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Phytochemical Analysis and Antibacterial Potential of *Stevia rebaudiana* (Bertoni, 1899) Leaf Extracts against *Aeromonas* Species: Influence of Extraction Methods and Solvents in Aquaculture Applications

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Abstract

Recent studies have explored *Stevia rebaudiana* Bertoni leaf extracts for their antibacterial potential and phytochemical content. However, the impact of extraction methods and solvents on aquaculture bacteria remains understudied. This research aimed to evaluate the antibacterial, radical scavenging, and phytochemical properties of *S. rebaudiana* extracts against *Aeromonas* species. Dried *S. rebaudiana* leaves were extracted using methanol (Mt) and ethanol (Et) through Soxhlet and maceration methods (SMt, SEt, MMt and MEt respectively). Soxhlet extraction yielded higher amounts (36.29% for Mt, 23.87% for Et) compared to maceration. Phytochemical analysis identified phenolics, flavonoids, alkaloids, saponin, tannin, and steroids in all extracts. Notably, MEt had elevated phenolic and flavonoid content, while SEt contained more tannins. MEt exhibited the strongest antioxidant activity (IC_{50} = 67.95µg/mL), aligning with its high phenolic and flavonoid levels. In antibacterial assays against *Aeromonas* strains, ethanol extract showed the largest zone of inhibition (ZOI) of 16.67mm for *A. salmonicida*, followed by methanol extract (15mm) at 250 mg/mL, using maceration and Soxhlet methods, respectively. However, none of the extracts displayed activity against *A. hydrophila*. This suggests that cold maceration is a cost-effective method that preserves heat-sensitive secondary metabolites within a shorter extraction time. In conclusion, this study highlights the significance of extraction techniques and solvents in obtaining potent antibacterial and antioxidant extracts from *S. rebaudiana* leaves. The findings emphasize the potential of these extracts in aquaculture practices and open avenues for further research in utilizing natural compounds for sustainable aquaculture strategies.

Keywords: *Stevia rebaudiana*, *Aeromonas,* Antioxidant, Anti-inflammatory, Secondary Metabolites, Maceration

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INTRODUCTION

Medicinal plants are an unavoidable component of human well-being. They produce a vast range of secondary metabolites, of which around 50,000 have been examined and their structures determined.[1] The plant's secondary metabolites have been used for ages to treat ailments, build immunity against a variety of bacterial and viral diseases and improve digestion.[2-5] The efficacy of these plant extracts, however, is dependent on the phytochemical composition, plant matrix, and operator expertise.[6] The extraction procedure is a vital stage in the process of identifying secondary metabolites from plant parts. The research has already shown that the biological activity of extracts obtained by different procedures might differ greatly, emphasizing the need to select the appropriate extraction technique.[7] Both conventional and unconventional extraction methods can be used to isolate active compounds from plants. The unconventional techniques include supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction, ultrasonic-assisted extraction etc. Most of the above-mentioned extraction methods require high temperatures. Despite the fact that higher temperatures encourage the release of more secondary metabolites, a possibility exists for the degradation of thermally unstable compounds.[8] However, most industries and laboratories are still using conventional methods like Soxhlet and cold maceration due to low cost, lower solvent consumption, low energy consumption and simple procedure.[9]

Researchers frequently analyse several extraction methods in terms of efficiency, yield, and cost-effectiveness to select the best methodology for their specific needs. Soxhlet extraction has been used for many years, although it is highly labour-intensive and requires significantly more solvents than the maceration approach.[10] The fundamental advantage of cold maceration, on the other hand, is that it helps maintain the volatile and thermolabile chemicals found in the original plant material.[11] Plant secondary metabolites are important in a variety of sectors, including aquaculture.[12] Because of the possible consequences for animal and human health, as well as the environment, the use of antimicrobials in aquaculture is a source of worry and continuous discussion. One of the major concerns associated with the use of antimicrobials in aquaculture is the potential development of antimicrobial resistance (AMR) bacteria that leads to treatment failures.[13] These AMR bacteria can spread through the environment, posing risks to other aquatic organisms and potentially impacting human health if transferred through the food chain. Disease prevention and mitigation are essential in aquaculture to ensure the health and welfare of the farmed species and the sustainability of the industry. It is proven that plant-based products can stimulate the fish immune system in a specific or nonspecific way.[14-17] There are numerous ways that medicinal plants have been used, such as a crude substance, an extract, or an active component. For fish farmers in particular, using crude plants has the advantage of requiring little effort to apply; however, there was occasionally a lesser impact than when the extract was utilised.[18]

Stevia rebaudiana is a herbaceous short-day plant of the Asteraceae family. It is the only species that belongs to genera *Stevia*, having sweetening, stevioside compound, found distributed in leaf, stem and flower and was considered as a sugar alternative in the early 1970s by Japanese people.[19,20] The major glycosides present in the plant are stevioside, rebaudioside A, rebaudioside C and dulcoside A with an order of sweetness of 210, 242, 30 and 30 times more than sucrose and the chemical composition varies according to cultivar and geographical area.[21,22] *Stevia* has gained popularity as a natural alternative to artificial sweeteners due to its therapeutic values like antioxidant, anticancerous, anti-inflammatory, antihyperglycemic and antimicrobial properties.[23-25] Recent research confirmed that the addition of stevioside could enhance liver antioxidant activity, immune functions and growth performance in juvenile mirror carp.[26] It has a promising natural remedy to avail disease management in the aquaculture field, as it is facing issues due to the use of antibiotics.

A scientific comparative study determining the superiority of one extraction method over another for extraction would be extremely beneficial to researchers and enterprises interested in *Stevia* processing and applications.

Such a study would shed light on the efficiency, yield, and quality of extracts obtained using various procedures. Thus, the primary goal of the study was to carry out preliminary phytochemical screening, investigate antioxidant activity, and assess the in vitro antibacterial activity of *S. rebaudiana* extracts produced by two different extraction methods, Soxhlet and maceration, against selected aquaculture pathogens.

MATERIALS AND METHODS

Plant material

Fresh and mature *Stevia rebaudiana* leaves were obtained from 'Stevia World Agrotech' in Bangalore, India, and identified by professionals from the Central Ayurveda Research Institute in Uttarahalli, Bangalore, India (Authentication No. SMPU/CARI/BNG/2021-22/2207). After collection, the leaves were cleaned using distilled water and dried for four days in the shade at room temperature before being milled into powder. For the current investigation, leaf powders were always made fresh.

Preparation of leaf extracts

For the present study, two different extraction methods, Maceration (cold extraction) and Soxhlet (hot extraction) were adopted using ethanol and methanol as solvents separately, to ensure the suitable method and solvents to assess. Thus, four extracts were prepared, ethanolic extract of Soxhlet (SEt) & Maceration (MEt) and methanolic extract of Soxhlet (SMt) & Maceration (MMt) and were used to screen against selected aquaculture pathogens.

Extraction by maceration

Approximately 20g of powdered dry leaf (as described above) was soaked in 100 mL methanol and ethanol separately in an enclosed conical flask for 72 hours. Then it was filtered through Whatman filter paper 1. Then the solvent was evaporated by a rotary vacuum evaporator at 60°C.[27]

Extraction by soxhlet

About 20 grams of the powdered leaf was placed in a thimble holder and about 200mL of the respective extraction solvent (methanol and ethanol) was filled in a round bottom flask. A rotary vacuum evaporator was used to evaporate and concentrate the solvent at 60°C.[28] The leftover concentrated residue from both extraction methods was placed in Petri dishes and let the solvent completely evaporate. The crude extracts were then stored at 4°C.

Screening of phytochemicals (qualitative)

All four extractions (SEt, MEt, SMt & MMt) methods were tested for the presence of bioactive compounds such as phenolics, flavonoids, alkaloids, saponin, steroids, anthraquinone, tannin, carbohydrates and protein using the standard protocol of Harborne (1998) with minor modification.[29]

Phytochemical analysis (Quantitative)

Determination of total phenolic content

The total phenolic content of the four extracts (SEt, MEt, SMt, and MMt) was evaluated using the Folin-Ciocalteu colourimetric method with gallic acid as a standard phenolic component.[30] The quantification was conducted using a calibration curve ($y = 0.0012x + 0.0013$, $R^2 = 0.997$) using various concentrations of gallic acid solutions such as 0, 100, 200, 300, 400 and 500 µg/mL and 1 mg/mL concentrations of all extracts were prepared. To 40 µl of each extract, 400 µl of Folin–Ciocalteu reagent (FCR) (10%)and 360 µl of Na_2CO_3(7%) were added for a total volume of 800 µl. The blue-coloured reaction mixture was thoroughly agitated and incubated for 30 minutes. The FCR reagent oxidises phenols in sample solutions, resulting in a dark blue colour, that is quantified using a UV-visible spectrophotometer at 760 nm against a reagent blank. The samples were prepared in triplicate for each analysis. The total phenolic content of the extracts was expressed in milligrams of gallic acid equivalents (GAE) per gram of dry weight of the sample (mg/g).

Determination of total flavonoid content

The total flavonoid content of all four extracts was measured using an aluminium chloride colourimetric assay with quercetin as a reference flavonoid component.[31] The quantification was conducted using a calibration curve ($y = 0.0081x + 0.0124$, $R^2 = 0.998$) using various concentrations of quercetin (0, 100,

200, 300, 400 and 500µg/mL) and 1 mg/mL concentrations of all extracts was prepared. Briefly, to 100 µl of each extract, 100 µl $AlCl_3$ (10%) and 100 µl of Na_2CO_3(1M) were added. The reaction mixture was thoroughly agitated and incubated for 45 minutes. Then, the absorbance was measured using a UV-visible spectrophotometer at 760 nm against blank. All the experiments were carried out in triplicates. The amount of flavonoids in the extracts was expressed as milligrams of quercetin equivalents (QE) per gram of sample dry weight (mg/g).

Determination of tannin content

Total tannin contents in ethanol and methanol extract of *S. rebaudiana* leaf prepared through the Soxhlet and maceration method were determined by colourimetric assay using tannic acid as a standard tannin compound.[32] The quantification was carried out by means of the calibration curve (y = 0.0017x + 0.0022, R^2 = 0.998) using various concentrations of tannic acid(0, 100, 200, 300, 400 and 500µg/mL) and 1 mg/mL concentration of all extracts were prepared. To about 1000 µl of each extract, 1000 µl Folin–Ciocalteu reagent (FCR) (10%) and 1000 µl of Na_2CO_3(35%) were added. The reaction mixture was thoroughly agitated and incubated for 30 minutes. Then, the absorbance was measured using a UV-visible spectrophotometer at 700 nm against blank. All the experiments were carried out in triplicates. The amount of tannin in the extracts was expressed as milligrams (mg) of tannic acid equivalents (TAE) per gram of sample dry weight (mg/g).

The GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on different extracts of *S. rebaudiana* leaves using GC-MSQP2010 SE SHIMADZU model. Spectroscopic detection by GC–MS involved a pressure of 76.2KPa Helium gas (99.995% purity) was employed as the carrier gas, with a flow rate of 4.3 mL/min. A very small amount (1 µL) of a diluted extract (prepared at 1% concentration) was introduced into the GC-MS system using the splitless injection technique (280°C). The compounds present in the sample were identified by comparing the acquired mass spectra to a mass spectral library provided by the National Institute of Standards and Technology (NIST).

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The free radicle scavenging activity of all four extracts of *S. rebaudiana* was assessed in terms of hydrogen donating ability using DPPH radicle. Ascorbic acid was used as a standard reference compound (All chemicals were analytical grade and procured from VASA Scientific, Bangalore). The assay was carried out according to the method of Sanna *et al.*[33] The radical scavenging activity was determined using the calibration curve (y = 2.6938x + 0.196, R^2 = 0.998) using various concentrations of ascorbic acid (0, 2,4, 6, 8, 10 and 12µg/mL). All four samples were prepared in methanol at different concentrations (0, 20, 40, 60, 80, 100, 120 µg/mL). To about 1000 µl of each extract, 3 mL of 0.1mM DPPH solution was added. The tubes containing the reaction mixture were thoroughly agitated and incubated for 30 minutes in the dark. The absorbance at 520 nm was measured using a UV-VIS spectrophotometer against blank. All of the experiments have been performed in triplicate. The percentage inhibition activity at different concentrations was determined and half maximal inhibitory concentration (IC_{50}) of extracts was calculated from the graph and compared with the standard.

Antibacterial assay

Two bacterial pathogens, *Aeromonas hydrophila* (MCC2052 1) and *Aeromonas salmonicida* (MCC2318 1) were selected for the present study as these are common fish pathogens. These isolates were obtained from the National Centre for Microbial Resource, Pune, India. The reference strains were cultured on nutrient agar slants, subcultured regularly and stored at -20°C. Nutrient agar (HiMedia) was used as media for subculture and antibacterial assay. The antimicrobial screening was performed in a laminar hood to avoid contamination by the test organisms. Neomycin (µg/disc) standard disc and 50µL of DMSO were used as the reference (positive and negative control, respectively). The agar well diffusion method was used to assess antibacterial activity.[34] Inoculums of test organisms (10^6 CFU/

mL) were spread on nutrient agar plates. Wells (6 mm diameter) were punched on the agar plates with sterile cork borer and filled with 50µl of various concentrations (50,100, 150, 200 and 250 mg/mL) of each extract. The plate was maintained at room temperature for 1 hour to allow for diffusion before being incubated at 30°C for 24 hours. Antimicrobial activity was determined by measuring the zone of inhibition against the test organisms. The growth was compared with both the positive and negative control. All experiments were conducted in triplicate.

Statistical analysis

The data were expressed as mean and standard deviation. The computational analysis was done by IBM SPSS software. The statistical significance was evaluated by One-way analysis of variance (ANOVA) followed by a post hoc test such as Duncan's test ($P<0.05$ was considered statistically significant).

RESULTS AND DISCUSSION

Percentage yield of extraction

The kind of extraction techniques/ solvents used and their extraction ability had a direct impact on the extraction yield.[35] The extraction efficiency is linked to the solvent volume, extraction time, as well as the impact on the environment and people. Therefore, selecting an effective and environmentally friendly extraction technique or solvent for the separation of phytochemicals from plant materials is crucial.[36] The percentage yield of extraction is given in Table 1. The findings showed that the percentage extractive yield was significantly influenced by the solvents and technique followed. The results proved that the soxhlet extraction was the most effective method resulting in an extraction yield of 36.29% and 23.87% for methanol and ethanol, respectively, compared to maceration. According to the results, soxhlet seems to be the suitable method of extraction of *S. rebaudiana* leaves. However, it should be noted that the high temperature used in the soxhlet method reduces the viscosity and surface tension of solvents, thereby allowing the solvents to penetrate deeply into the plant material and also permitting the co-extraction of the fibres. These add to the dry extract weight. The exposure of plant material to several rinses of warm solvent may also be the cause of the maximum extraction.[37] According to the results, methanol solvent showed better yield when compared to ethanol in both methods. Methanol is more polar than ethanol, hence favours more extraction yield. Truong *et al.*[38] also showed similar results to the present study. *Severinia buxifoliua* extraction yield was maximum when methanol was used as the extraction solvent.[38] This might be a result of the high concentrations of polar phytochemicals found in plant material, which are soluble in highly polar solvents like methanol and ethanol.

Table 1. Yield of extraction of *S. rebaudiana*

Method of extraction	Yield (%)
Methanol maceration (MMt)	8.43 ± 0.58
Ethanol maceration (MEt)	7.13 ± 0.09
Methanol Soxhlet (SMt)	36.29 ± 0.38
Ethanol Soxhlet (SEt)	23.87 ± 0.72

Phytochemical screening (qualitative)

The phytochemical composition analysis (qualitative) of *S.rebaudiana* leaf extracts showed the presence of phenolic compounds, saponins, flavonoids, steroids, carbohydrates, alkaloids, proteins and tannins as summarized in Table 2. While anthraquinones were absent in all extracts. The phenolic and flavonoids have proven to have antibacterial and antioxidant activity.[39] So that their presence in all extracts could be responsible for the respective biological activities. Even though the different extraction methods can influence the potency of extracts.

Phytochemical analysis (quantitative)

Phenolic compounds are known to possess antioxidant, antimicrobial and anti-inflammatory properties.[40-42] According to the present study, all extracts had a high phenolic content that ranged from 45.25 to 61.69 mg GAE/g dry extract (Table 3). Plant extracts abundant in phenolic compounds or individual isolates can serve as natural additives for food preservation. Their incorporation can extend the longevity of edibles by suppressing the proliferation of spoilage microorganisms and slowing oxidative processes.[43] Thus, it can be an indication that *Stevia* is being

Table 2. Phytochemical screening of *S.rebaudiana* leaf extracts

Phytochemical component	Extraction type				Test followed
	MEt	MMt	SEt	SMt	
Phenolic compounds	+	+	+	+	Ferric chloride test
Alkaloid	+	+	+	+	Draganodorff's Test
Saponin	+	+	+	+	Froth test
Flavonoids	+	+	+	+	Lead acetate test
Tannin	+	+	+	+	Ferric chloride test
Steroid	+	+	+	+	Salkowski test
Anthraquinone	–	–	–	–	Borntrager's test
Carbohydrate	+	+	+	+	Molisch's test
Protein	+	+	+	+	Million's test

Table 3. Quantification of phytochemicals from different extracts of *S. rebaudiana*

Extracts	Phytochemical Parameters (mg/g)				
	Total phenolic content	Total flavonoid content	Tannin content	Carbohydrate	Protein
MEt	61.69±1.53^{a}	22.62±0.28^{a}	10.90±0.81^{a}	107.07±0.43^{b}	204.99±1.67^{a}
MMt	54.42±1.50^{b}	14.55±0.07^{c}	6.58 ±0.52^{b}	111.07±0.86^{a}	193.33±1.67^{b}
SEt	45.25±1.49^{c}	12.45±0.17^{d}	5.41±0.53^{bc}	110.61± 0.93^{a}	175.55±1.67^{c}
SMt	52.80±1.25^{b}	18.71±0.11^{b}	5.02±0.30^{c}	100.08 ± 0.30^{c}	171.48±1.92^{d}

Mean values in the same column with different alphabets were significantly different (p<0.05)

used in the food industry. The findings showed that MEt had the highest phenolic concentration of 61.69±1.52 mg GAE/g followed by MMt, 54.42±1.5 mg GAE/g, SMt, 52.80±1.25 mg GAE/g and SEt, 45.25±1.49 mg GAE/g; having a notable difference between them ($p < 0.05$) (Table 3). The total phenolic concentration could serve as a basis for a screening of antioxidant activity since their hydroxyl groups facilitate their ability to scavenge free radicals.[44] Similar results obtained from the studies conducted by Shukla *et al.*[45] have shown that the ethanolic leaf extract of *S. rebaudiana* possesses a phenolic content of 61.50 mg GAE/g. The findings suggest that phenolic compounds from *Stevia* can be readily extracted using ethanol and can be used to serve as a basis for a screening of antioxidant activity since their hydroxyl groups facilitate their ability to scavenge free radicals.

Flavonoids are one of the most studied groups of plant phenol with notable medicinal action. In the present study, all extracts had a high flavonoid content that ranged from 13.12 to 22.52 mg QE/g of dry extract (Table 3). The findings showed that MEt had the highest flavonoid concentration of 22.62±0.28 mg GAE/g followed by SMt (18.71±0.11 mg QE /g), MMt (14.55±0.07 mg QE /g) and SEt (12.45±0.17mg QE/g); with a notable difference between them ($p < 0.05$). The findings from this current investigation are in accordance with Zaidan *et al.*,[46] who showed that the leaf ethanol extract of *S. rebaudiana* has the highest flavonoid content of TFC of 10.91 mg QE/g.[46] Some flavonoids, such as flavones and flavonols, are also employed in cosmetic formulation because of their anti-ageing and anti-inflammatory characteristics.[47,48] Quercetin, protocatechuic acid and ferulic acid is the most abundant flavonoid in *Stevia* extracts.[49] Quercetin is found to have biological activities like apoptosis induction, inhibiting angiogenesis and modifying cell cycles.[50] This is evidence of the pharmacological importance of *Stevia.*

In the present investigation, all extracts had a high tannin content that ranged from 5.48 to 11.25 mg GAE/g of dry extract (Table 3). The findings showed that MEt had the highest tannin concentration of 10.90±0.81 mg GAE/g followed by MMt (6.58±0.52 mg GAE/g), SEt (5.41±0.53

Table 4. Compounds identified in the leaf extracts of *S. rebaudiana* and their significant biological activities

Class	Compounds	Rt (min)	Reported biological activities	Reference
Alkene	1-Dodecene	9.31		
	2-Dodecene	9.31		
	1-tetradecene	9.31		
	1-Heptadecene	13.90		
	1-Pentadecen	13.90		
	1-Tridecene	13.90		
	2-Phytene	16.57	Antimicrobial, antioxidant and cytotoxic	Larayetan et al.[60]; Hasan et al.[61]
	1-Octadecene	16.00		
Fatty alcohol	1-Dodecanol	9.31	Antibacterial	Zhang et al.[62]
	1-Decanol, 2-hexyl	20.60		
Alkane	Tridecane	9.41		
	Undecane	9.41		
	Hexadecane	9.41		
	Dodecane, 4,6-dimethyl	10.34		
	Dodecane, 3,7-dimethyl	10.34		
	Tetradecane	10.34	Antimicrobial	Nasr et al.[63]
	Undecane, 3,8-dimethyl	10.34		
	Undecane, 3,7-dimethyl	10.91		
	Octadecane	12.78		
	Heptadecane	12.78		
	1-Octadecyne	16.57		
	Nonadecane	15.38	Antioxidant	Kazemi M.[64]
	Tetratetracontane	21.17		
	Tetracosane	21.17		
	Octocosane	21.17		
	Docosane	21.17		
	Heneicosane	15.38	Antioxidant Antimicrobial	Vanitha et al.[65]; Mohammadi et al.[66]
Aromatic carboxylic acid	Benzoic acid	14.64	Antimicrobial Antioxidant	Park et al.[67] Velika, Kron[68]
Ester	Dibutyl phthalate	18.04	Antibacterial	Bi et al.[69]
Carboxylic acid	Phthalic acid	18.04		
Fatty acids	Pentadecaflurooctanoic acid	20.60		
	Eicosanoic acid	18.26		
	Docosanoic acid	18.26		
	n-hexadecanoic acid	18.26	Antibacterial Antioxidant, nematicide	Abubakar, Majinda[70]
Terpenoids	Neophytadiene	16.57	Antibacterial, anti-inflammatory	Bhardwaj et al.[71]

mg GAE/g) and SMt (5.02±0.30mg GAE/g); with considerable difference between them ($p < 0.05$). The highest yield of tannin in MEt could be due to the release of additional hydrogen bonds between tannins and protein as a result of prolonged exposure of the sample to solvent by maceration.[51] Similar findings reported by Farahmandfar *et al.*[52] in *Arum maculatum* leaf extract, showed the highest tannin (5.33mg/g) obtained by the maceration method using ethanol: water(1:1) as solvent. Due to the fact that tannin is a polyphenol component, polar solvents can

easily extract it. The findings of Naima *et al*.[53] witnessed that ethanol is the best solvent to extract condensed tannins from *Acacia mollissima* barks with an extraction yield of 18.50±0.06 Cya/g.

The results of the quantitative analysis showed that maceration is an effective method for extracting secondary metabolites like phenolics, flavonoids and tannin. The soaking of leaf powder for an extended period allowed the above-mentioned polar compounds to dissolve in alcohol. However, in Soxhlet, the continuous refluxing of solvents over the samples may lead to the degradation of some heat-sensitive secondary metabolites and lower their composition in it.[54] As per the current study, ethanol was able to extract phytochemicals in terms of quantity. The possible reason is that it is more polar than methanol, making them for strong hydrogen bonds and dipole-dipole interaction with polar compounds in plants. Also, the fact that ethanol is safer for extraction without causing a higher risk to human health.[55] The findings indicated that the maceration method is the simple and low-cost way and ethanol is the best solvent to extract secondary metabolites such as phenolics, flavonoids and tannin from *S. rebaudiana* leaf.

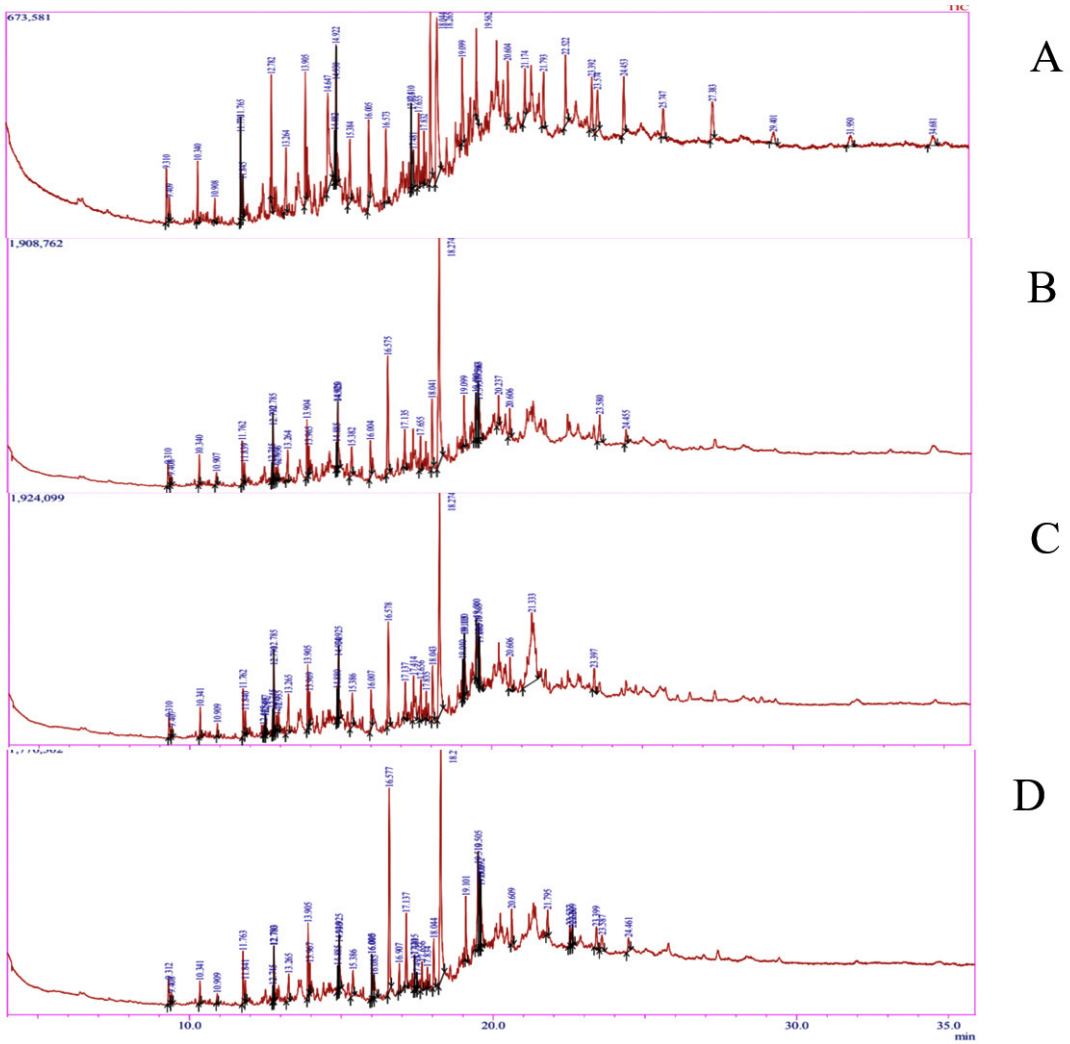

Figure 1. GC-MS chromatogram of leaf extracts of *S. rebaudiana*. (A-SEt; B-SMt; C-MMt; D-MEt)

GC-MS analysis of *S. rebaudiana*

GC-MS analysis of four different extracts of *S. rebaudiana* showed that the chemical constituents belong to different classes like alkane, alkene, fatty alcohol, fatty acids and carboxylic acid. The chromatogram is depicted in Figure 1. A total of 34 similar compounds were identified from all four extracts, which are listed in Table 4. The abundant compound from all extracts was n-hexadecanoic acid. The antibacterial and antioxidant of n-Hexadecanoic acid have been reported in many research works.[56] Neophytadiene, a diterpenoid was also identified from all extracts with various quantities. The highest amount was obtained from MEt with a relative percentage composition of 12%, followed by SMt (8.94%). Previous research has proven the antibacterial and anti-inflammatory

Table 5. Antioxidant activity of different extracts of *S. rebaudiana*

Concen. (µg/mL)	MEt	MMt	SEt	SMt
0	0.00±0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}
20	14.83 ± 0.00^{f}	10.86 ± 0.06^{f}	10.94 ± 0.00^{f}	4.62 ± 0.18^{f}
40	29.21 ± 0.00^{e}	20.04 ± 0.06^{e}	22.04 ± 0.06^{e}	18.81 ± 0.16^{e}
60	45.92 ± 0.06^{d}	33.60 ± 0.10^{d}	32.18 ± 0.06^{d}	27.92 ± 0.14^{d}
80	60.56 ± 0.00^{c}	43.30 ± 0.23^{c}	44.99 ± 0.06^{c}	40.66 ± 0.11^{c}
100	71.68 ± 0.00^{b}	54.27 ± 0.00^{b}	62.90 ± 0.00^{b}	51.88 ± 0.09^{b}
120	87.34 ± 0.00^{a}	61.54 ± 0.06^{a}	69.72 ± 0.06^{a}	66.41 ± 0.09^{a}
IC_{50}	67.95	94.31	75.39	95.40
IC_{50} standard (Ascorbic acid)	18.49			

Mean values in the same column with different alphabets were significantly different ($p<0.05$)

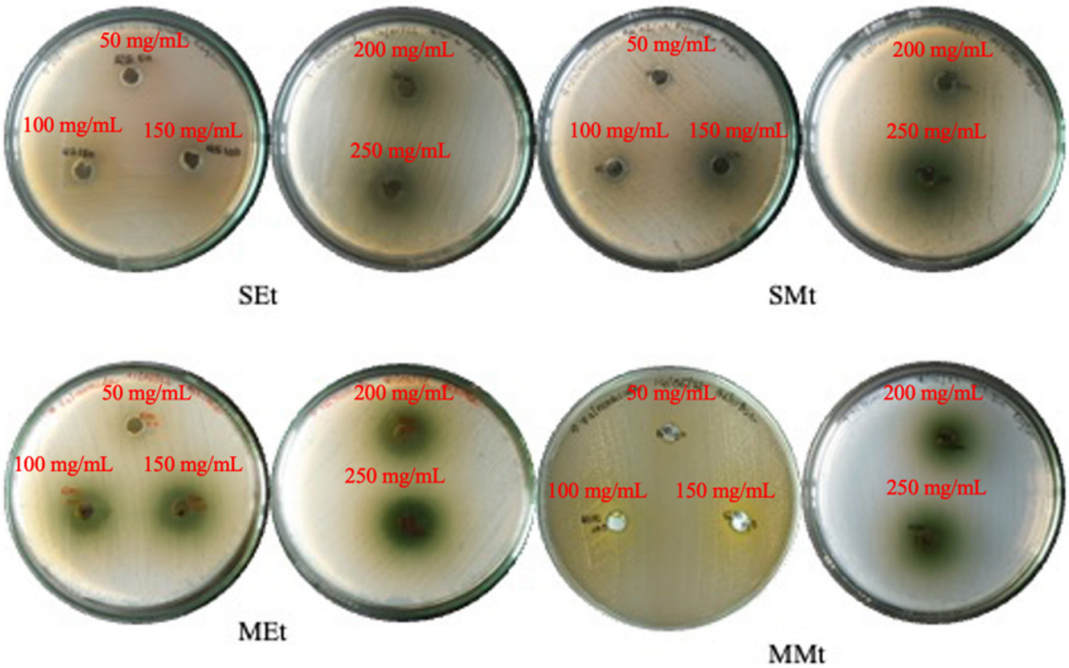

Figure 2. Antibacterial activity of *S. rebaudiana* leaf extracts against *A. salmonicida* (Zone of Inhibition)

Table 6. Antibacterial activity of various extracts of *S. rebaudiana* against *A. salmonicida*

Concen. (mg/mL)	SEt (mm)	SMt (mm)	MEt (mm)	EMt (mm)
50	7.33 ± 0.58^{d}	8.33 ± 0.58^{d}	8.33 ± 0.58^{d}	0.00 ± 0.00^{c}
100	10.00 ± 1.00^{c}	11.00 ± 0.00^{c}	12.00 ± 1.00^{c}	0.00 ± 0.00^{c}
150	12.00 ± 1.00^{b}	12.67 ± 0.58^{b}	13.67 ± 0.58^{b}	10.00 ± 1.00^{b}
200	13.33 ± 0.58^{ab}	13.67 ± 1.15^{ab}	15.33 ± 0.58^{a}	10.33 ± 0.58^{b}
250	14.67± 0.58^{a}	15.00 ± 1.00^{a}	16.67 ± 1.15^{a}	11.67 ± 0.58^{a}

Mean values in the same column with different alphabets were significantly different ($p<0.05$)

activities of neophytadiene. Al-Rajhi *et al.*[57] have found that high neophytadiene content in *Mentha pulegium* directly correlated with the increased antibacterial activity against *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* etc. There are few reports on the occurrence of phthalates in plants. GC-MS results also revealed the existence of dibutyl phthalate in all four extracts of *S. rebaudiana.* It is reported that plant-derived dibutyl phthalate is having many biological properties such as antibacterial activities.[58] Dibutyl phthalate isolated from *Begonia malabarica* showed significant antibacterial activity against *E. coli, Staphylococcus epidermis, Streptococcus pneumoniae, Micrococcus luteus, Vibrio cholerae* and *Shigella flexneri.*[59] The majority of these compounds have been discovered to have significant biological activity, like antibacterial, antioxidant, and anti-inflammatory action against specific diseases or pathogens.

DPPH radicle scavenging assay

The phytochemical analysis of the current study has revealed the existence of phenolics and flavonoids in all four extracts, which is directly correlated with antioxidant activity. Hence the analysis of radicle scavenging activity is a mandatory tool to determine the antioxidant activity of four extracts of *S. rebaudiana*. The antioxidant activity of natural products can be analysed in terms of electron donation ability by using 2,2-diphenyl-1- picrylhydrazyl (DPPH) radicle scavenging assay. The present study revealed that all extracts showed concentration-dependent inhibition of free radicles. As reported by previous studies the antioxidant potential of extracts was found to be lower than ascorbic acid.[72] The percentage inhibition was significantly different among all concentrations for all four extracts ($p < 0.05$). Among all extracts, MEt showed the highest antioxidant activity in terms of inhibition of free radicle with IC50 of 67.95µg/mL. This is followed by SEt (75.39µg/mL), MMt (94.31µg/mL) and SMt (95.40µg/mL) (Table 5). The greatest capacity of superoxide radicle scavenging of MEt can be correlated with high phenolics and flavonoid content. In the study conducted by Ahmad et al.[73], the methanolic extract of *S. rebaudiana* leaf (obtained by maceration method) showed percentage inhibition of 77.68% for the DPPH scavenging assay, which was in close agreement with the results of the current investigation. A similar trend of DPPH scavenging was observed for ethanol extracts of *Buhinia purpurea* leaf obtained by maceration and soxhlet method.[74] Their research found that macerated extract outperformed soxhlet in terms of scavenging activity with percentage inhibition of 88.6±3.0 and 71.4±0.9 % at 40µg/mL, respectively. According to the findings of the current study, ethanolic leaf extract of *S. rebaudiana* obtained by the maceration method can be employed as a readily available source of natural antioxidants with positive health effects. In view of the fact that maceration permits extraction at room temperature and subsequent solvent removal at decreased pressure, ensuring the recovery of more antioxidant compounds of interest (phenolics) without the risk of denaturation or other changes brought on by high temperatures as in hot extraction techniques like soxhlet.[75]

Antibacterial assay

The bactericidal activity of *S. rebaudiana* leaf extracts against *A. hydrophila and A. salmonicida* is depicted in Table 6. The

outcome revealed that all extracts showed concentration-dependent antibacterial activity against *A. salmonicida* (Figure 2). However, none of the extracts demonstrated a significant zone of inhibition when used in different concentrations against *A. hydrophila*. The possible reason for not showing antibacterial activity may be due to the formation of biofilms by this particular species. Previous studies have shown that the formation of biofilms, which promote the spread of antibiotic-resistant genes among bacteria in biofilms than in planktonic cells, is a key element in the onset of chronic infections and drug resistance in *A. hydrophila*. The study conducted by Bakhtiari *et al.*[76] witnessed that 19 strains of *A. hydrophila* were resistant to 75% of studied antibiotics.[76] Similar findings were reported by Jayaraman *et al.*[77] with chloroform and aqueous extract of *S.rebaudiana* leaf.[77] Only a few studies reported the antibacterial activity of *S. rebaudiana* against fish bacterial pathogens such as *Aeromonas* spp.

Maximum antibacterial activity (DZI) against *A. salmonicida* was demonstrated by MEt (16.67±1.15 mm) and SMt (15.00±1.00 mm) at 250 mg/mL. SEt showed DZI of 14.67±0.58 mm and MMt showed 11.67±0.58 mm. Statistically, there was a significant difference in the inhibitory activity among SEt and SMt at 50, 100 and 150 mg/mL against *A. salmonicida* ($p < 0.05$). Previous studies have reported the antibacterial activity of ethanol, methanol and chloroform extracts of *S. rebaudiana* are effective against *Pseudomonas aeruginosa, Escherichia coli* and *S. aureus.*[78] The solubility, concentration and composition of secondary metabolites might be responsible for the antibacterial activity of different extracts against *A. salmonicida*. At the same time, there was no significant difference between 200 and 250 mg/mL concentration of all extracts against *A. salmonicida.* The variation in the activity among different extracts might be caused by their chemical makeup.[79] The bioactive compounds such as phenolics, flavonoids, tannins, alkaloids and saponins can hinder the growth and metabolism of microorganisms in a negative way. Phenolics and flavonoids can inhibit bacterial peptidogly can synthesis and modify membrane permeability.[80] Alkaloids are proven to be intercalated with nuclear DNA resulting in cell death.[81] Tannins are able to inactivate cell envelop transport protein and adhesins.[82] The presence of volatile phytochemicals detected by GC-MS analysis is proven to have antibacterial, antioxidant and anti-inflammatory activities. The findings of the current work have proven that *S. rebaudiana* could serve as a source of a potential antimicrobial agent against fish pathogens such as *A. salmonicida* as the usage of natural plant extracts is so widespread.[83] The overall data of this study were consistent with previous results.

CONCLUSION

Plant extracts possess combined antioxidant and antibacterial attributes, rendering them valuable across medical, cosmetic, and food sectors. They offer natural alternatives to synthetic counterparts, reducing dependence on chemical additives. Our study highlights maceration as the optimal method for efficiently extracting phenolics, flavonoids, and tannins from *S. rebaudiana* leaves. Ethanol extracts from maceration, rich in phytochemicals, effectively inhibit fish pathogens, like *A. salmonicida*. Plant-derived compounds, such as alkaloids, terpenoids, tannins, and flavonoids, underpin the antibacterial potential. These disrupt bacterial structure, enzymes, and metabolism, curtailing growth. Phenolic compounds, by quenching free radicals and reducing agents, limit radical chain reactions. While plant extracts find utility in medicine, cosmetics, and food, their antibacterial efficacy varies based on species, extraction methods, and bacterial strains. Our ongoing research underscores the ease and cost-effectiveness of cold maceration in preserving heat-sensitive metabolites during *S. rebaudiana* extraction. Further investigation is vital to decipher mechanisms and optimize plant extracts for antibacterial use. In conclusion, *S. rebaudiana* extracts emerge as a viable resource for pioneering aquaculture antibacterial treatments.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AR and KV designed the experiments, analyzed the data and wrote the manuscript. AR performed the experiment and collected the data. KV performed supervision and revised the manuscript. Both authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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