



**Antibacterial Activity of *Rubus multibreatus* Le'vl Plant Extracts Against
*Staphylococcus aureus***

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Abstract

The genus *Rubus* is a family of shrubs and mostly edible berries such as rasp berries, blue berries, strawberries. Of the 2208 species, 331 are documented [3] and about 139 native to China, and being a wild plant, it can grow anywhere except for Antarctica [1] and with a wide variety as well as fairly similar phyto-components[2]. *Rubus multibreatus* is commonly known in China as Da wu pao, its leaves used in teas and to locally treat stomach upsets, haemorrhoids and rheumatism, [1]. Over years, due to a variety of intraspecies, it has acquired different synonyms, which include; *R.mallodes*, *R.andropoga*, *R.clinocephalus*, *R.pluribracteatus*, *R.terminalia* and *R.macroptera* [4].

Keywords: Bacterial inhibition; solvent extraction; flavonoids; true tannins; Gallic acid.

Abbreviations

S.aureus- *Staphylococcus aureus*

E.coli-*Escherichia coli*

S.tphi-*Salmonella typhimurium*

MIC-minimum inhibitory concentration

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MBC-minimum bactericidal concentration

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

qPCR-quantitative polymerase chain reaction

EtOAc- ethyl acetate

EtOH-ethanol

DCM- dichloromethane

n-but-Butanol

1. Introduction

Even though the plant has been reported to have medicinal properties, published work on the subject is meagre, expressing the major challenges in knowledge on traditional medicine and the current digital era in new drug formulation. Journals on traditional medicine in Chinese medical universities have given insight on possible findings on the topic, which was in sync with this research. Presence of flavonoids [55,56]; kaempferol and quercetin [56] via various extraction procedures, as well as claims of analgesic and anti-inflammatory properties of the plant [57] Library thesis research done by Sichuan agricultural university students supervised by prof He to demonstrate reduced diarrhoea in mice infected with *Salmonella typhimurium* prompted this research.

S.aureus is a gram positive cocci bacteria that can colonize the nasal cavity, skin, eyes, hence can cause infections like sinusitis, tonsillitis, pharyngitis, pneumonia, keratitis, arthritis and skin infections. It generally invades via bacterial colonisation, biofilm formation, capsase polysaccharide protein binding, and production of toxins [9]. It's commonly acquired from contaminated food, post-surgery infections and may cause osteomyelitis, toxic shock and lethal pneumonia [5,6,7,8,9] The bacteria has also been found in dusty places in typical residences [10]. *S.aureus* toxins can cause cellular lysis, such as the α haemolysin, which interferes with movement of K^+ ions, causing an influx of Ca^{2+} ions, and activation of cytokines IL-1 β , IL-6, IL-8, Haemolysin β , which inhibits IL-8 causing chronic skin infections and mastitis in animals, production of hyaluronidase, which degrades hyaluronic acid on extracellular tissues such as skin, skeletal tissues, umbilical cord, lungs, heart valves, brain, blood and liver [5,6], Leucotoxins: HIgA, HIgB, HIgC, LukE, Luk D and Luk AB, which together with Luk PVL (Panton valentine leucocidin) cause lysis to neutrophils and macrophages, hence causing chronic lung and skin infections. Super antigens/enterotoxins that can cause toxic shock syndrome due to food poisoning by α -leucocidal hemolysin, in contaminated food, that can withstand 100°C and can cause diarrhoea, hypotension and MODS (multiple organ dysfunction syndrome). It is also IgE specific, causing severe sinusitis and asthma [6,8] Staphylococcal enterotoxins A, B, C, D can also exist in dust. [10] SpA binding protein in *S.aureus* binds to IgG via peptidoglycan 5 to form biofilms to colonise it's host and avoid macrophage attack, aided by clumping binding and adherent genes; SpA, SpB, fnbA, fnbB, ebps, ica A,B,C,D[8,7]

2. Materials and methods

- ▶ S.aureus TCH1516 (ATCCBAA-1717) (USA300)
- ▶ E.coli serotype 078,
- ▶ S.Typhi ATCC14028

The Leaves of *R.multibreatus* were harvested and dried in a shade under sanitary conditions, ground and stored in an air-tight enclosure. Small portions of about 40g were macerated with about 1.2L of 90%EtOH under ultrasonic treatment, thrice, every 30 minutes, vacuum filtered, and concentrated using different solvents; DCM, EtOAc, and n-But. All three solvent extracts, after separation from the solvents, via a rotary evaporator, (pressure>0.04, Temp=55°C), were used to test for antimicrobial activity using the three bacteria species; S.aureus, E.coli and S.typhi. The EtOAc extract showed the best activity for S.aureus at a MBC of 12.5 mg/ml. All 3 drug extracts were dissolved in 2% DMSO, of the total sterile 1% saline water used to make drug dilution concentrations of 12.5, 6.25, 3.125 mg/ml. To avoid interference by DMSO used to dissolve the extracts, from absorbance data, only <2% was required to make a solution, before diluting with the liquid media, and the same highest amount of DMSO calculated to be present in the diluted drug portions was always added to the positive control for uniformity. Further, only S.aureus was used as the positive control. Bacteria was cultured using liquid broth and was diluted to 10^7 , which was considered suitable, with about 300-350 CFUs. This was used alongside treatment with drug concentrations of 2MIC, 1MIC and 0.5MIC, of the ethyl acetate plant extract and was observed at OD=600nm every two hours, to obtain a growth curve to portray the effect of the drug on the growth of bacteria. The growth curve experiment was done again using another set of 2MIC, 1MIC and 0.5MIC drug solutions only, without bacteria, to serve as negative controls due to the colour intensity of the drug, which made it difficult to compare with the positive and negative controls which only had liquid media. The OD values at 600nm were taken every two hours, for 12 hours, and then after 24h and 36hours.

Chemical tests for phytochemical constituents were carried out on the ethyl acetate extract to determine the general constituents in it. Acid hydrolysis of 2% NaOH by HCl to test for flavonoids, dilute ethanol and FeCl₃ to test for oleoresins, the Salkowski test for triterpenes and sterols using chloroform and H₂SO₄, the Borntrager's test for anthraquinones using H₂SO₄ and ether, anthocyanins test using 2N HCl and ammonia, Libermann's test for phenolic acids, Aqueous FeCl₃ to test for tannins and the broth test for Saponins.

General flavonoid extraction procedure was implied by aqueous extraction of the powdered leaves, separation of flavonoids from tannins using 2M lead acetate to get the supernate, dilution with distilled water and acidification with HCl to remove sugars, ethanolic precipitation of flavonoid crystals and fractional crystallisation to dry the crystals. Tannins and ellagic acid were extracted via 90% ethanol ultrasound treatment and vacuum filtration, then separated using ether, to get the supernate true tannins solution and ellagic acid precipitate solution. The two extracts were dried using a rotary evaporator under high pressure and optimum temperature. SDS-PAGE of total proteins was carried out on total flavonoids, tannins and Gallic acid extracts on page gels to decipher the extent of bacterial inhibition by the extracts and to determine the drug with the best activity at given concentrations. The samples were subjected to ultrasonic crushing while in ice, centrifuged, and the supernate solution mixed with protein booster at a volume ratio of 1:3, loading buffer: sample, heated for 5mins at 100°C

to reduce proteases, then stored at -4°C overnight, before electrophoresis.

QPCR for the 16S S.aureus gene was carried out to illustrate bacterial clearance using flavonoid and gallic acid extracts. After centrifugation to remove liquid media, in a sterile laminar flow bench, trizol was used to crack RNA, and CHCl₃ to separate RNA from the organic phase, Isopropyl alcohol to precipitate RNA in the separated ionic phase. It was then washed with cold 75% ethanol and dried, then each sample dissolved in 20µl RNase water. The concentration and purity of extracted RNA was measured by a Nanophotometer. RNA was then transcribed to c-DNA using an RNA kit and heating cycles of (25°C,5mins), (42°C,60mins) and (70°C,5mins). Primers were then mixed with c-DNA and amplified in four steps; 1 cycle pre-incubation at 95°C for 5mins, 40 cycles of amplification 60°C for 30mins, 1 cycle of melting at 95°C, and 1 cycle of cooling at 50°C for 30mins.

2.1 Extract preparation

From the EtOAc extract, which was the only portion that showed activity at 12.5mg/ml, chemical tests consistent with results from other leaf extracts in other of Rosacea species

Confirmed the presence of flavonoids, oleoresins, tannins and phenolic acids.

Amounts of concentrated extracts by DCM, EtOAc and n-but from the 40g macerated in ethanol

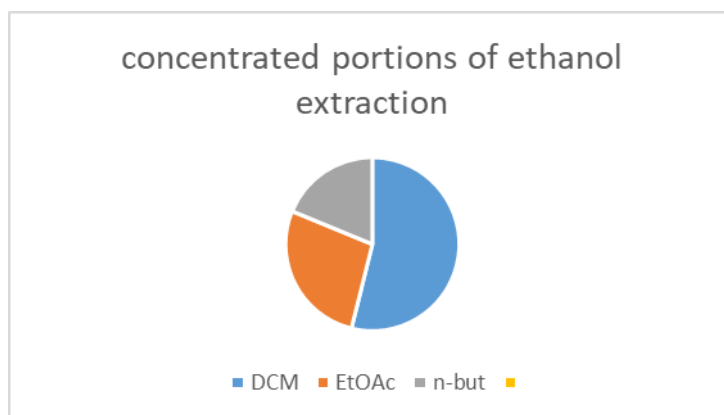


Figure 1: Amount of extracted portions after concentrating using different solvents.

Extracted ellagitannins from the macerated 40g were also separated into Gallic acid extract and tannins extract, while flavonoid crystals were separately extracted using another 40g via aqueous extraction

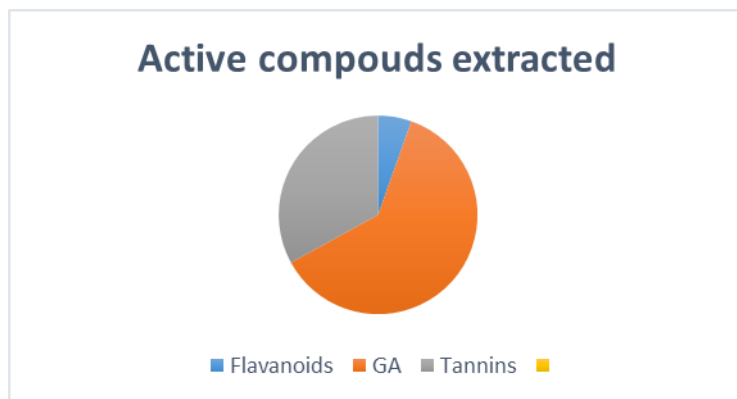


Figure 2: Amount of extracted metabolites using the same amount of powdered leaves.

3. Results

3.1 Bacterial Inhibition

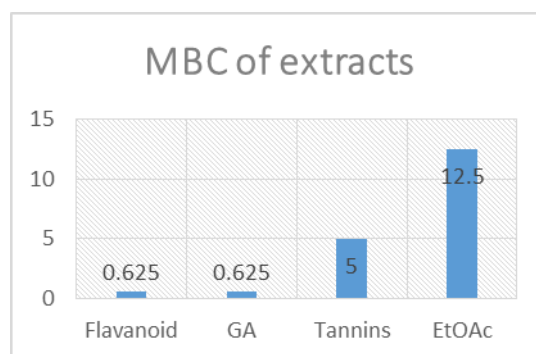


Figure 3: MBC of the extracted metabolites.

It was observed that extraction of tannins, gallic acid and flavonoids separately improved activity as opposed to solvent extraction by ethyl acetate in general. This could be attributed to separation of hydrophobic true tannins extract, which in fact aided the bacteria to grow, while at high concentration. The MIC of both gallic acid and flavonoids was determined to be 0.625mg/ml while though there was inhibition by tannins at 0.625mg/ml, the most concentrated portion of 10mg/ml showed more bacterial colonies as compared to the 10^7 bacterial dilution spread. However, the more diluted portions of 5,2.5,1.25, and 0.625mg/ml did inhibit bacterial growth.

Growth curve experiment for the EtOAc extract was done to show the growth of bacteria over 36hours, and high concentrations did improve the antimicrobial activity of the plant extract.

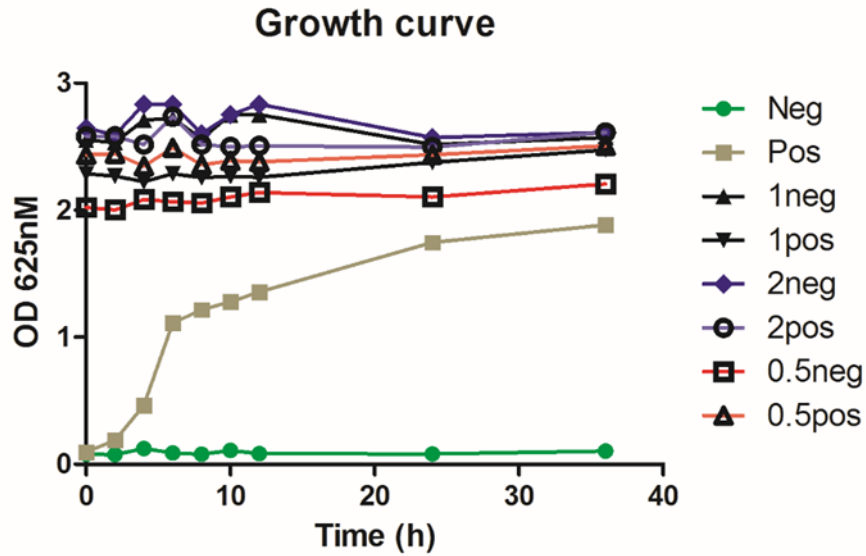


Figure 4: Negative control, positive control, 1MIC negative, 1MIC positive, 2MIC negative, 2MIC positive, 0.5MIC negative, 0.5MIC positive.

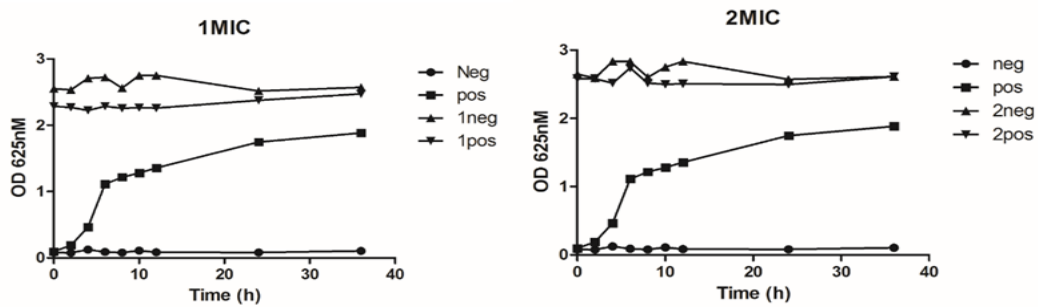


Figure 5: (1MIC negative, 1MIC positive, Positive control, negative control), (2MIC negative, 2MIC positive, positive control and negative control)

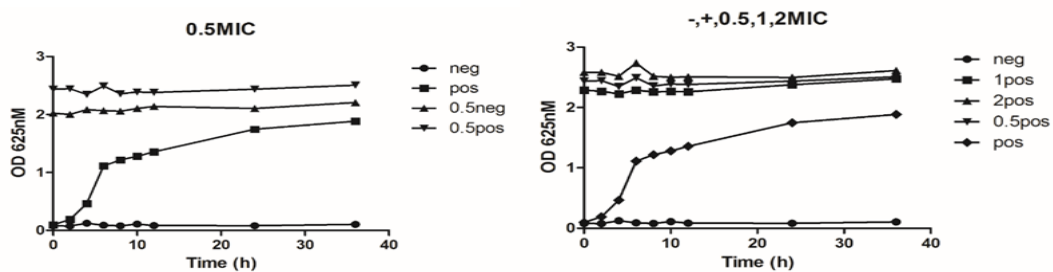


Figure 6: (0.5MIC negative, 0.5 MIC positive, positive control and negative control), (2MIC positive, 1MIC positive, 0.5MIC positive, positive control and negative control).

SDS-PAGE of total proteins proved that Gallic acid inhibited the bacteria completely, even at low doses, as compared to flavonoids, however, flavonoids inhibited the bacteria with increased dosage. Higher concentrations of tannins showed an increase in bacterial proteins compared to the bacteria itself.

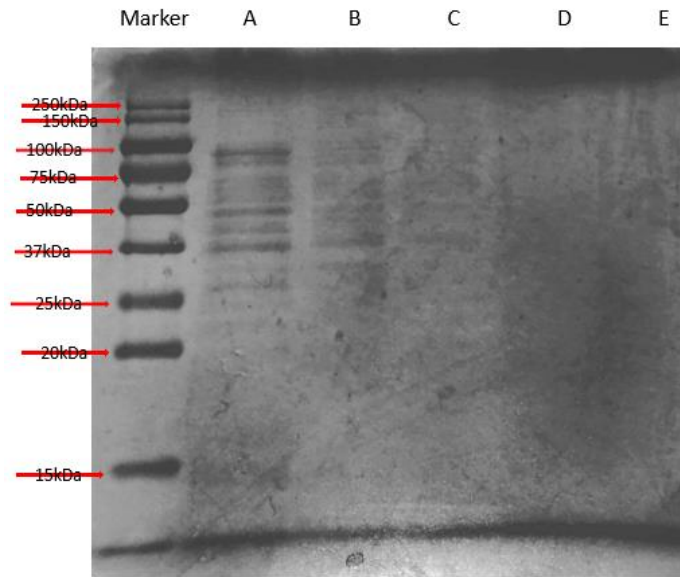


Figure 7: SDS-PAGE of marker, positive control(A), and flavonoids (B, C, D, E) at concentrations; 0.78125,1.5625, 3.125, and 6.25mg/ml respectively.

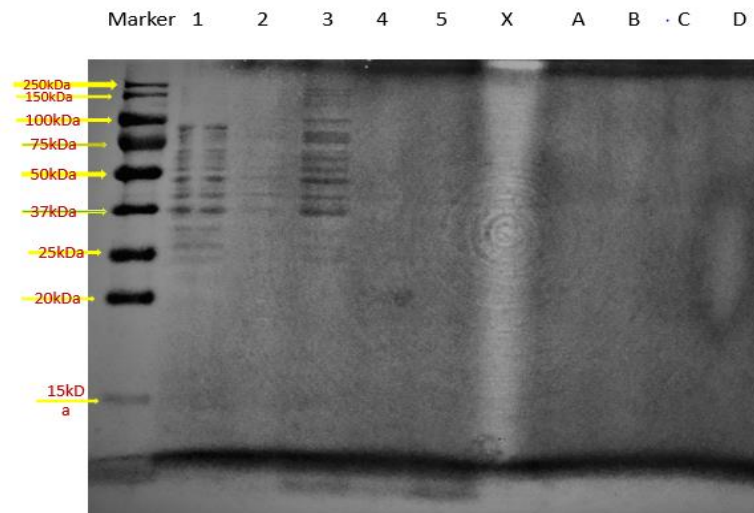


Figure 8: (From L-R; SDS-PAGE of the marker, positive control [1], (tannins; [2,3,4,5], at concentrations 0.78125, 1.5625, 3.125 and 6.25mg/ml respectively, a separation gap(X), and Gallic acid (A, B, C, D) at concentrations 0.78125, 1.5625, 3.125 and 6.25mg/ml respectively).

The 16S Staphylococcal gene, and two MRSA genes; mecA and lukS/F-PV were used to test the effectiveness of flavonoids and Gallic acid at both low concentrations(1MIC,0.5MIC,0.25MIC) ;0.78125mg/ml,

0.39063mg/ml, 0.1953mg/ml and higher concentrations at (2MIC,3MIC and 4MIC) ;1.5625mg/ml,3.125mg/ml,6.25mg/ml. At 2MIC and above drug solutions, the concentration of all three genes was null. Both the 16S and LukS/F-PV gene were expressed at concentrations of 35.00 while the mecA gene was expressed at a concentration of 33.00. Gallic acid was the most effective for 16S and mecA gene by a reduction in gene expression from 35.00 and 33.00 to almost null at 1MIC, and flavonoids from 35.00 to 23.68 and from 33.00 to 32.73 respectively. On the other hand, flavonoids reduced the expression of LukS/F-PV from 35.00 to 24.47, and Gallic acid from 35.00 to 31.68

The 16S gene; F-(5' AGG CCC GGG AAC GTA TTC AC 3'), R-(5' GAG GAA GGT GGG GAT GAC CT 3'), [16]

MecA gene; F-(5'GTA GAA ATG ACT GAA CGT CCG ATA A 3'), R-(5'CCA ATT CCA CAT TGT TTC GGT CTA A 3'), [19]

LukS/PV gene; F-(5'ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A 3'), R-(5'GCA TCA AGT GTA TTG GAT AGC AAA A 3') [19]

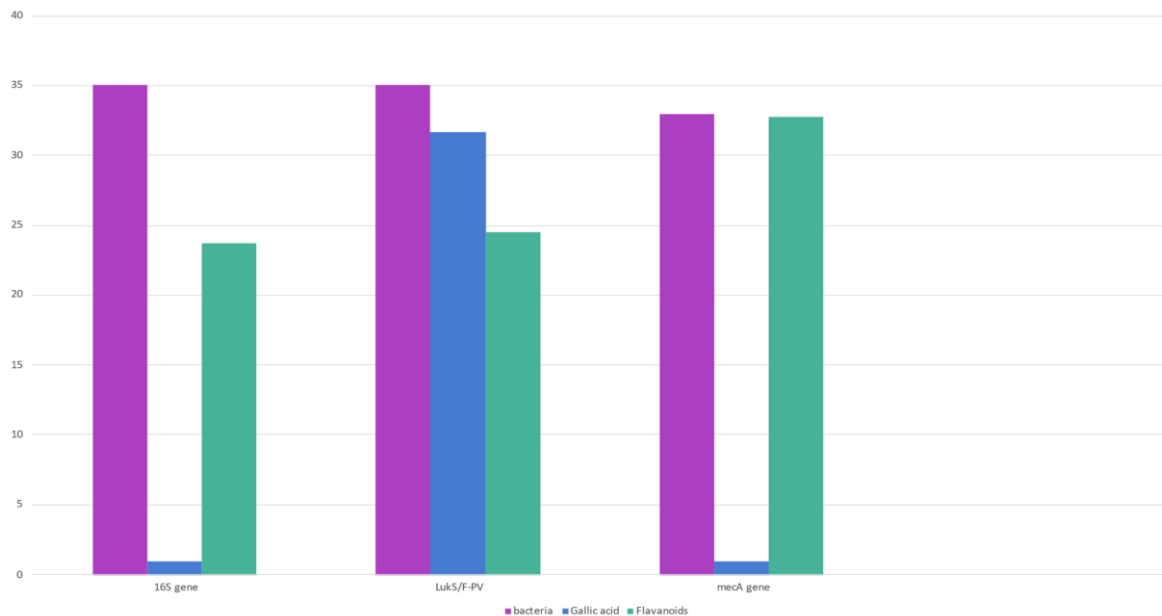


Figure 9: Gene expression of the positive control and after treatment with different metabolites.

HPLC analysis of the leaf extract, based on the chemical tests from ethyl acetate extract was done to confirm presence of 6 compounds; ferulic acid>caffeic acid>gallic acid>ellagic acid>kaempferol>quercetin, with absence of rutin, in descending order of concentration quotient.

	Content (%)	Content µg/g
Ferulic acid	0.0415	415.1
Caffeic acid	0.03	300.0
Gallic acid	0.0207	207.3
Ellagic acid	0.0108	108.1
Kaempferol	0.0043	43.4
Quercetin	0.0022	21.9
Rutin	Not detected	

Figure 10: Table for the concentration of compounds in the Ethyl acetate extract.

Materials

HPLC grade methanol (mobile phase)

0.1% formic acid (mobile phase)

Trifluoroacetic acid (mobile phase)

Ultra-pure water

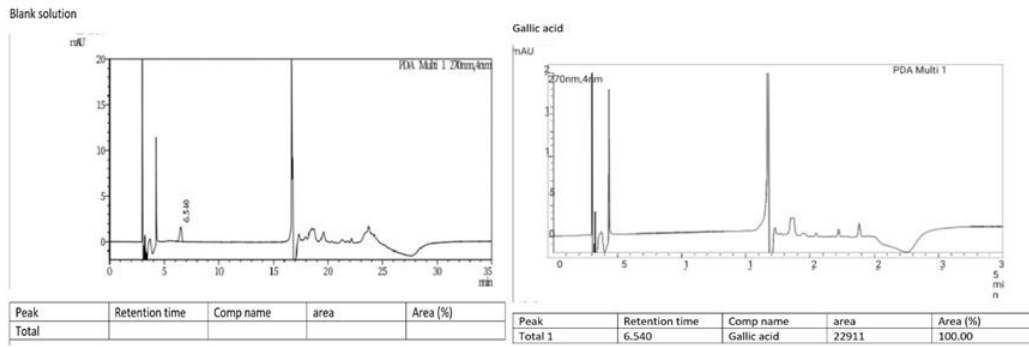
Pure gallic acid, caffeic acid, ferulic acid, ellagic acid, rutin, quercetin, kaempferol

Chromatographic detection

0.1% formic acid was used as the mobile phase A and acetonitrile as mobile phase B, with a constant flow rate of 1.0ml/min. The detection wavelength($\lambda=270\text{nm}$), column temp 30°C, injection volume 20µL.

The leaf extract was dissolved in methanol, subjected to ultrasonic dissolution, filtered by 0.45µM microporous membrane, filtrate 2.5mg/ml reserved. 0.5µg/ml of the control (gallic acid) was prepared using 5mg of gallic acid compound, dissolved in 25ml methanol, carbinol was added to 1ml of the solution, and 0.5µg/ml reference sample was made.

The content of each of the compounds was calculated from the peak area.



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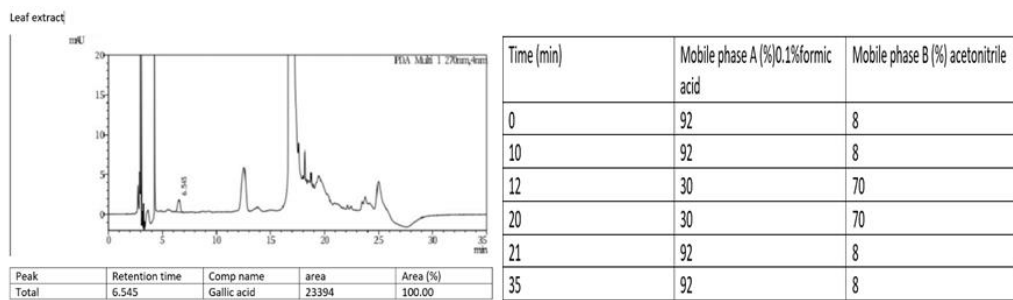
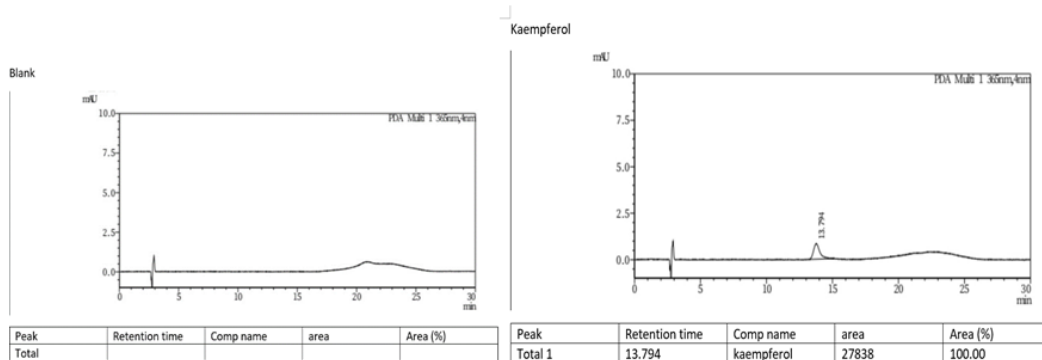


Figure 11: HPLC analysis for Gallic acid.

Kaempferol-75mg of the leaf extract was dissolved in methanol, subjected to ultrasonic dissolution, filtered by 0.45µm microporous membrane. A reference kaempferol 5mg sample was dissolved in methanol to make a 0.5µg/ml sample. 0.1% formic acid was used as the mobile phase A and methanol as mobile phase B. The flowrate was 1.0ml/min, detection λ=365nm, column temperature 30°C, injection volume 20µl. Concentration was calculated per peak area.



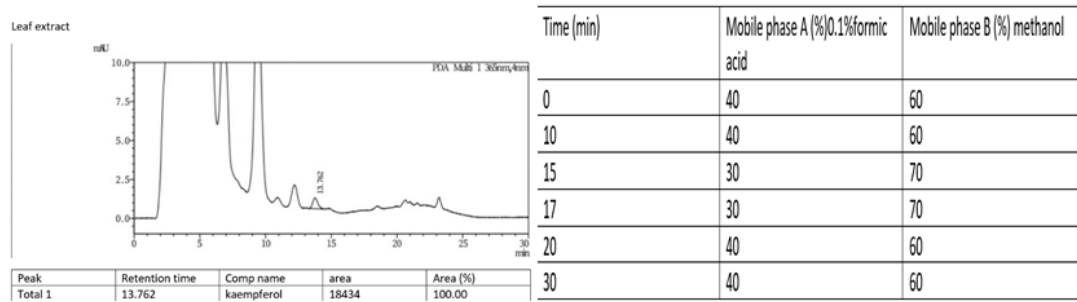


Figure 12: HPLC analysis for Kaempferol.

Quercetin-0.1% of trifluoroacetic acid and methanol 50:50 was used as the mobile phase, with a flow rate of 1.0ml/min, detection $\lambda=370\text{nm}$, column temperature 30°C , injection volume $20\mu\text{L}$.

200mg of leaf sample was dissolved with methanol via ultrasonic treatment in a 20ml flask and filtered with $0.45\mu\text{M}$ microporous membrane. 5mg of quercetin was dissolved in 25ml methanol. 1ml of the solution was topped up with more methanol to scale in a separate 200ml bottle to make up a control solution of $0.25\mu\text{g/ml}$.

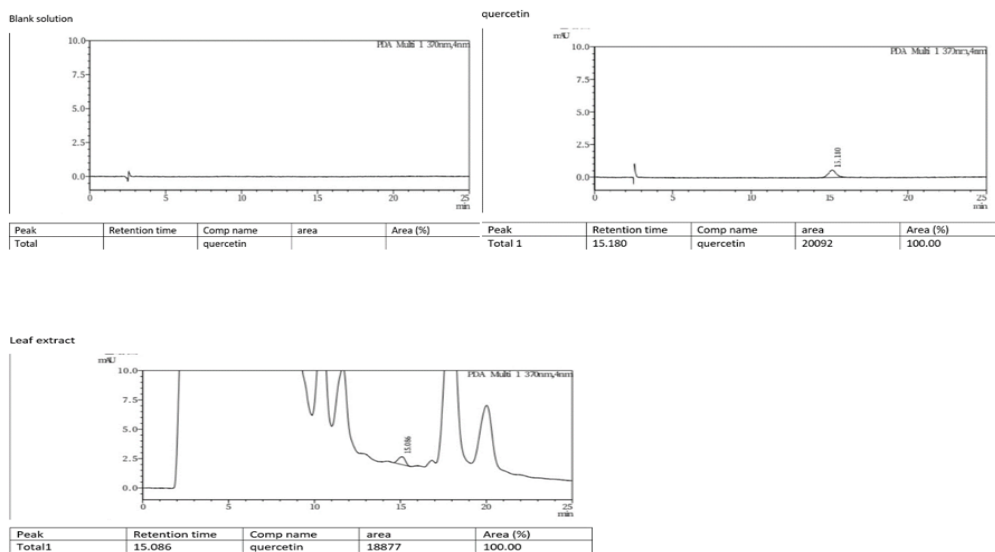


Figure 13: HPLC analysis for Quercetin.

Ellagic acid-0.1% formic acid was used as the mobile phase A, and methanol as mobile phase B. A constant flow rate of 1.0ml/min, detection $\lambda=254\text{nm}$, column temperature of 30°C , injection volume of $20\mu\text{L}$. 25mg of the leaf extract was dissolved using methanol in a 10ml flask, filtered by a $0.45\mu\text{m}$ microporous membrane. The control 5mg Ellagic acid was dissolved by methanol to scale in a 25ml flask, put in a 10ml flask and dissolved to scale, to a final concentration of $0.3\mu\text{g/ml}$.

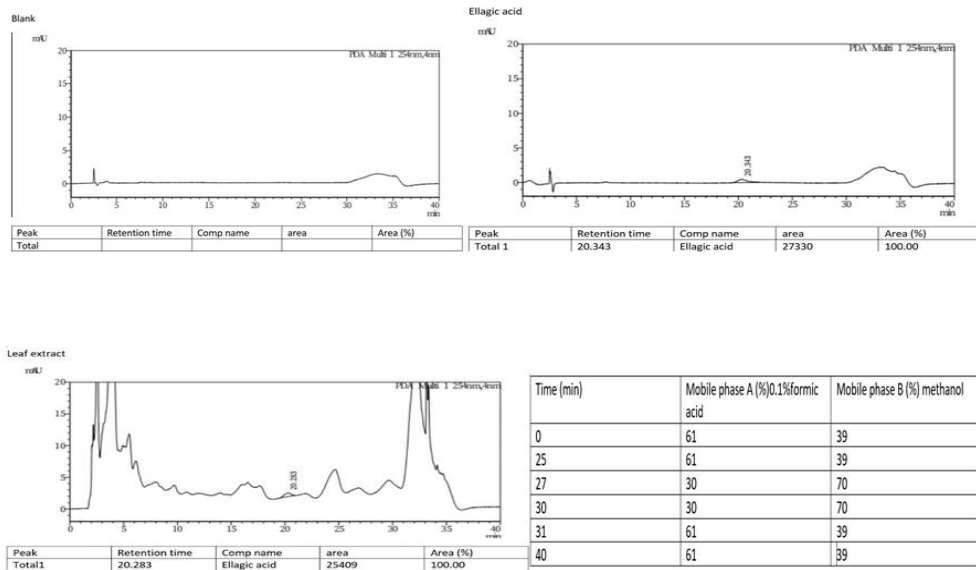


Figure 14: HPLC analysis for Ellagic acid.

Caffeic acid-0.1% aqueous trifluoroacetic acid was used as mobile phase A and methanol as mobile phase B, a flowrate of 1ml/min, detection $\lambda=323\text{nm}$, column temperature= 30°C , injection volume= $20\mu\text{L}$

10mg of the leaf extract was dissolved in methanol in a 10ml flask via ultrasonic treatment, filtered by a $0.45\mu\text{m}$ microporous membrane to make a filtrate with concentration 1mg/ml. 5mg of the control caffeic acid was diluted with methanol in a 25ml flask via ultrasound and diluted to scale. 0.3ml of the solution was dissolved in 10ml methanol to a final concentration of $0.3\mu\text{g/ml}$.

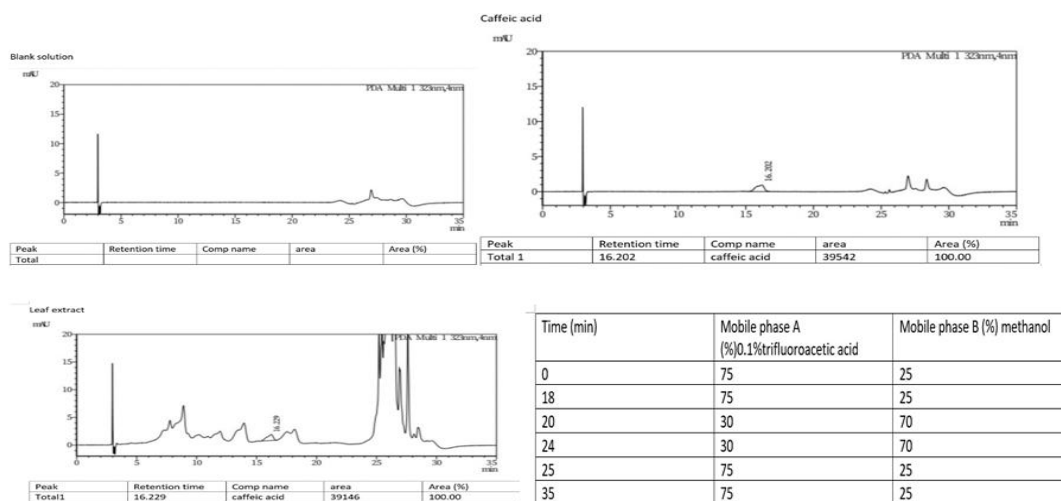


Figure 15: HPLC analysis for Caffeic acid.

Ferulic acid and rutin-0.1% aqueous trifluoroacetic acid was used as the mobile phase A and acetonitrile as mobile phase B. The flowrate was 1mm/min, detection $\lambda=254\text{nm}$, column temperature 30°C , injection

volume=20µL. 25mg of the leaf extract was dissolved in 10ml ethanol via ultrasonic treatment, filtered by a 0.45µm microporous membrane to give a filtrate with concentration 2.5mg/ml. Reference 5mg of ferulic acid and rutin was dissolved in 25ml methanol via ultrasound, 1ml of the solution was diluted with 20ml methanol to get 10µg/ml and then again 1ml of the latter solution to 10ml methanol to get a final concentration of 1µg/ml.

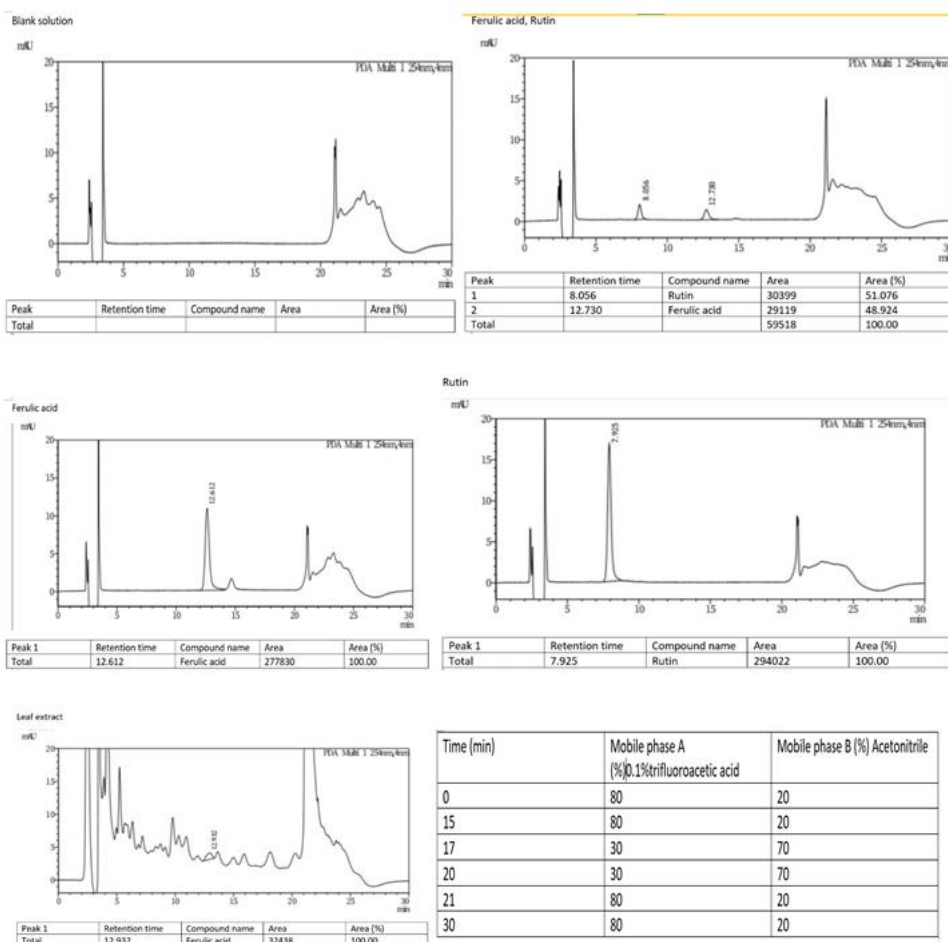


Figure 16: HPLC analysis for ferulic acid and rutin.

All six of the confirmed metabolites have been described to have antibiotic (and, or) antifungal properties, whether as pure compounds or combined with commercial drugs to increase antimicrobial activity. Gallic acid, could inhibit *S.aureus*, *E.coli*, *B.subtilis*[27], more active than caffeic and chlorogenic acid against inhibition of *S.aureus*[36], inhibit *S.aureus* strains when combined with pyrogallol[27], inhibit fungal sporulation[27]and had a synergic effect while combined with norfloxacin against *S.aureus*, and antifungal activity against both *C.albicans* and *C.tropicalis* while combined with fluconazole. Caffeic acid could inhibit *C.albicans* [11,40], *S.aureus* both MSSA and MRSA without being strain specific, as well as a synergic effect while combined with antibiotics to inhibit *S.epidermitis*, *B.cereus*, *M.luteus*, *L.monocytogenes*, *K.pneumoniae*, *S.marcescens*,

P.mirabilis, *E.coli*, *P.aeruginosa* and *C.albicans*[40]. Caffeic acid also inhibited MRSA more than gallic acid [48]. Caffeic acid was reported to inhibit *S.aureus* by inhibiting production of α -hemolysin[36], treatment of wound infections caused by *S.aureus*, *Klebsiella pneumoniae* and most active against *S.epidermitis*[42], a combination of caffeic acid and UV treatment to inhibit gram negative bacteria, which are resistant to UV treatment alone[41].Inhibition by caffeic acid esters towards *S.aureus*, with increasing alkyl chains[34], prevention of wilting in plants and inhibition of biofilm formation by *R.solanacearum*, activation of disease resistant enzyme and synthesis of lignin[51]. Caffeic acid was especially described to have antimicrobial, antifungal and antiviral activity, especially for *S.aureus* and many other bacterial and fungal species; *S.epidermitis*, *E.coli*, *P.aeruginosa*, *C.tropicalis*, *C.albicans*, *C.freundii*, *E.aerogens*, *E.cloacae*, *K.oxytoca*, *P.hauseri* and *P.mirabilis* [49]. Leaf extract of kaempferol by ethyl acetate concentrate had both antibiotic and antifungal activity against *S.aureus*, *P.aeruginosa*, *S.typhimurin*, *C.albicans*,*C.parapsilos* and *C.neoformans*[33], anticlumping action towards biofilms of *S.aureus*[47].Quercetin has contrasting reports on whether or not it's more effective on gram positive bacteria than on gram negative. It showed less activity towards gram positive bacteria, but had inhibitory effects on *E.coli* and *P.aeruginosa*[31],activity against *E.coli* too was confirmed but no activity towards *P.aeruginosa* and moderate activity to VRSA and *S.saprophyticus*[29], although anticlumping action to inhibit biofilm formation was reported[47]and on both MRSA,MSSA [32], and inhibition of *S.aureus* and *P.aeruginosa*, without inhibition of beneficial bacteria; *S.flexneri* and *L.casei* var shirota at 500mg/ml(Renu Narendra). Difluoroquercetin was strain specific in antibiofilm formation in *S.aureus* and resistant to gram negative bacteria[43],and stronger activity on gram positive bacteria than on gram negative in chicken cecum, although it inhibited both types; *P.aeruginosa*, *E.coli*, *S.aureus* and *S.typhimurin*. This was attributed to cell wall damage, inhibition of ATP in gram positive bacteria [30] and inhibition of β lactamases in *S.epidermitis* while used with amoxicillin[45].Ellagic acid has been reported to have antibiofilm activity against *S.aureus*[47], inhibit MRSA from pomegranate rind extract[38], various strains of *H Pylori* both in vitro and in vivo for gastric tissues, [35] inhibition of oral bacteria, with high specificity to insoluble *Streptococcus mutans* by inhibiting the hydrophobic glucan production[39] and inhibition of adherence and biofilm formation of *Streptococcus agalactiae*[44]. Activity of ferric acid by reduction of Fe^{3+} to Fe^{2+} ions was reported to have antibiotic properties. Bacterial absorption of Fe^{3+} ions leading to reduction into Fe^{2+} could inhibit bacteria, however further oxidation by a superoxide molecule could lead to formation of an $OH\cdot$ radical, which has unselective sterilizing property [37]. Ferric acid was also used in treatment of *P.aeruginosa*, in cephalosporin antibiotics[53], and in nanoparticle form, which is cheaper than silver nanoparticles [52], although less effective at low concentrations but more sensitive to *E.coli* than *S.typhi* [28],green synthesis of ferric acid from plants also showed activity against *E.coli* but more effective against *S.aureus*[54]

4. Discussion of results

Although prior experiments done proved diarrhea relief from water extracts of the plant in mice injected with *Salmonella typhimurium*, the extracts was more sensitive to *S.aureus* at smaller doses as compared to the latter. Medical significance of antibiotic effects by a given compound should be within small dosages, however the claims to relief diarrhea cannot be ignored and can be attributed to presence of phyto-compounds found in the plant. More research on the inflammatory cytokines involved in the treatment using various bacteria would express the medicinal efficacy and specificity in treatment therapy. Presence of flavonoids, namely quercetin

and kaempferol was proved to be in sync with the research done by Guizhou medical university [56], however this doesn't clarify whether or not other flavonoids may be present in the extract and could have better antibacterial effects. Other compounds such as the phenolic acids; gallic acid, ellagic acid, caffeic acid and ferulic acid were also present and using different extraction procedures showed better activity at lower dosages (gallic and ellagic acid extract) as compared to total flavonoids. This however does not refute research showing that flavonoids have good antibacterial effects, as the total flavonoids extract also showed better activity towards Luk S/F panton valentine gene (figure 9) as compared to the gallic acid extract. Different extraction methods, different compound concentrations owing to intra-species variants and environmental factors may greatly affect the composition and amounts of metabolites extracted globally. Storage of extracts is still questionable on the general antibiotic effects and compounds stability due to possible loss of activity with storage.

5. Conclusion

The ethylacetate concentrate of the ethanolic leaf extract of *Rubus multibreatus* was effective at inhibiting *S.aureus* more than *E.coli* and *S.typhi*. This was attributed to presence of flavonoids, and phenolic acids in ellagitannins. Presence of gallic acid, kaempferol, quercetin, ellagic acid, caffeic and ferulic acid could be attested to the antibiotic effects. The non-toxic nature of the plant as used in making teas and traditional remedies for various ailments could be an important marker in development of less toxic antibiotics, combating antibiotic resistance in new drug development projects, and incorporation of natural medicine in the general diet to enhance disease resistance. In-vivo experiments are ongoing as well as further experiments on the pure compounds to determine their antibiotic effects.

6. Limitations of the experiments

- Drug solubility of the leaf extracts was the major constraint as DMSO could interfere with bacterial inhibition thus giving misleading results. The amount of DMSO used $\leq 2\%$ of the broth solution and the highest calculated amount of DMSO present in the highest treatment group must always be added to the positive control.
- Plants have a very wide variety of compounds, proteins and lignin, making pure compound extraction difficult. Repetition and huge amounts of extracts is required. This could result in environmental degradation and the plant could ultimately die as a result of harvesting a lot of samples.
- During sample collection, it is mandatory to have a botanist present to avoid picking from the wrong plant, which would undoubtedly result in misleading results. This specific plant has various close species and the pure wild plant not subjected to genetic modification is preferred for uniformity in research.
- Plant samples should be collected during early spring only, when metabolites are highly concentrated and stored in dry sanitary conditions to avoid contamination especially by fungus.
- Information on traditional medicine is very challenging to acquire, as most of the research is guarded by traditional healers and is hardly published. Most medical journals on the same are also not available in English and since the plant is available across the globe, it can be assumed that many indigenous tribes continue to use it to treat disease, but this research never gets published.

7. Conflict of interest

There was no conflict of interest in this study.

8. Funding/supervision

This work was supervised by Prof He Changliang and done in collaboration with Shimadzu Lab Solutions.

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