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Original article

Insights on the antioxidant, antidiabetic, antiamnesic, cytotoxic, thrombolytic and antibiofilm activities of *Stevia rebaudiana* leaves

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Abstract. *Background and Aim*: The antioxidant, antidiabetic, antiamnesic, cytotoxic, thrombolytic and biofilm inhibition activities of seven fractions (methanol, ethanol, ethyl acetate, *n*-hexane, *n*-butanol, chloroform and aqueous) of *Stevia rebaudiana* leaves were determined. *Methodology:* The antioxidant contents and activity was measured as total phenolic contents (TPC), total flavonoid contents (TFC) and free radical scavenging assays. Anti-diabetic and antiamnesic efficacies were tested in terms of glycation, alpha amylase and acetylcholinesterase inhibitions. DNA damage protection and hemolysis analysis were done to evaluate cytotoxicity. In addition thrombolytic and antibiofilm studies were performed by prescribed methods. *Results*: The *n*-butanol fraction had highest TPC, whereas, optimal TFC and antioxidant activity were observed in methanol fraction. Ethyl acetate, aqueous and chloroform fractions showed maximum antiglycation, alpha amylase inhibitory and acetylcholinesterase inhibitory activities respectively. In cytotoxic profile, hemolytic activity was in the range of 4-47%, with *n*-butanol being the most potent hemolytic agent. All fractions prevented DNA damage except ethanol, *n*-butanol and aqueous fractions. The greatest biofilm inhibition was produced by *n*-hexane extract for *Pasteurella multocida* strain and aqueous fraction for S*taphylococcus aureus* strain. Highest (27%) thrombolytic activity was studied in *n*-hexane sample. *Conclusion*: The current study highlighted significant antioxidant, antidiabetic, antiamnesic, cytotoxic, thrombolytic and antibiofilm activities of *Stevia rebaudiana* leaves that might be significant for the management and treatment of various diseases.

Keywords: *Stevia rebaudiana, Pasteurella multocida,* alpha damylase, acetylcholinesterase, thrombolytic activity

Introduction

Natural plant products play an important role to prevent and treat different diseases. About 80% population of the world depends on natural medicinal sources and the demand of medicinal plants is increasing day by day. In recent years, the trend of natural dietary supplements and herbal extracts has increased due to their strong health benefits and less toxic effects. Phytoconstituents such as flavonoids, carothenoids, proteins, enzymes, peptides and vitamins have significant biological activity and used as secondary metabolite. Plants secondary metabolite is unique to specific species and they increase their capacity to persist and compete against different environmental conditions including oxidative stress, radiations and infections (1, 2).

Stevia rebaudiana (Asteraceae) is an herbaceous perennial shrub. More than 100 phytochemicals have been found in *Stevia* family. *Stevia* leave, stem and flowers consist of diterpene, glycosides, isosteviol, stevioside, rebaudiosides, steviolbioside and dulcoside. Steviosides and rebaudiosides impart sweet taste to stevia leaves, as these are about 250 to 300 times sweeter than sucrose. Leaves contain minerals, vitamins, phenols, flavonoids. Glycosides present in Stevia

leaves include stevioside, rebaudioside, steviolbioside, and isosteviol, that impart sweetened perception and is a value-added food product. These glycosides have therapeutic potentials against numerous ailments (3, -5) and are not only a significant food source but are also used in cosmetic industry (6,7).

Free radicals, hydroxyl radicals and superoxide anion radicals in *S. rebaudiana* leaves (8) act as anti-oxidants, antimicrobial and chemo-therapeutic agents (9,10) for the treatment of many diseases such as candidacies, high blood pressure, wounds, sores, gum diseases, microbial infections, dental caries, obesity, dementia, neuropathies, diabetes mellitus and heart diseases (11, 12). Previously (13, 14), stevia extract demonstrated antidiabetic, hypoglycemic, antihypertensive, anti-inflammatory, anticarcinogenic, and immuno-modulatory properties. *Stevia rebaudiana* has various beneficial properties against hyperglycemia because it helps in the treatment of diabetes, weight control and dental caries. *Stevia* leaf extracts are commonly used as hypoglycemic agents because they are nontoxic and have very low caloric value. Its long-term use decreases the intensity of cardiovascular diseases in chronic diabetic patients. The hypoglycemic ability of *Stevia rebaudiana* is due to direct effect of steviosides on pancreatic cells due to which they secrete more insulin. *Stevia* contain honey like flavor which makes it tasty alternative to glucose-spiking sweeteners like sugar and corn syrup (8, 13).

There is no comprehensive analysis of diverse therapeutic potentials of *S. rebaudiana* leaves extracts prepared in different solvents. Therefore, present study was conducted to evaluate the antioxidant, antidiabetic, antiamnesic, cytotoxic, thrombolytic and anti-biofilm activities of *Stevia rebaudiana leaves* in various organic solvents.

Materials and Methods

Plant material

S. rebaudiana (Asteraceae) leaves procured from Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad, Pakistan were identified and authenticated at the Department of Botany, University of Agriculture, Faisalabad,

Extraction and fractionation

Conventional maceration technique was employed for extraction of bioactive compounds based upon their separation in different solvents. Extracts (145 g) of powdered samples prepared in methanol (15) were partitioned into different solvents such as *n*-hexane (25 g), *n*-butanol (20 g), ethanol (60 g), ethyl acetate (5 g) and chloroform (10 g) along with aqueous extract (10 g).

Anti-oxidant contents and activity

Total Phenolic Contents (TPC) were measured by Folin- Ciocalteu reagent and results were expressed in terms of mg gallic acid equivalent/100 g dry weight (16). Aluminum chloride colorimetric technique was used for total flavonoid contents (TFC) estimation (17) and TFC was expressed as gram catechin equivalents/100 gram dry weight. The anti-oxidant activity of extracts assessed by DPPH radical scavenging assay (18) and percent radical scavenging concentration was calculated by using the following formula: Radical Scavenging (IC_{50}%) = 100 x (A blank – A sample / A blank).

Antidiabetic activity

Antiglycation activity

Bovine serum albumin (BSA) dissolved in 67 m*M* phosphate buffer (pH 7.4), glucose (50 mg/mL dissolved in 67 m*M* phosphate buffer; pH 7.4) and test sample were incubated at 37°C (19). The negative control preparations had reaction mixture without plant extract or synthetic inhibitor and positive control had synthetic drug metformin as inhibitor. For blank incubation, BSA was replaced by buffer solution. Inhibition (percentage) was estimated as: 100 - [(OD (sample) / OD (blank)] x 100].

Alpha amylase inhibition assay

Plant sample and porcine pancreatic alpha amylase (Sigma Aldrich) at a concentration of 0.5 mg/mL (20 m*M* phosphate buffer, pH 6.9) were incubated at

25°C for 90 minutes. Starch solution (20 m*M* phosphate buffer, pH 6.9) was added, incubated at 25°C for 30 minutes and heated in boiling water with 3, 5 dinitro-salicylic acid (DNS) reagent. Absorbance was measured at 540 nm (20). As positive control, synthetic inhibitor acarbose and as negative control, reaction mixture without sample was used. For blank sample, enzyme was replaced by buffer solution. Percent inhibition was calculated as: (Absorbance of control- Absorbance of test sample)/ Absorbance of control x 100.

Antiamnesic activity - Acetylcholinesterase inhibitory assay

Plant sample (30 µL), 2.8 mL (0.1M, pH 8) phosphate buffer, 30 µL acetylcholinesterase (AcheE) and 100 µL of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) stock solution were added. After incubation for 15 minutes at 25°C, 30 µL acetylcholine iodide was added. Absorbance was measured at 412 nm. As positive control, synthetic inhibitor (amigra) and as negative control, reaction mixture without sample was used (21). Percentage inhibition was calculated as: (Absorbance of control-Absorbance of test sample)/Absorbance of control x 100.

Cytotoxic potential

DNA damage protection assay

Reaction mixture (supercoiled pBR322 plasmid DNA and plant sample) was incubated at 37°C for 5 minutes, following by the addition of Fenton reagent (H_2O_2, ascorbic acid, $FeCl_3$). Mixture was again incubated at 37°C for 30 minutes and analyzed by 1% agarose gel electrophoresis. The positive control contained plasmid DNA and Fenton reagent, while the negative control consisted of only the plasmid DNA. The results were analyzed by trans-illuminator (22).

Hemolytic activity

Blood samples were centrifuged at 1500 rpm for three minutes, plasma was discarded and pellets were washed three times with PBS (pH 7.4) by centrifugation at 1500 rpm for 5 minutes. The cells were suspended in normal saline. RBCs suspension was mixed with test sample, incubated at 37°C for 30 minutes. Then centrifuged at 1500 rpm for 5 minutes. Supernatant was diluted with sterile PBS. 0.1% Triton X-100 was the positive and PBS was negative control. Absorbance at 576 nm was measured (23) and hemolytic inhibition (%) was calculated with the following formula: % Hemolysis = (Absorbance of test sample − Absorbance of negative control/Absorbance of positive control − Absorbance of negative control) × 100.

Thrombolytic activity

Blood samples in pre-weight micro-centrifuge tubes (We) were incubated at 37°C for 45 minutes for clot formation. Serum was removed and tubes with clot were again weighted (Wm) to calculate weight of clot before lysis. Weight of clots (Wc) was determined as = Wm - We Plant sample was added in tubes and incubated at 37°C for 90 minutes for clot lysis. Tubes were inverted and left overnight. Tubes were weighted to calculate the weight of clot (W1) after lysis. After lysis weights of clots were determined by taking difference between weight of clot after lysis (W1) and weight of empty tubes (We). Wc = W1 − We. Then percentage of clot lysis activity was determined by using following formula: Clot lysis (%) = Wb - W1/ Wb x 100. Where (Wb) is weight of clot before lysis and W1 is weight of clot after lysis (24). Streptokinase and distilled water were applied as positive and negative control respectively.

Biofilm inhibitory activity

Nutrient broth, plant extracts and bacterial strains were added in 96 wells microliter plate and incubated overnight at 37°C. Titer plates were washed 3-times with phosphate buffer saline, air dried and fixed with methanol for 2-3 minutes and 100 µL of crystal violet strain was added. 100 µL of glacial acetic acid was used to solubilize the crystal violet. Absorbance was taken with plate reader at 630 nm. Ampicillin and nutrient broth were used as positive control while nutrient broth and bacterial strain were used as negative control. Percentage inhibition was calculated as: (OD of control-OD of sample)/OD of control x 100 (25, 26).

Data analysis

The results were analyzed statistically by analysis of variance (ANOVA) using SPSS (Statistical Packages for Social Sciences, version 16.0, 2003 ® SPSS Inc., Chicago, IL, USA) software.

Results and discussion

Antioxidant contents and activity

Phenols and flavonoids possess a broad range of biological and pharmacological activities.

Inactivation of ROS, prooxidative transition metal ions chelation, free radicals scavenging and hydro peroxidase reduction are the major mechanisms of antioxidant activities of plants (9, 34). Significant ($p>0.01$) difference in TPC, TFC and free radicle scavenging activities among all the extracts and fractions was observed (table 1). TPC were in the range of 6.72 - 12.70 (gallic acid equivalent). The *n*-butanol showed highest contents. TPC in descending order were: *n*-hexane> methanol > ethyl acetate > aqueous > ethanol> chloroform. Previously, Singh *et al.* (27) studied the methanol extracts of *S. rebaudiana* leaves and reported 11.04±3.16 (gallic acid equivalent) TPC. TFC and free radical scavenging potential of *S.rebaudiana* leave fractions were in the range of 2.73±1.44 (quercetin %) and concentration dependent 82.36 % respectively. The methanol extract showed highest TFC and DPPH activity. TFC in descending order were: chloroform > ethanol > aqueous > *n*-hexane > ethyl acetate > *n*-butanol. DPPH in descending order were: aqueous > ethanol > *n*-butanol > chloroform > ethyl acetate > *n*-hexane. In a previous study on *S.rebaudiana* leaf extracts, TFC were 21.73 - 31.99 g catechin equivalents and DPPH activity was in the range of 23.12 - 66.23%. Similarly, the percentage inhibitions of DPPH radical with methanol extract of *Stevia* leaves and callus were found as 33.17% and 56.82%. In DPPH assay, callus showed higher antioxidant activity compared to the *Stevia* leaves. Chelation process prevents free radical generation and *Stevia* extract has highest chelating ability. Antioxidant activity of *Stevia* extracts is mostly related to phenolic and flavonoid contents. Aqueous leaves extract of *Stevia rebaudiana* significantly increases vitamin C and E levels. So, leaves extract of *Stevia rebaudiana* is used as natural antioxidants in various industries (28).

Table 1 Antioxidant profile

Extract/ Fractions	TPC	TFC	DPPH activity (IC_{50})
Methanol	9.46 ± 0.043	26.45 ± 0.047*	61.16 ± 0.007*
Ethanol	7.50 ± 0.04	24.27 ± 0.029	52.82 ± 0.01
Ethyl acetate	9.06 ± 0.027	17.45 ± 0.034	36.12 ± 0.019
Chloroform	6.72 ± 0.04	24.32 ± 0.022	38.71 ± 0.021
n-hexane	11.14 ± 0.024	18.05 ± 0.03	23.12 ± 0.013
n-butanol	12.70 ± 0.021*	16.23 ± 0.048	50.54 ± 0.019
Aqueous	7.64 ± 0.044	21.41 ± 0.01	55.91 ± 0.011
BHT	-	-	66.23 ± 0.023

Data expressed as mean ± standard deviation. TPC: total phenolic contents expressed mg gallic acid equivalent /100 g dry weight; TFC: total flavonoid contents expressed as g catechin equivalents/100 g dry weight; DPPH: 2, 2-diphenyl l-l-l-picrylhydrazyl, BHT: Butylated hydroxy toluene (positive control).

*significant ($P<0.05$).

Antidiabetic activity

Antidiabetic potentials were assessed in terms of glycation and alpha amylase inhibitions (table 2). Ethyl acetate showed maximum antiglycation activity (53.22%). Antiglycation activity of *S. rebaudiana* leaves fractions in descending order were: *n*-butanol > chloroform > ethanol > methanol > *n*-hexane > aqueous. Various studies highlighted the importance of glycation inhibition by plants (29). Results of present study were not in accordance to the previously reported 76.80% glycation inhibition by *S. rebaudiana* leaves extracts (30).

To assess the role of stevia extracts in reducing glucose absorption, alpha amylase inhibitory assay was performed. Aqueous fraction showed highest alpha amylase inhibitory activity (59.17). Enzyme inhibitory activity of other solvents in descending order was: chloroform > ethyl acetate > *n*-hexane > *n*-butanol > ethanol > methanol. Previously, Singh *et al.* (31)

Table 2 Antidiabetic, Antiamnesic, hemolytic and thrombolytic profiles

Extracts/fractions	Antidiabetic activity (% inhibition)		Antiamnesic activity (% inhibition)	Hemolytic activity	Thrombolytic activity
	Glycation	Alpha amylase	AChE		
Methanol	35.56	30.42	34	8 ±1.59	14
Ethanol	37.33	34.17	1.1	10 ± 2.78	9.3
Ethyl acetate	53.22	50.0	18.7	27 ± 0.98	18
Chloroform	37.41	51.25	34.1	9 ± 1.12	26
***n*-hexane**	31.53	49.58	2.6	17 ± 2.16	27
***n*-butanol**	44.26	43.33	11.8	47 ± 3.23	11
Aqueous	24.09	59.17	5.8	4 ± 2.26	22
Synthetic inhibitor	52.33	36.25	59.9	-	-
Triton-X	-	-	-	82.13 ± 4.43	-
Streptokinase	-	-	-	-	61

Data presented as percentage or mean (%) ± SE.

AChE: Acetylcholinesterase, Metformin (synthetic glycation inhibitor), acarbose (synthetic alpha amylase inhibitor), amigra (synthetic acetylcholinesterase inhibitor) were used as positive controls. Triton-X and streptokinase were positive controls for hemolytic and thrombolytic assays respectively.

conducted similar study with aqueous extracts of *S. rebaudiana*. They reported significant alpha amylase inhibition. It is suggested that stevia leaves can be used to develop efficacious therapeutic drug for diabetes mellitus treatment.

Antiamnesic activity

Repression of acetylcholinesterase (AChE) activity is a common antiamnesic practice to manage different neurological disorders. Plants are versatile source of AChE inhibitory compounds and most of the recent research focus on the use of plant extracts as cheap natural medicine worldwide (32).

Chloroform fraction showed maximum AChE inhibition (34.1). Inhibitory activity of remaining solvents in descending order was: methanol > ethyl acetate > *n*-butanol > aqueous > *n*-hexane > ethanol. Zhao *et al*. (33) investigated the role of *Stevia* residue extract against oxidative stress in mice. Being good source of chlorogenic acids, extracts significantly increased the antioxidant enzymes concentrations and decreased acetylcholinesterase (AChE) activity. It was concluded that *S. rebaudiana* can be a used to inhibit or mitigate oxidation stress and age-induced neurodegeneration.

Cytotoxic activity

DNA damage assay

Hydroxyl ions are strong oxidants that impart damage to DNA and protein (34). The current research exposed the DNA protection ability of chemical composites present in organic fractions of *S.rebaudiana* (figure 1). Ethanol, *n*-butanol and aqueous extract/fractions made smear bands (lane 2, 6, 7) indicating DNA damage. While all other extract/fractions exhibited the clear bands exhibiting no DNA damage. Fenton reaction in mitochondrial electron transport chain produces free radicals near DNA molecules. Polyphenol-rich Stevia extracts may decrease oxidative stress. In current study, antioxidant role of stevia leaves extracts supports data documented earlier. Previously, Ghanta *et al*. (12) conducted analogous analysis for *S. rebaudiana* crude extracts and documented DNA preventive actions of methanol and ethyl acetate extracts of *S. rebaudiana*.

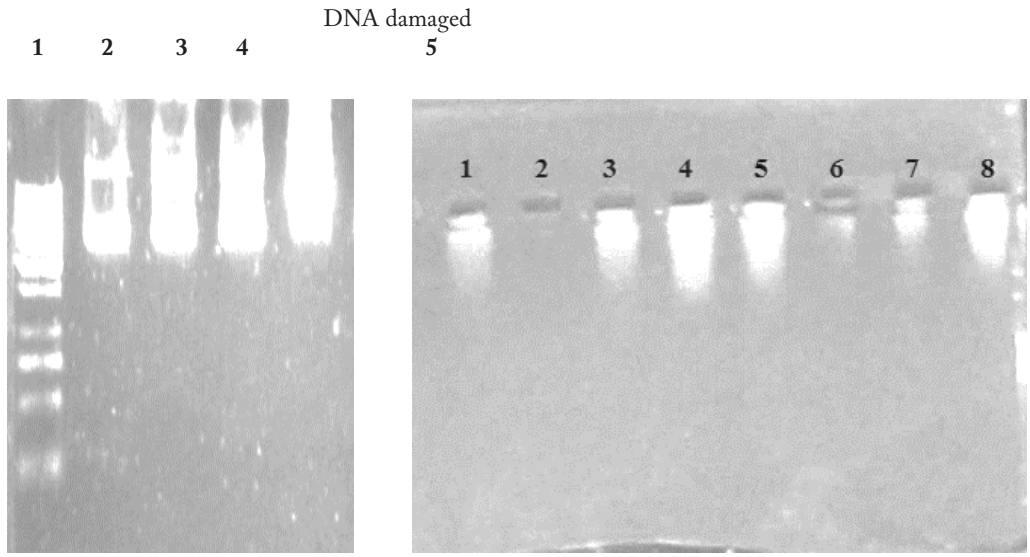

Figure 1(a-b) Agarose gel electrophoresis of DNA-damage protection assay
a: Lane1: ladder; Lane2: positive control; Lane 3: negative control ($FeSO_4$); Lane 4 and 5: negative control (H_2O_2), **b**: The Lanes 1-7: plasmid DNA incubated with methanol, ethanol, ethyl acetate, chloroform, *n*-hexane, *n*-butanol, aqueous fraction and treated with H_2O_2.

Hemolytic activity

Results exhibited least toxic effects of *S. rebaudiana* leaves extracts in terms of hemolysis of red blood cells (RBC). Long term use of plants-based medicines can cause destruction of RBC. In hemolytic assay (table 2), *n*-butanol showed maximam hemolytic activity (47%) as compared to other samples. Hemolytic activity in ascending order was as aqueous < methanol < chloroform < ethanol < *n*-hexane < ethyl acetate. respectively. Limited data is available regarding hemolytic activity of stevia plant. Sansano et al. (35) studied the effect of *Stevia rebaudiana* Bertoni on the hemolytic potential of *Listeria* monocytogenes. Exposure to 2.5 % (*w/v*) stevia concentration reduced the hemolytic capability of *L. monocytogenes*. The prospect of minimizing the pathogenic potential of *L. monocytogenes* (hemolysis) by stevia should be established in further studies. Inferences highlighted in current research reflects the potential application of stevia as a successful herbal remedy in practice.

Thrombolytic activity

Fibrinogen potential is destroyed by plasmin formation of thrombin occurs (the motivated form of plasminogen). Various thrombolytic medications are made for the treatment of heart diseases such as myocardial infarction. Thrombolytic activity of plant samples was compared Streptokinase, a potent thrombolytic agent. The *n*-hexane fraction displayed highest thrombolysis and for other solvents in ascending order it was as; ethanol < *n*-butanol < methanol < ethyl acetate < aqueous < chloroform. Our results (table 2) are justified by significant antioxidant components in *S. rebaudiana*. Myint *et al*. (7) attributed the thrombolytic activity of medicinal plants to the presence of polyphenols.

Biofilm inhibitory assay

Two bacterial strains *Staphylococcus aureus* and *Pasteurella multocida* were used for antibiofilm assay. *n*-hexane fraction showed maximam (87.05%) percentage inhibition (figure 2) and methanol showed

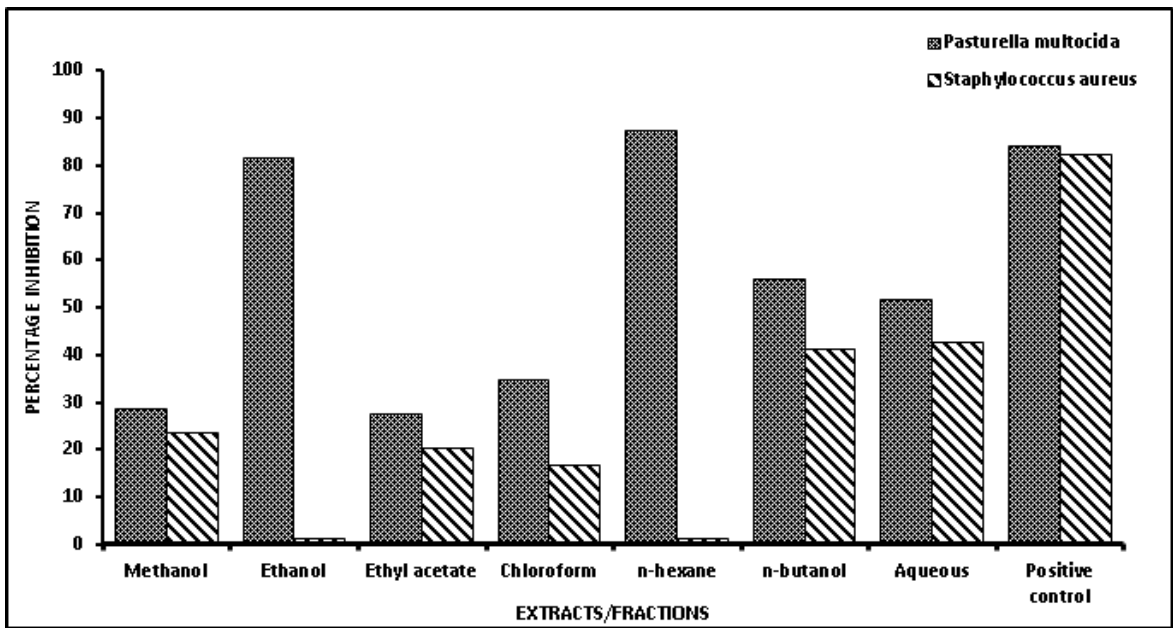

Figure 2 Biofilm (Quantitative) Assay
Data presented as percentage inhibition. Positive control: Ampicillin.

minimum (-2.421%) inhibition of *Pasturella multocida* strain. Percentage inhibition of other fractions for same strain in ascending order were methanol < ethyl acetate < chloroform < aqueous < *n*-butanol < ethanol. For *Staphylococcus aureus*, aqueous fraction showed maximum (42.47%) inhibition. Other fractions showed percentage inhibition in descending order as *n*-butanol > methanol > ethyl acetate > chloroform > ethanol > *n*-hexane. Confocal images of minimum and maximum biofilm inhibitions are presented in figure 3 (a-d).

Moselhy *et al*. (9) studied the bactericidal activity by using acetone and aqeous extracts of *Stevia rebau-*

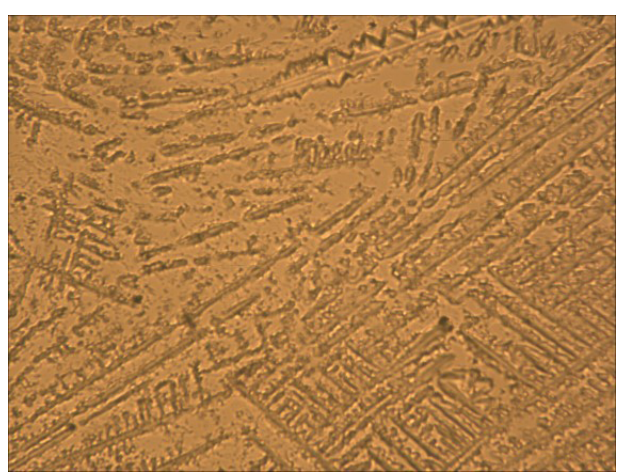

Figure 3(a-d). Confocal images of antibiofilm assay
a: maximum inhibition of *Pasturella multocida* by *n*-hexane fraction, b: minimum inhibition of *Pasturella multocida* by ethyl acetate fraction, c: maximum inhibition of *Staphylococcus aureus* by aqeoues extract, d: minimum inhibition of *Staphylococcus aureus* by ethanol fraction

diana leaves toward five bacterial strains. They showed that *S. rebaudiana* acetone extract has the ability to inhibit the growth of certain bacteria which explains its usage in the cure of various pathogenic diseases.

Conclusion

The current study highlighted significant antioxidant, antidiabetic, antiamnesic, cytotoxic, thrombolytic and antibiofilm activities of *Stevia rebaudiana* leaves that might be significant for the management and treatment of various diseases. All these results are quite thought-provoking. Multifaced benefits of *S. rebaudiana* make it worth investigation further.

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Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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