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Antibacterial and Antifungal Activity of *Stevia rebaudiana* (Asteraceae) Leaf Extract *in vitro* Condition

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ABSTRUCT: In the present communication we studied antimicrobial activity of *Stevia* plant extract under in *vitro* condition against plant phytopathogens, which is stored in the culture collection of the Institute of Phytopathology and Biodiversity. The three different extracts of leaves namely, chloroform, acetone and absolute ethyl alcohol were tested against *Ralstonia solanacearum*, *Pseudomonas syringae pv. actinidiae* (PSA), *Erwinia amylovora* bacteria strains and to the fungal plant pathogens: *Alternaria alternata, Colletetrichum gloeosporioides* and *Fusarium moniliforma*. The largest zone of inhibition for *R. solanacearum* KhPe90 strain was measured 15mm with chloroform extract and 18mm by the ethyl alcohol extract, but 5mm has shown acetone. The maximum inhibition zone for chloroform extract against PSA strains were 18mm and 20 mm were observed for ethyl alcohol extract, while for acetone extract has shown 8mm. For test strains of *E. amylovora* diameter of lyses area was 12mm with action of chloroform extract and 15mm with absolute ethyl alcohol, while in this case acetone extract showed minimum zone of inhibition (8mm). The observation of 98 hour old cultures of *A. alternate* has shown that mycelia were grown about 15mm with action of absolute ethyl alcohol extract. Chloroform had impacted on this pathogen, mycelia diameter was 18mm and with acetone was 20mm, while in this same time, control culture had 40mm diameter mycelia. The high inhibitory effect on the growth of *F. moniliforma* culture had chloroform extract (mycelia was 14mm), next was absolute ethyl alcohol extract (25mm) and last one was acetone (35mm). On the growth of *C. gloeosporioides* test culture had high inhibitory effect of chloroform extract (growth of mycelia was 18mm), followed by the absolute ethyl alcohol extract (22mm) and last one was acetone (25mm).

Thus, the studies have shown high antibacterial and antifungal activity of some extracts of *Stevia rebaudiana* leaves against test plant pathogens. It needs further study based on *in vivo* condition.

Keywords: *Stevia* extract; Antimicrobial activity; plant pathogen.

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INTRODUCTION

Nowadays, the control of plant diseases is a global problem. It is noteworthy that the biological activities have little place in the novel mechanisms of the action in the incidence of the plant diseases. At the same time, the number of resistant microorganisms significantly increases (Sundin and Bender 1996). The screening of plant extracts has been of great interest to scientists in the search for new drugs for greater effective treatment of several diseases (Dimayuga, 1991). Therefore, plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (Diallo, 1999); (Erdogrul, 2002). The antimicrobial action of the plant phytochemicals, the same "natural antibiotics" used in ecology, medicine and agriculture (Venkanna, 2012).

In this regard, one of the potent members of the Asteraceae family is *Stevia rebaudiana* (commonly referred to as Honey leaf, Candy leaf and Sweet leaf), which is popular in the world not only as a low-calorie, medicinal, natural sweetener, also known for its antioxidant activity of leaf extract (Pinheiro, 1987; and Takahashi, 2001). It is rich in terpenes, flavonoids, phenols and flavonoids, which cause the antimicrobial properties of the plant (Taware, 2010). The phytochemicals present in *Stevia rebaudiana* are austroinullin, β carotene, dulcoside, niacin, rebaudi oxides, riboflavin, steviol, stevioside and tiamin Crammer, 1986). *Stevia* is also proved to inhibit the growth of certain bacteria and other infectious organisms hence used against wounds sores and gum disease.

It may also explain while the herb is advocated for anyone who is susceptible to yeast infections or reoccurring streptococcal infections, two conditions that seem to be aggravated by white sugar consumption. The biological activity of *Stevia* compounds was studied by Tomita *et al.* (1997). *Stevia* has been cultivated in different countries since the previous century. *Stevia* was introduced in Georgia in the 80s of XX century (Sivaram & Mukundam, 2003), (Geuns, 2011).

The aims of our research is to investigate the antibacterial and antifungal activity of the extract of *Stevia rebaudiana* leaves against some plant pathogen using various solvents.

MATERIALS AND METHODS

The study was conducted at the Department of Plant Disease Monitoring, Diagnostics and Molecular Biology of the Institute of Phytopathology and Biodiversity of Batumi Shota Rustaveli State University, and the Western Georgian Regional Center of Chromatography.

Stevia leaves were collected from the different stages of the plant grown in the experimental field of the Institute of Phytopathology and Biodiversity of Batumi Shota Rustaveli State University.

In the trial, 24 bacterial (*Ralstonia solanacearum* 17 - BBGStr.54; AcP61; KhP9; KoPe19; KhP34; AkhP80; AcP98; NCPPB 325; NCPPB3725; NCPPB4214; KhPe90; KhP6; KhT88; KoT64; KoT47; KoP18; AkhP81; *Pseudomonas syringae pv. Actinidiae* 3 - NCPPB3738; KW1; KW2; *Erwinia Amylovora* 4 - NCPP 4359 ERW1; ERW2 (122); ERW3) and three fungal isolates - *Alternaria alternate*; *Colletetrichum gloeosporioides*; *Fusarium moniliforma* were used.

They were obtained from the culture collection of our institute and from the National Collection of Plant Pathogenic Bacteria (NCPPB). The pure cultures were maintained by routine sub-culturing at one-week interval in modified SMSA medium, Casamino acid-Peptone-Glucose (CPG) medium, modified NSA, KB and potato dextrose agar slants respectively for bacterial and fungi.

Preparation of plant extract: The fractions have been obtained from10 grams of green dried *Stevia* leaf, extracted by the SFE method with solvent of chloroform, acetone and absolute ethanol (Ruslan Davitadze and Aleko Kalandia 2018).

Assay for antimicrobial activity: Agar-well bioassay was employed for testing antibacterial activity of *Stevia rebaudiana* leaves (Linday, 1962). Each extract were made to final concentration of 10mg/ml. All extracts were subjected to antimicrobial assay by measuring the diameter of zone of inhibition (IZD) using disc diffusion method (Valgas, 2007). Nutrient sucrose agar and potato dextrose agar plates were prepared by pouring 20ml each in sterile Petri dishes for bacterial and fungal assay respectively and allowed to solidify. 0.4 ml of 10^{-4} dilution of 24 hours old bacterial and 48 hours old fungal cultures were used so as to ensure the concentration of these organisms to contain approximately 1×10^6 CFU/ml (Birt, 2001). The antifungal and bacterial assay for each of the extracts against all microorganisms tested was performed in triplicates.

RESULT AND DISCUSSION

In vitro, the diffusion method was used to determine the antimicrobial sensitivity of extracts obtained from *Stevia* leaves. To accomplish this task, we implemented several series of experiments. Petri dishes were at a temperature of 28°C, and the observation was carried out for 24-48 hours. The quality of the tested extract activity was determined by the size of the lysis area around the bacterium strain after adding 20 ml of the extract. The antibacterial activities of the solvent extracts of *Stevia rebaudiana* showed significant variations, as shown in Table 1.

To actioned of the *Stevia* leaf extracts on the growth of bacterial strains, it was shown that ethyl alcohol and chloroform had the highest inhibitory activity (measured by the zone of inhibition). The largest inhibition zone for 24h culture of *Ralstonia solanacearum* was 15mm with chloroform extract and 18mm by the ethyl alcohol, but 6mm has shown acetone extract. The high inhibition zone for chloroform extract against *Pseudomonas syringae* pv. *actinidiae* strains was 18mm and for ethyl alcohol extract was observed 20 mm, while for acetone extract has shown 8mm. For test strains of *Erwinia amylovora* the diameter of lysis area was 12mm with to action of chloroform extract and 15mm with absolute ethyl alcohol, in these cases acetone has shown also low activity, maximal inhibition zone was 8mm. Among the three extracts tested, ethyl alcohol extract had greater antibacterial potential, followed by chloroform extract, then the other extracts. As in Fig. 1 and Fig. 2 shown the largest zones of inhibition were observed for ethyl alcohol extract against *Pseudomonas syringae* pv. *actinidiae* KW1 strains (20 mm) and *Ralstonia solanacearum* KhT88 (18 mm).

Table 1: Determination of antibacterial activity of the extracts of *Stevia rebaudiana* leaves by diameter inhibited zone (mm) on test culture.

Test organism		Solvents			
		Chloroform	Acetone	absolute ethyl alcohol	control
Ralstonia solanacearum					
1	BBGStr.54	8mm	3mm	10mm	-
2	AcP61	5mm	2mm	10mm	-
3	KhP9	7mm	5mm	8mm	-
4	KoPe19	8mm	3mm	6mm	-
5	KhP34	10mm	4mm	10mm	-
6	AkhP80	12mm	3mm	10mm	-
7	AcP98	6mm	6mm	8mm	-
8	NCPPB 325	10mm	2mm	10mm	-
9	NCPPB3725	2mm	2mm	12mm	-
10	NCPPB4214	2mm	5mm	14mm	-
11	KhPe90	15mm	5mm	18mm	-
12	KhP6	5mm	3mm	12mm	-
13	KhT88	2mm	3mm	18mm	-
14	KoT64	4mm	4mm	15mm	-
15	KoT47	8mm	6mm	8mm	-
16	KoP18	3mm	2mm	10mm	-
17	AkhP81	2mm	3mm	12mm	-
Pseudomonas syringae pv. Actinidiae					
1	**NCPPB3738**	**18mm**	**8mm**	**18mm**	-
2	**KW1**	**15mm**	**7mm**	**20mm**	-
3	**KW2**	**18mm**	**7mm**	**18mm**	-
Erwinia Amylovora					
1	NCPP 4359	10mm	7mm	10mm	-
2	ERW1	12mm	6mm	15mm	-
3	ERW2 (122)	8mm	6mm	12mm	-
4	ERW3	10mm	8mm	15mm	-

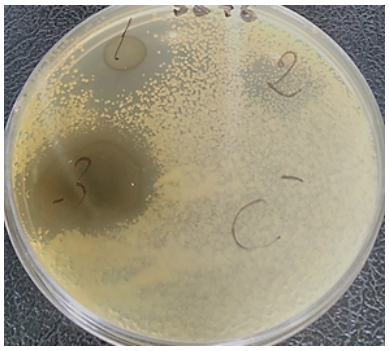

Fig. 1. *Pseudomonas syringae pv. Actinidiae* – strain KW1; 1- result of chloroform 1% extract; 2- result of acetone 1% extract, 3- result of absolute ethyl alcohol 1% extract; 4-Control.

The fungal pathogens - *Alternaria alternata*, *Colletetrichum gloeosporioides* and *Fusarium moniliforma* were used to study the antifungal activity of extracts of stevia leaves by dimension mycelium growth zone (mm). The antifungal activities of the solvent extracts of *Stevia rebaudiana* also varied significantly among the test organisms as we can see in Table 2.

The observation of 98 hour old cultures of *Alternaria alternate* has shown that mycelia were grown 15 mm to action of absolute ethyl alcohol extract. Chloroform had impacted 96 culture of *Alternaria alternate* was grown 18mm and with acetone was 20mm, while in this same time control culture had 40mm diameter mycelia.

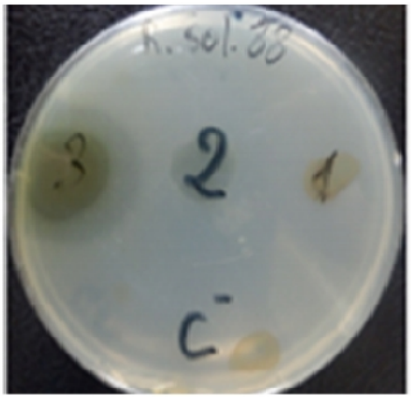

Fig. 2. *Ralstonia solanacearum* strain KhT88; 1- result of chloroform 1% extract; 2- result of acetone 1% extract, 3- result of absolute ethyl alcohol 1% extract; 4-Control.

Table 2: Determination of antifungal activity of the leaves extracts of *Stevia rebaudiana* by measuring mycelial growth zone (mm).

N	Time of observation	Solvents			
		Chloroform	Acetone	absolute ethyl alcohol	Control
1.	*Alternaria alternata*				
	48 hour	8mm	10mm	5mm	15mm
	96hour	18mm	20mm	15mm	40mm
2.	*Fusariom moniliforma*				
	48hour	8mm	18mm	17mm	25mm
	96hour	14mm	35mm	25mm	60mm
3.	*Colletetrichum gloeosporioides*				
	48hour	13mm	20mm	20mm	30mm
	96hour	18mm	25mm	22mm	50mm

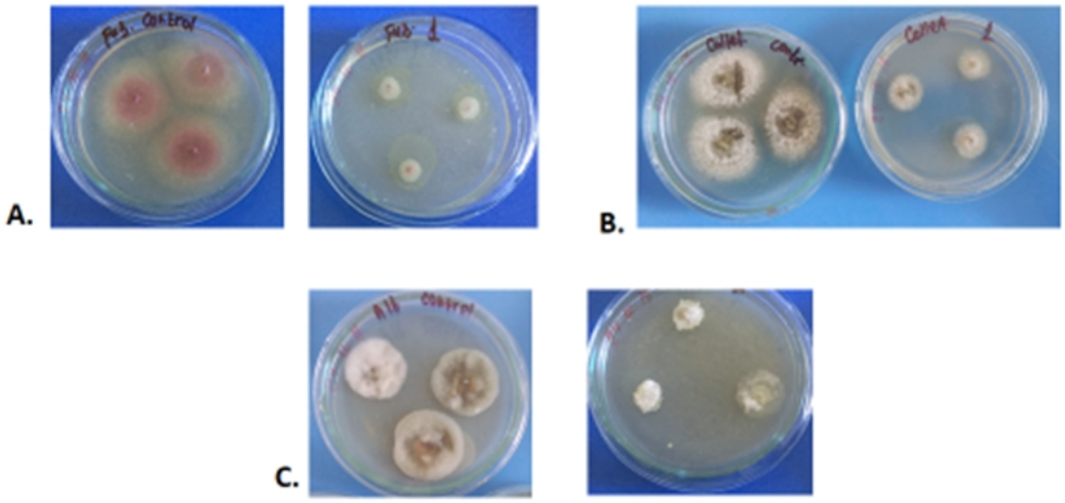

Fig. 3. A. The results of activity of the 1% chloroform extracts on *Fusarium moniliforma* culture, Right -test culture (14mm); left – control(60mm); B. The result of activity of the 1% chloroform extracts on *Colletetrichum gloeosporioides* culture, Right -test culture 18mm; left – control (50mm); C. The results of activity of the 1% absolute ethyl alcohol extracts on *Alternaria alternate* culture, Right -test culture (15mm); left – control (40mm).

Among all the three extracts the absolute ethyl alcohol had higher inhibited effect against fungal pathogen *Alternaria alternate.* The high inhibitory effect on the growth of *Fusarium moniliforma* culture had chloroform extract (diameter mycelia was 14mm), next was absolute ethyl alcohol extract (25mm) and last one was acetone (35mm). On the growth of *Colletetrichum gloeosporioides* test culture had high inhibitory effect of chloroform extract (the growth of mycelia was 18mm), followed by the absolute ethyl alcohol extract (22mm) and last one was acetone (25mm), (Fig. 3).

The results showed that sensitive fungal pathogens are characterized by the reduced growth compared to the control (mycelium is weak or absent) and by deformed spores or mycelium (Fig. 4). They were characterized not only by weak growth but also by different colors. Microscopic examination showed that the test wall of the microorganism dissolved, and the hyphae became highly deformed.

Thus, the studies have shown high antibacterial and antifungal activity of chloroform and absolute ethyl alcohol solvent extracts of *Stevia rebaudiana* leaves against test plant pathogen, but this study is undergoing and need to be confirmed using *in vivo* models.

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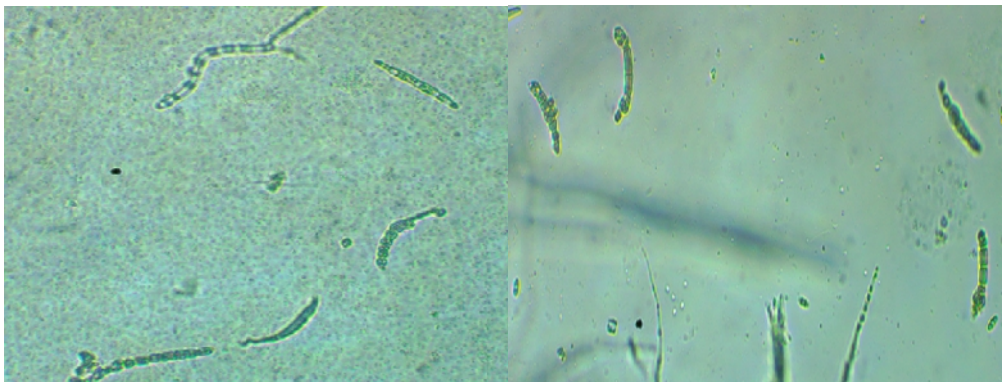

Fig. 4. Deformed spores of *Fusarium moniliforme*under the microscope after the action of the 1% extracts of chloroform.

Thus, the studies have shown high antibacterial and antifungal activity of chloroform and absolute ethyl alcohol solvent extracts of *Stevia rebaudiana* leaves against test plant pathogen, but this study is undergoing and need to be confirmed using *in vivo* models.

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