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Exploring the Pharmacological Potential of *Smilax china*

Fatma Hussain*, Sunder Unab and Muhammad Shahid
Department of Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad-38040, Pakistan

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Abstract. *Smilax china* (China root) has been mostly used in conventional medications for the treatment of numerous ailments. In current study, root extracts and fractions of *S. china* were assessed for antioxidant, flavonoid, phenol contents, antidiabetic (antiglycation, alpha amylase, acetylcholinesterase inhibition), cytotoxicity (DNA protective, hemolytic tests), thrombolysis and antibiofilm efficacies. The methanolic extract was fractionated into *n*-butanol, *n*-hexane, chloroform, ethanol, ethyl acetate solvents along with aqueous fraction. Results indicated that *n*-hexane had maximum DPPH scavenging potential (34%, $P<0.05$), while highest flavonoid (22.6 mg CE, $P<0.05$) and phenols (24.06 mg GAE, $P<0.05$) were observed in ethyl acetate and *n*-butanol respectively. Ethanol sample exhibited significant glycation inhibition (70%; $P<0.05$), whereas methanol and ethanol fractions indicated maximum alpha amylase (29.7%; $P<0.05$) and acetylcholinesterase (7.49%; $P<0.05$) inhibitions. Impairment of DNA was blocked by four solvents extracts; i.e. ethanol, ethyl acetate, *n*-hexane, *n*-butanol, whereas, *n*-hexane, aqueous fraction and *n*-butanol fractions showed optimum antihemolytic (20%), antifibrinolytic (32%) and anti-microbial (58%) potentials. It is recommended that *Smilax china* has tremendous potential to become a natural medicine supplement. However, it warrants further exploration in clinical trials with animal and human models.

Keywords: *S. china* root, oxidative stress, antidiabetic, cytotoxicity, antimicrobial

Introduction

Medicinal plants, enriched with different bioactive compounds can eliminate numerous diseases. These secondary metabolites include alkaloids, vitamins, sterols, saponins, glycosides, carotenoids, flavonoids, quinones, polyphenols and tannins. All over the world many plant species have been used due to their biological actions. Plants containing biologically active compounds have a medicinal history as long as the history of mankind. Different types of drugs are synthesized from medicinal plants because these are cheaper raw materials and accessible to poor communities (Unuofin and Lebelo, 2020). *Smilax china* L. (Smilacaceae) is a climbing herb found in moderate and humid regions worldwide. Chemical constituents of *S. china* are sugars, glycosides, starch, fat, saponins, smilacin, tannin, cinchonin, terpenes, alkaloids, flavonoids and gum. Polysaccharides isolated from *Smilax china* L. exhibited anti-inflammatory potentials by regulated biosignaling pathways (Zhang *et al*., 2019). Recently, it was studied by Tettey *et al*. (2020a) that *Smilax china* leaf extracts suppress pro-inflammatory adhesion response in human umbilical vein endothelial cells and proliferation of HeLa cells. Earlier, Yang *et al*. (2019) described that *Smilax china* usage in the diet helps to reduce weight gain, enhances lipolysis and beta oxidation as well as reduces lipogenesis in animal models. The underlying mechanism may be the activation of 5' AMP-activated protein kinase.

From roots, various compounds such as engeletin, kaempferol, kaemperol 7-*O*-beta-Dglucopyranoside, isoengeletin, dihydrokaempferol, dihydrokaempferol-5-*O*-P-D-glucopyranoside, rutin, kaempferol-5-*O*-beta-D-glucopyranoside,3,5,4 trihydroxystibene, beta-sdaucosterol, vanillic acid and beta sitosterol have been identified (Xu *et al*., 2019) due to which it exhibits biological activities against oxidative stress, diabetes mellitus, coagulation, microbial diseases, mutations, cancer and inflammation. It is most effective against kidney disease, acute bacterial dysentery, syphilis, stiffness of bone joints, dermal ailments, hepatic diseases, male fertility issues (Qadir *et al*., 2020; Saraswathi *et al*., 2012). Polyphenols present in *Smilax china* may destroy bacterial cell membrane and cell wall. This mechanism is speculated to be responsible for the potent antimicrobial activity of the plant, making it a promising therapeutic option as compared to synthetic drugs to combat microbial infections (Xu *et al*., 2019). Tettey and colleagues described endothelial-dependent vasodilation by *Smilax china* through PI3K and Akt signaling pathways (Tettey *et al*., 2020b).

Phenolic compounds of *S. china* are effective to treat hyperglycemia by increasing insulin secretion (Bhati

*Author for correspondence; E-mail: fatmauaf@yahoo.com

et al., 2011). *S. china* has the potential to eliminate the harmful effects of nicotine due to its antioxidant components. It enhances the activity of hepatic cytochrome P450 2A6 (Kim *et al*., 2014). Ahmad *et al*. (2019) used various fractions of *Smilax china* to assess antioxidant, phenolic, flavonoid components and enzyme inhibitory potentials. Results indicated maximal bioactivities by most of the samples. Recently, Feng *et al*. (2020) studied that flavonoids in *S. china* root inhibit inflammatory responses and it involves mediation by toll-like receptor (TLR-4) signaling process. Although diverse bioactivities of *S. china* root have already been explored, the knowledge about biological activities of *S. china* extracts using different polarity-based organic solvents is limited. So, this study was designed to investigate antioxidant, antidiabetic, cytotoxic, thrombolytic and antibiofilm activities of *S. china* roots. The current study presents a high level of originality concerning to pharmacological efficacies of *Smilax china* root. Six solvents along with aqueous extracts were used for the research, an experimental setup that might not be used for this plant previously. The bioactivities assessed are related to multiple pathological conditions such as oxidative stress, diabetes mellitus, microbial infections, DNA mutations, cytotoxicity and heart diseases. Findings have the potential to be used within multifactorial treatment domains.

Materials and Methods

Sample collection and preparation. *S. china* root were collected and methanol (MeOH) extracts were prepared (Nasir *et al*., 2012). Briefly, 100 g powdered sample and 1 L methanol were incubated for 3 days at ambient temperature. After filtration with Whatman number 1 filter paper, residues were extracted in MeOH twice by the same procedure. Then semi solid viscous extracts (filtrate) were dried on the water bath. This procedure was done thrice after four days.

Fractionation of methanolic extracts was done into different solvents such as *n*-hexane, *n*-butanol, ethanol, methanol, ethyl acetate and chloroform at the ratio of 1:10:10. (extract: dist. water: solvent) in separating funnel (Mehmood *et al*., 2012). Yields obtained were as follows: *n*-hexane (25 g), *n*-butanol (20 g), ethanol (60 g), ethyl acetate (5 g), chloroform (10 g) and aqueous (10 g).

Equipments. Following equipment were used: spectrophotometer (UV-188 Shimadzu, Japan), incubator (89L Korea), weighing balance (ATY224, Shimadzu, Japan), water bath (WB-22-P Germany), gel document system (AlphaImager^{TM} Gel Imaging system, Alpha Innotech, Germany), micro centrifuge machine (H1500FR, Cyprus).

Chemicals, reagents. Laboratory grade chemicals, reagents and enzymes from Sigma-Aldrich, Germany were used. These included methanol, *n*-hexane, *n*-butanol, ethanol, ethyl acetate, chloroform, DPPH (2, 2-diphenyl l-picrylhydrazyl, $AlCl_3$, potassium acetate, Folin- Ciocalteu reagent, sodium carbonate, glucose, bovine serum albumin, Na_2HPO_4, starch, alpha-amylase enzyme (porcine pancreatic), DNS (dinitro-salicylic acid) reagent, DTNB (5, 5-dithio-bis-2-nitrobenzoic acid), acetylcholinesterase enzyme (*Electrophorus electricus*), acetylcholine iodide, physostigmine, metformin hydrocholoride, acarbose, supercoiled pBR322 plasmid DNA, Fenton's reagent, agarose, indigo dye, nutrient broth and ampicillin. Microbial strains were procured from Industrial Biotechnology Laboratory, Department of Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad.

Antioxidant potential. *DPPH radical scavenging assay.* Approximately 40 µL sample was mixed with 300 µL DPPH (2, 2-diphenyl l-picrylhydrazyl) (Souri *et al*., 2008) and after incubation (30 min) absorbance was measured (517 nm). Calculation: % radical Scavenging (IC_{50}) = 100 × [$OD_{(control)}$ – $OD_{(sample)}$ / $OD_{(control)}$].

Antioxidant content. *Total flavonoid estimation by aluminium chloride colourimetric assay.* 0.5 mL of fractions, 0.1 mL of 10% $AlCl_3$, potassium acetate (1 M, 0.1 mL), distilled H_2O were mixed, incubated at ambient temperature (30 min) and absorbance at 415 nm was measured. Data were calculated as mg catechin equivalents (CE)/100 g dry weight (Siddique *et al*., 2010).

Total polyphenol estimation by Folin- Ciocalteu (FC) assay. According to Chahardehi *et al*. (2009), 100 µL FC reagent, 20 µL sample, 1% Na_2CO_3 (300 µL), 1.58 mL dis. H_2O were combined and incubated (30 min). Absorbance (760 nm) was used to calculate and present phenol concentrations (mg gallic acid equivalents (GAE)/100 g dry weight).

Anti-diabetic activity. *Assay of protein glycation inhibitory potential.* Antiglycation activity was estimated by the prescribed technique (Matsuda *et al*., 2003).

Glucose (100 mg/ 2 mL) solution, bovine serum albumin solution (prepared in 67 mM phosphate buffer of pH 7.4) and test samples (67 mM phosphate buffer; pH 7.4) each about 250 µL were mixed and placed at 37 °C for eleven days along with positive control or PC (metformin) and negative control or NP (reaction mixture without synthetic inhibitor or plant extract). Absorbance was taken at 440 nm. % inhibition: 100 × (control NP optical density – sample optical density$_{(sample)}$ / control NP optical density).

Assay of alpha amylase inhibitory potential. Following the reported method (Apostolidis *et al*., 2006), 100 µL of 20 mM phosphate buffer (pH 6.9) with 1 mg/ 2 mL α-amylase (Porcine pancreatic enzyme, Sigma Aldrich) with 100 µL extracts was incubated for 90 min at 25 °C. After the addition of 100 µL starch solution and incubation (30 min) DNS (dinitro-salicylic acid) reagent (5 mL) was mixed in it, heated in the water bath (5 min). The positive control was glucobay (acarbose) and negative control had mixture but no plant sample extract. Absorbance at a wavelength of 540 nm was measured. Inhibition was estimated as: (control sample absorbance – test sample absorbance $_{(sample)}$ / control sample absorbance) × 100.

Acetylcholinesterase inhibitory potential. The mixture had 2.8 mL buffer (15.6 g Na_2HPO_4 dissolved in 750 mL dis. H_2O), 100 µL DTNB (5, 5-dithio-bis-2-nitrobenzoic acid) stock solution, 30 µL acetylcholinesterase enzyme (Electrophorus electricus Sigma-Aldrich Germany) and 30 µL test sample. Incubated (15 min) at 25 °C and mixed with 30 µL acetylcholine iodide as substrate (Rahman *et al*., 2005). As a positive control, physostigmine and as a negative control, reaction mixture without sample was used. After taking absorbance at 412 nm, the inhibition percentage was estimated as: (control sample absorbance – test sample absorbance $_{(sample)}$ / control sample absorbance) × 100.

Cytotoxic activity. ***DNA protection test.*** Test samples (10 µL) were mixed with super coiled pBR322 plasmid DNA and kept for 5 min at 37 °C followed by the addition of 10 µL Fenton's reagent (Ruma *et al*., 2013). After incubation for 30 min, the analysis was done by running 1% electrophoresis (agarose gel). Positive control had Fenton's reagent and plasmid, while negative control was plasmid only. The bands that appeared on the gel were examined by the gel document system.

Assay of Anti-hemolyzing potential. Blood samples (3 µL) were centrifuged (1500 rpm). Pellets of blood clots were washed with phosphate buffer saline (PBS; pH 7.4) thrice and centrifuged. Suspended blood was mixed with 20 µL extracts and incubated together (30 min; 37 °C). After centrifugation (1500 rpm, time: 5 min), phosphate buffer was added to the supernatant (Powell *et al*., 2000). The absorbance taken at 576 nm was used for calculations: % hemolytic effect =

$$\frac{\text{Sample solution absorbance} - \text{negative control solution absorbance}}{\text{Positive control solution absorbance} - \text{negative control solution absorbance}} \times 100$$

Determination of thrombolytic effect. Clot (C) lysis ability was estimated by previously reported protocol (Hossain *et al*., 2012). At 37 °C, after clot formation in 2 mL blood, serum was separated. The weight of C before lysis (Wb) was calculated from the weight of unfilled tubes and filled tubes. Sample (100 µL) was added and incubated overnight at above-mentioned temperature. Weight of tubes was measured and C weight after lysis (W_1) was calculated from the difference in weights of unfilled and filled tubes (having clot). Calculation: Clot lysis % = 100 × (Wb – W_1/ Wb). Positive (citric acid) and negative (distilled water) controls were used.

Biofilm inhibitory activity. *Staphylococcus aureus* and *Pasteurella multocida* were used. For 24 h nutrient broth, extracts, micro-organisms (100 µL each) were incubated (37 ºC). Then stained with 2% crystal indigo dye (Sabir *et al*., 2014). Positive control contained ampicillin and nutrient broth. The inhibitory percentage was determined after measuring absorbance (630 nm): (Optical density of control minus Optical density of sample/Optical density of control) × 100.

Statistical analysis. All data was analyzed by SPSS software (version 22) and results were expressed as significant when $P<0.05$.

Results and Discussion

Antioxidant profile. Free radicals are generated in the human body during various metabolic processes and imbalance between free radicals and inherent antioxidants leads to pathological conditions. Antioxidants present in medicinal plants offer protection from diseases. Phytochemicals like flavonoids, polyphenols, alkaloids, etc. are predominant antioxidant

constitutes present in it. All of these are responsible to eradicate free radicals that are postulated to confer numerous diseases (Unuofin and Lebelo, 2020).

Results of the antioxidant profile are presented in Fig. 1. Highest DPPH scavenging ability (34 %; P<0.05) was exhibited by *n*-hexane fraction and results were as: aqueous < ethyl acetate < methanol < ethanol < chloroform = *n* butanol. Previously, *S. china* root extract in methanol exhibited a 7.4 µg/mL IC_{50} value, while assay also showed high levels of DPPH free radical scavenging action in ethyl acetate, butanol and aqueous extracts (Lee *et al*., 2001). Similarly, Seo *et al*. (2012) reported 30 and 70 µg/mL IC50 values in methanolic and water extracts of *S. china* root respectively. Kim *et al*. (2013) reported 10% DPPH radical trapping efficacy in water extracts of *S. china* root. Ahmad *et al*. (2019) documented 79.33±0.15% (methanol), 67.55±0.26% (chloroform), 59.26±0.13% (*n*-butanol) and 52.96±0.58% (benzene) radical trapping potentials of *S. china* root extracts. Results of antioxidant activities in the current study are contradictory to those reported earlier. These variations may be due to a different range of bioactive constituents distributed in organic fractions of *S. china*, method of extraction, post-extraction processing, and plant maturity (growth phase) stage (Rojo-Gutiérrez *et al*., 2020).

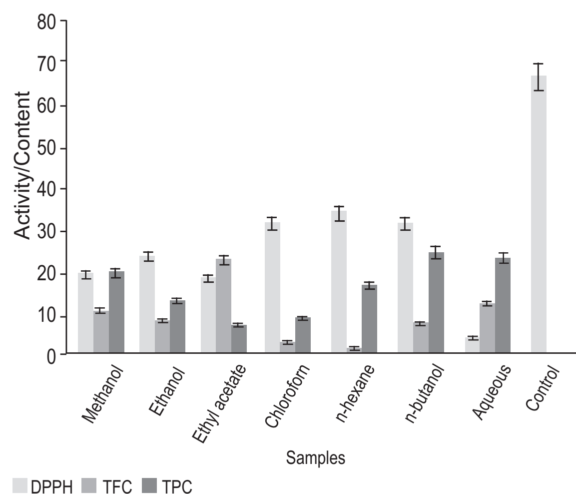

Fig. 1. Antioxidant profile of *S. china* root extracts and fractions. [data is represented as percentage or mean±S.D (standard deviation). DPPH: 2, 2-diphenyl l-l- l-picrylhydrazyl; TFC: total flavonoid contents (mg CE/100 g dry weight); TPC: total phenolic contents (mg GAE /100 g dry weight), BHT: butylated hydroxy toluene (positive control for DPPH assay)]

Highest flavonoid contents (22.6 mg/100 g CE; P<0.05) were found in ethyl acetate fraction and other fractions showed TFC in ascending order as: *n*-hexane < chloroform < *n*-butanol < ethanol < methanol < aqueous. Ahmad *et al*. (2019) observed 41.71-96.80 mg quercetin equivalent/g TFC in ethyl acetate and *n*-hexane fractions of *S. china* root. Contrary to that, Kim *et al*. (2013) reported 1.25 mg quercetin equivalents/g FC in *S. china* root water extracts.

TPC was 6.86 to 24.06 mg/100 g GAE (P<0.05) and ascending tendency was: ethyl acetate < chloroform < ethanol < *n*-hexane < methanol < aqueous < *n*-butanol. In a previous study, Hee-Sun *et al*. (2006) estimated 36 mg GAE/g and Shim (2011) found 1954.97±6.82 µg GAE/mL polyphenols in ethanolic and water extracts of *S. china* root. Our results are partially following those reported previously that water extracts of *S. china* roots have 39.50 mg GAE/g total phenols (Ahmad *et al*., 2019). The major difference in results may be due to the fact that phenolic contents in *S. china* root are varied depending upon different extraction methods, processing, units of measurement and sample age (Rojo-Gutiérrez *et al*., 2020).

Antidiabetic activity. Anti-glycation efficacy, alpha amylase and acetylcholinesterase inhibitory activities were measured. Proteins amino groups react with reducing sugars and as a result, advanced glycation endproducts (AGEs) are formed. These compounds play a significant role in the manifestation of complications present in diabetic patients. Acetylcholinesterase (AChE) is an utmost significant enzyme for cholinergic neural system involved in the breakdown of acetylcholine and choline esters. Inhibitors of AChE have impact on cholinergic transmission with significant therapeutic efficacy. Alpha amylase inhibition leads to cure diabetes as it is responsible for catalyzing carbohydrate in the human body (Alam *et al*., 2019; Mathew and Subramanian, 2014).

Results of the antidiabetic profile are summarized in Fig. 2. Significant inhibition of AGEs ranged from 26 to 70% (P<0.05). Inhibition (%) in downward direction was: ethanol > ethyl acetate > chloroform > *n*-hexane > aqueous > methanol > *n*-butanol. The comparable outcome was revealed by another study showing 60.53±3.21% glycation inhibition by *Smilax glabra*

(Choi *et al*., 2015) and during a glycation inhibition assay, *S. china* showed 145±8 µg/mL IC_{50} value (Lee *et al*., 2017). Methanol fraction exhibited optimum (29.7%; P<0.05) alpha amylase inhibitory potential while other fractions displayed this activity with rising order as: aqueous < ethanol < ethyl acetate < *n*-hexane < *n*-butanol < chloroform. In a previous study, *S. china* root powdered sample reconstituted in dimethyl sulphoxide inhibited alpha amylase activity by 80% (Xiao-Ping *et al*., 2010). This higher alpha amylase restriction factor was due to the use of isolated specific active constituent, sarsasapogenin from *S. china* rather than extracts and fractions as these may be containing many other compounds. Similarly, methanol fraction of *Smilax excels* leaf inhibited alpha amylase activity up to 98.5% (Dehghan *et al*., 2016). AChE inhibition percentage of *S. china* was in the range of 2.07-7.49% (P<0.05) and these results are not in agreement with those reported by another study. Ahmad *et al*. (2019) stated that benzene fraction of *S. china* root samples revealed 82 percent inhibition of acetylcholinesterase.

Diabetes leads to progressive neuro-degenerative complications. Plant-based AChE inhibitors offer a major therapeutic option to deal with antineurotic effects (Abhijit *et al*., 2017). The phytoconstituents such as alkaloids, kaempferol and sarsasapogenin were responsible for the antidiabetic potential of *S. china*

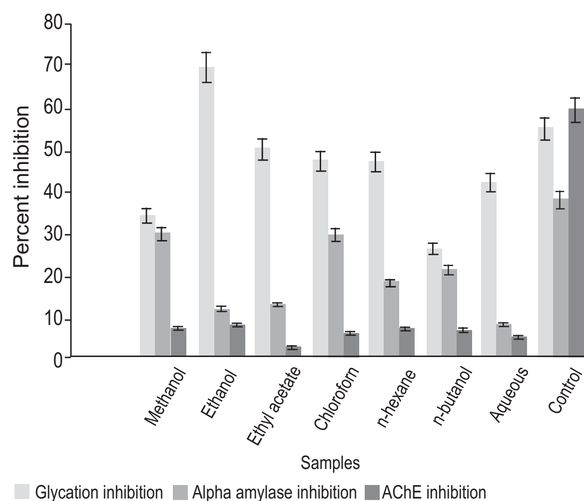

Fig. 2. Antidiabetic profile of *S. china* root extracts and fractions. [data is represented as percentage ± S.D. Positive controls: metformin (antiglycation), glucobay (alpha amylase inhibitory assay), physostigmine (acetylcholinesterase inhibitory assay)]

(Shahrajabian *et al*., 2019; Xiao-Ping *et al*., 2010; Lee *et al*., 2001).

Cytotoxic activity. *DNA damage protection assay.* Hydroxyl ions as strong oxidants damage the DNA and protein by changing their nature and metal-binding sites. The action of hydrogen peroxide creates free radicals. Sedative life-style, harmful environmental agents and dietary habits are factors associated with DNA damage (Barzilai and Yamamoto, 2004). The current research work evaluated the DNA damage protection and free radicals eradicating activity of bioactive constituents distributed in organic fractions of *S. china*.

As it is shown in Fig. 3, methanol, chloroform and aqueous fractions did not protect DNA strand breakage against H_2O_2 induced damage through Fenton's reaction. It is believed that the presence of flavonoids protects the cell membrane and DNA strand as the structure of flavonoids has hydroxyl group in B-ring but there is rare evidence in literature regarding the DNA protecting role of *S. china* root extracts. Oxidative DNA damage protectors of plant origin offer the therapeutic interventional strategies for diverse clinical disorders such as cancer, cataract formation, myocardial ischemia, Alzheimer's disease, etc. (Sultana *et al*., 1995).

Hemolytic activity. Erythrocyte membrane includes fatty acids which are more prone to free-radical induced peroxidation. Hemolysis is mostly triggered by oxidation reactions that cause lipid peroxidation. Secondary plant metabolites are excellent protectors of lipid peroxidation and results of hemolytic activity are conferred in Fig. 4. As far as the literature review could ascertain, screening of *S. china* hemolytic activity has never been reported. Therefore, the data presented here could be assumed as the first record for the literature. *n*-hexane fraction showed maximum (20%) hemolytic effect, while remaining fractions exhibited ascending trend as: ethyl acetate < ethanol < methanol < *n*-butanol < aqueous < chloroform. Earlier, *Smilax macrophylla* leaves showed % lysis in the range of 3.41-8.48, comparable with results of a previous study (Zubair *et al*., 2017). Hirota *et al*. (2015) stated that ethanol, chloroform and *n*-hexane extracts of aerial parts of *Smilax larvata* showed 40 to 100% hemolysis. The hemolytic activity exhibited by extracts of *Smilax* genera is due to the saponins (Zubair *et al*., 2017; Hirota *et al*., 2015).

Thrombolytic activity. The prevalence of blood clotting problems in blood circulation is increasing day by day.

Emboli in blood vessels block the blood flow leading to low oxygen tension and blood in tissues. Plasmin blocks the fibrinogen activity as a result of which thrombin is formed. Results of fibrinolytic activity are shown in Fig. 4. In the following research, prominent clot lysis (32%) was exhibited by an aqueous fraction. Thrombolytic potential in the ascending pattern was: ethyl acetate < *n*-butanol < chloroform < *n*-hexane < ethanol < methanol. No previous exploration was found on the clot lysis ability of *S. china*. Literature survey

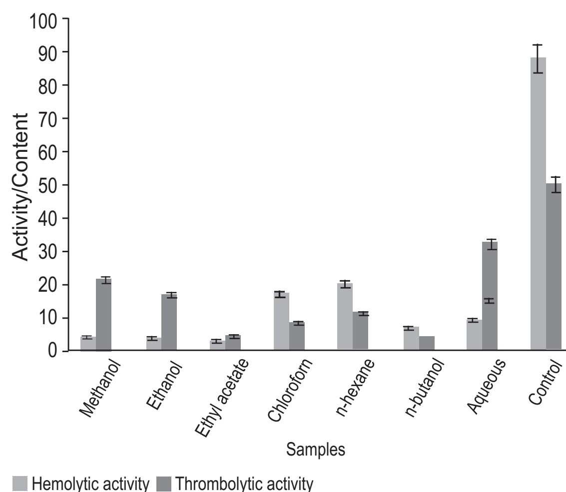

Fig. 4. Hemolytic and thrombolytic assay of *S. china* root extracts and fractions. [data is presented in percentage ± S.D. Positive control (ampicillin). Positive controls: citric acid (thrombolytic assay) and triton-x (hemolytic assay)]

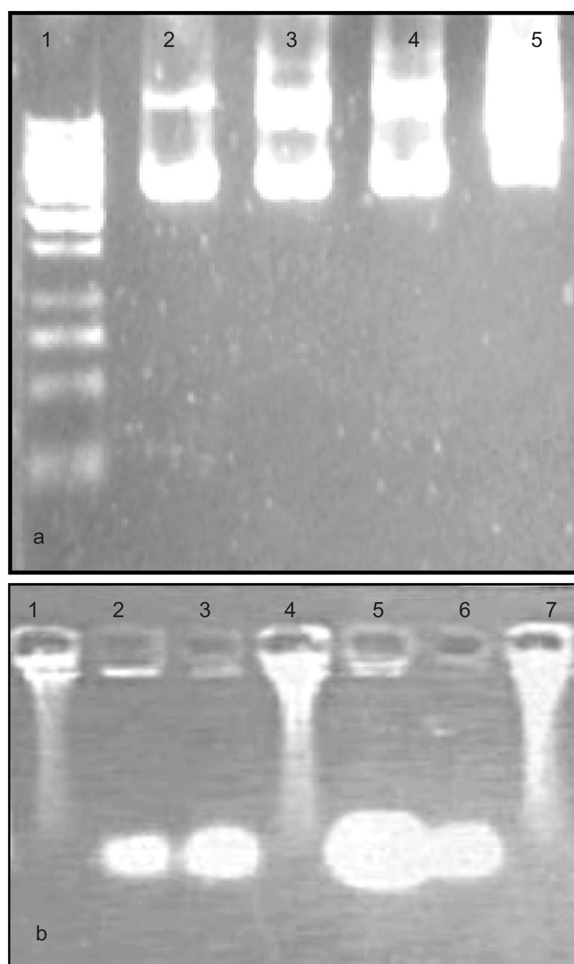

Fig. 3. DNA-damage protection electrophoretic analysis **(a)** Controls. 1: ladder, 2: positive control, 3:negative control ($FeSO_4$), 4 and 5: negative control (H_2O_2), **(b)** Samples: 1: DNA + methanol extract, 2: DNA + ethanol extract, 3: DNA + ethyl acetate extract, 4: DNA + chloroform extract, 5: DNA + *n*-hexane extract, 6: DNA + *n*-butanol extract, 7: DNA + aqueous extract.

of *Smilax zeylanica* indicated that its ethanolic leaf extract had marked clot lysis ability i.e. 43.35% (Hossen *et al*., 2014). Thrombolytic agents present in medicinal plants imply the clot lysis potential by activating plasminogen that further dissolves fibrin meshwork (Ali *et al*., 2013).

Biofilm inhibitory assay. Biofilms are microbial colonies that grow and adhere to a particular place. The presence of biofilms in industrial equipment contaminates the food products. The discovery of the natural antibiofilm agents is the prerequisite. Results of antibiofilm activity are depicted in Fig. 5. Optimal and nominal growth-inhibitory potentials of tested strains are presented in Fig. 6. Maximum microbial growth inhibitions were shown by *n*-butanol (58%) and ethyl acetate (56.29%) fractions respectively. Ascending trend of inhibition was revealed by all samples. The antibacterial potential of different extracts of *S. china* is attributed to its phytonutrients. Similarly, in another study, Nawi *et al.* (2010) investigated that *S. china* root extract of methanol inhibited *S. aureus* growth (2.00±0 mm inhibition zone).

The use of *S. china* roots offers the promising strategy to fight against oxidative stress, diabetic complications, cancer, neurodegenerative disorders, bacterial infections and so forth. There is limited research work on this

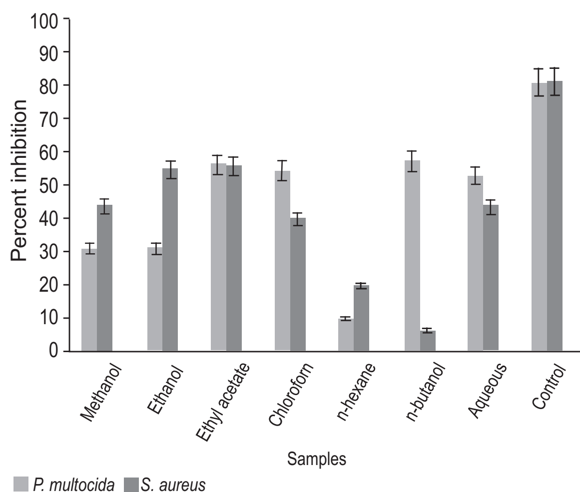

Fig. 5. Biofilm inhibition assay of *S. china* root extracts and fractions. [data is presented in percentage ± S.D. Positive control (ampicillin)]

plant, so there is further need to explore *S. china* bioactive constituents and their medicinal efficacies.

Conflict of Interest. The authors declare no conflict of interest.

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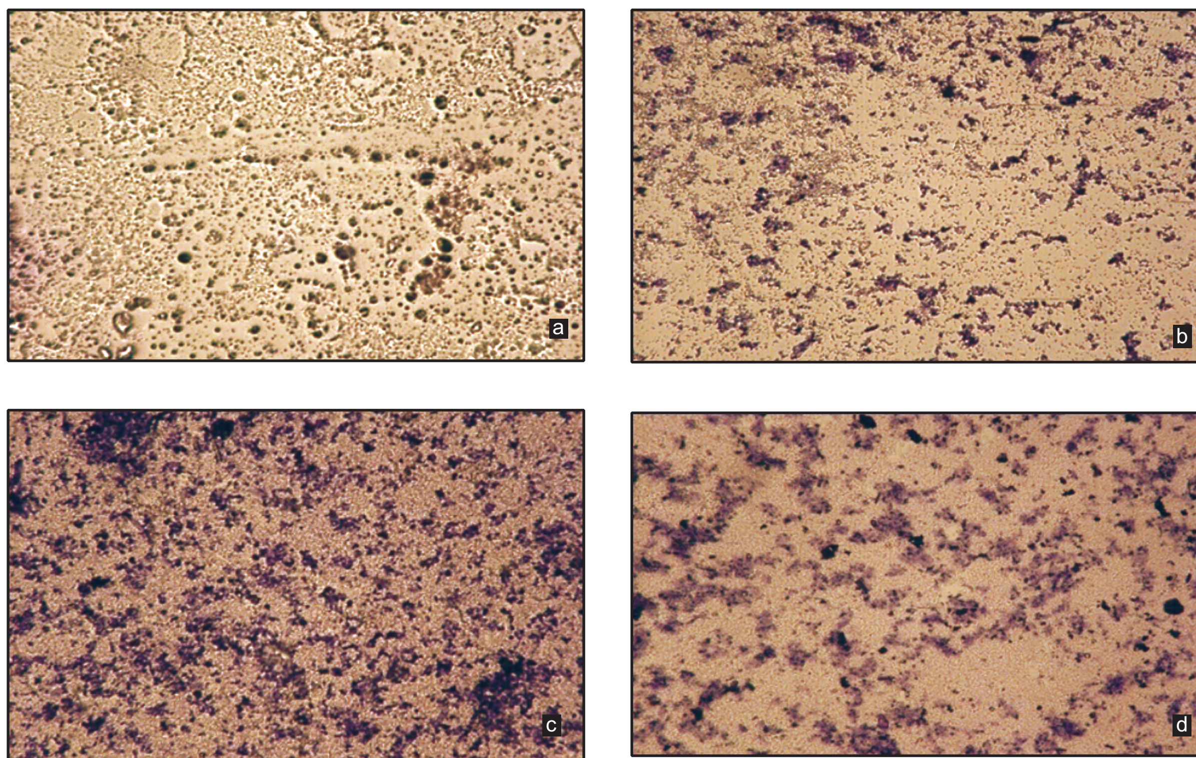

Fig. 6. Biofilm inhibition assay
a and **c** = Minimal microbial growth inhibition indicated by emerging *S. aureus P. multocida* colonization respectively,
b and **d** = Maximal microbial growth inhibition indicated by diminished colonies of *S. aureus* and *P. multocida* respectively.

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