Arg
PM6	E04	-219	Gly-Cys
PM6	F02	-174	Gly-Trp
PM6	F03	-221	Gly-Tyr
PM6	G03	-219	Ile-Ala
PM6	G04	-153	Ile-Arg
PM7	A06	-175	Lys-Ala
PM7	A07	-185	Lys-Arg
PM7	B03	-147	Lys-Th
PM7	B05	-217	Lys-Tyr
PM7	B07	-201	Met-Arg
PM7	B09	-174	Met-Gln
PM7	D07	-275	Pro-Gly
PM7	E06	-127	Ser-Phe
PM7	E07	-195	Ser-Pro
PM7	E08	-139	Ser-Ser
PM7	F06	-228	Trp-Ala
PM7	F07	-291	Trp-Arg
PM7	G06	-143	Tyr-Gln
PM7	G07	-94	Tyr-Glu
PM7	G08	-89	Tyr-Gly
PM8	A03	-91	Ala-Asp
PM8	A06	-153	Ala-Met
PM8	B01	-193	Gly-Asp
PM8	B04	-207	His-Glu
PM8	C07	-143	Gln-Glu
PM8	C08	-129	Phe-Met
PM8	H01	-194	Gly-Gly-Ala
PM8	H03	-189	Gly-Gly-Gly
PM8	H05	-85	Gly-Gly-Leu
PM8	H06	-192	Gly-Gly-Phe
PM8	H08	-122	Gly-Phe-Phe

Note: *Biolog readings: represents the differences between the control bacteria and the treated bacteria. Negative values indicate that the control bacteria had greater respiration rate than the treated bacteria. C: carbon, N: nitrogen, P: phosphorus and S: sulfur.

DISCUSSION:

RC extract showed bacteristatic effect on *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29247); this enabled the detection of any inhibition in the cell growth by monitoring the changes in the level of respiration between treated and untreated cells. The selected bacteria were used as representatives for elucidating the phenotypic and biochemical profiling of Gram positive and negative bacteria treated with RC extract. The results of PM analysis of *E. coli* and *S. aureus* treated cells provided a good example for RC antimicrobial activity when compared with PM results of untreated cells. Both Gram positive and negative bacteria showed similar response to RC extract, up to 80% similarity. This granted clue on the common antibacterial mechanisms pursued by RC extract on both Gram negative and positive bacteria. Moreover, there was a very good agreement to the results obtained by SEM and TEM observations. The PM results showed that RC extract had a clear ability to inhibit bacterial growth by direct effect on the assimilation of different metabolites. PM readings for untreated cells showed that these cells grew normally in PM plates while PM readings for treated cells indicated clear defects in the growth of bacteria using peptide metabolism pathways. The other clear defect was detected in the metabolism pathways of carbon source metabolites. The detected features by SEM and TEM observations that were characterized by changes in cell wall, which may be due to an increase in cell wall permeability, could be explained by the defect in the peptide metabolic pathways. RC extract affected this pathway more than other pathways in a significant way. Peptidoglycan is composed of peptides (like L-alanine and D-alanine) plus glycan chain (polysaccharide, like N-acetyl D glucosamin), [29]. Moreover, peptidoglycan prevents bacteria from undergoing an osmotic lyses. Therefore, it is an ideal target for selective toxicity [30]. The results showed that RC extract inhibited the assimilation of N-acetyl D glucosamin together with L- and D-alanine. Therefore, RC extract could be a highly promising antibacterial agent that can hit bacteria at targets different from those hit by commercially available drugs. For example, the beta-lactam antibiotics like penicillin and apmicillin inhibit bacterial growth by binding to and inhibiting enzymes needed for the synthesis of the peptidoglycan wall [31]. Cycloserine is an analog of D-alanine and blocks the incorporation of D-alanine into the peptide bridges in the bacterial cell wall [32]. Additionally, RC extract was shown to inhibit the utilization of sulfur metabolite pathways. Sulfur is an essential component of cells [29]. In *E. coli*, among the 4,500 genes in the genome, more than one hundred genes are directly involved in some steps of sulfur metabolism [33]. Besides, sulfur metabolic pathways of pathogenic bacteria hold importance for its biological implications as well as the presence of potential enzymes that act as good targets for many future antimicrobial drugs [34]. Therefore, sulfur pathways affected by RC extract represent an additional target signifying the importance of RC extract as a candidate for new drug(s) against new targets in both Gram positive and negative bacteria. Furthermore, RC extract showed significant inhibition to the metabolic pathways that utilize other nitrogen sources and nutritional supplement. The blocking of these pathways may give an overview on the effective antibacterial activity of RC extract in this study. However, it is not known whether single or multiple antibacterial components in RC extract are responsible for the currently shown phenotypic profiling, but the wide range of metabolic pathways affected by RC extract suggested strongly that more than one component shares the antibacterial activity of RC extract. Nevertheless, the current phenotypic profiling of RC extract can give clues on the novelty of the

responsible antibacterial components as well as provided a metabolic guide, or metabolic finger print, for the future work on isolating and testing the function of the antibacterial components observed in RC extract.

CONCLUSION:

It was concluded that RC extract using acid methanol solvent provided potent antimicrobial effects against the tested Gram negative and Gram positive bacteria. RC extract showed that it is capable of hitting a wide range of metabolic pathways which were clarified through using state-of-the-art Biolog Phenotype Microarray. RC inhibited bacterial growth through peptide, carbon, nd sulfur–based pathways of bacterial growth. Hence, this study acts as a comprehensive platform for further studies to elucidate the components responsible for such remarkable and broad antibacterial mechanisms found in RC. Moreover, the phenotypic profiling of antibacterial activity of RC have shown that using extracts of functional foods or medicinal plants might be more robust in counteracting microbial resistance against antimicrobials. This phenomenon is explained by that extracts of functional food or medicinal plants possess a wide range of components that act in parallel or in synergistic way in exerting the antibacterial effect. RC extract was found to be an excellent example of the potency hidden in using certain functional foods as such or as extracts in combating bacteria without fearing of rapid development of bacterial resistance due to the lack of mono-acting antibacterial effect in these extracts. Therefore, this study serves as the current endeavor to find potent natural antibacterial products from functional food as we are heading consistently to the post-antibiotic era due to the ever increasing microbial resistance against current antibiotics.

Authors' contributions: RR and AS did the sampling, research, design, and conducted all the assays of the current study. F and AS conducted the research planning, statistical and technical design.

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