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# Anticoagulant, Antiherpetic and Antibacterial Activities of Sulphated Polysaccharide from Indian Medicinal Plant *Tridax procumbens* L. (Asteraceae)

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**Abstract** The sulphated polysaccharide from the widespread *Tridax procumbens* plant was studied for the anticoagulant, antiherpetic and antibacterial activity. The anticoagulant activity was determined by the activated partial thromboplastin time assay. The sulphated polysaccharide from *T. procumbens* represented potent anticoagulant reaching the efficacy to heparin and chondroitin sulphate. Moreover, the sulphated polysaccharide extracted from *T. procumbens* was found non-toxic on Vero cell lines up to the concentration of 200 μg/ml. Sulphated polysaccharide exhibited detectable antiviral effect towards HSV-1 with $IC_{50}$ value 100–150 μg/ml. Furthermore, sulphated polysaccharide from *T. procumbens* was highly inhibitory against the bacterial strains *Vibrio alginolyticus* and *Vibrio harveyi* isolated from oil sardine.

**Keywords** *Tridax procumbens* · Sulphated polysaccharide · aPTT · HSV-I

## Introduction

From very early times, medicinal plants have been a rich source of biologically active compounds and play an important role in drug discovery. Researchers have cast a sharper eye on natural products to get medicinally important compounds from plants. Mehta et al. [1] reported antioxidant elements from seeds of *Emblica officinalis* as detected by laser-induced breakdown spectroscopy. It was found that *E. officinalis* extract effected antioxidant enzymes and has the potential acted as an agent to boost the antioxidant system in the diabetic animal model. Rai et al. [2] proved that freeze-dried rhizome powder of *Curcuma longa* dissolved in milk increased high-density lipoprotein and haemoglobin with significant decrease in the levels of blood glucose, lipid profile and hepatoprotective enzymes in diabetic rats and therefore can be used as antidiabetic dietary supplement. Rai et al. [3] explored flavonoids from aqueous extract of medicinal

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plant *Cynodon dactylon* with marked antioxidant efficacy on diabetes-induced diabetic rats. Furthermore, aqueous extract of *C. dactylon* showed protective role against carbofuran-induced oxidative stress thereby inhibiting level of acetylcholinesterase in the brain of model rats [4]. Moreover, ethanolic extract of *C. dactylon* finds its application as antidiabetic agent of high potential in diabetic models against hepatic complications [5].

Many medicinal plants have been reported with hypoglycaemic effects as they contain terpenoids, iridoid, glycosides, flavonoids and other phenolic compounds [6]. It was reported that aqueous extract of *Trichosanthes dioica* showed remarkable effect on severe diabetes mellitus by decreasing blood glucose, postprandial glucose, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, urine sugar and urine protein [6]. Researchers have extensively explored antidiabetic effect of aqueous extract of *T. dioica* fruits in diabetic rats by inhibiting CYP450 metabolising gene expression levels [7]. Various reports have also suggested that polyphenolic compounds, such as flavonoids, flavonols and terpenoids of plant origin, possess antioxidant activity and find the application in place of the synthetic antioxidants. Sharma et al. [8] reported that aqueous extracts of leaves of some medicinal plants such as *T. dioica*, fruits of *Moringa oleifera* and *Ficus benghalensis* as well as seeds of *E. officinalis* were rich sources of phenolics, flavonoids and flavonol contents and hence proved good candidates as natural antioxidants.

Different plant parts like root, stem, flower, fruit, twigs exudates and modified plant of medicinal plants represent a rich source of antimicrobial agents. Mishra et al. [9] investigated antioxidant and antistaphylococcal activities of different solvent extracts of *Bauhinia variegata*, *Tinospora cardifolia* and *Piper longum* and found that phytochemicals play as potential antioxidants and antimicrobials. Further, they reported that acetone extract of bark and petroleum ether and ethanol extract of leaf of *Cinnamomum zeylanicum* exhibited complete inhibition of growth of two species of dematiaceous moulds, *Alternaria solani* and *Curvularia lunata* [10].

Sulphated polysaccharides are a class of molecules characterised by a plethora of biological activities with often favourable tolerability profiles in animals and humans. Sulphated polysaccharides are widely distributed among marine plants and animals. Sulphated polysaccharides have a broad range of important bioactivities comprising antioxidant, anticoagulant and antithrombotic activities. They are also known to increase the resistance to some virus and inhibit some tumour development [11]. These properties mainly depend on the presence and spatial positioning of their sulpho groups.

Anticoagulant activity is the most widely studied properties of sulphated polysaccharides. Sulphated polysaccharides are either extracted from marine algae [12], invertebrates [13] or obtained by chemical sulphation of natural polysaccharides.

*Tridax procumbens* L. (Asteraceae) is best known as a widespread weed and pest plant. It is widespread throughout India and is employed as indigenous medicine for a variety of ailments, including jaundice and liver disorders [14]. It is commonly known as “Ghamra” in Hindi and in English “Coat buttons” because of the appearance of its flowers. It has been extensively used as Indian traditional medicine as antifungal, antibacterial and insect repellent in bronchial catarrh, diarrhoea and dysentery [15]. It possesses wound healing activity and promotes hair growth [14]. It has also been known for antioxidant properties [16] and antidiabetic properties [17]. Aqueous extract of *T. procumbens* has been studied for decreasing arterial blood pressure at greater level.

The objective of the study was to demonstrate that terrestrial plant *T. procumbens* exhibit potent anticoagulant, antiviral and antibacterial properties. Therefore, in the present study,

the chemical structure, anticoagulant, cytotoxic, antiviral and antibacterial properties of sulphated polysaccharides isolated from *T. procumbens* were studied.

## Materials and Methods

### Materials

*T. procumbens* L. (Asteraceae) leaves were collected from the fields of SRM University washed and dried at room temperature. Standard heparin, chondroitin sulphate and dermatan sulphate were purchased from the Sigma-Aldrich Chemie (Steinheim, Germany). Platelin L-activated partial thromboplastin time (aPTT) reagent was from Biomed (H.P., India) and toluidine blue and 1,3-diaminopropane were purchased from Himedia (Mumbai, India). All other solvents and chemicals were of analytical grade. HSV-I viruses were obtained from National Institute of Virology, Pune, India, and Vero cell lines were obtained from National Center for Cell Sciences, Pune, India.

### Extraction and Purification

The procedure of Holick et al. [18] was followed in the present study to extract sulphated polysaccharides from the leaves of *T. procumbens* by treating it with cetylpyridinium chloride (CPC). One hundred grams of dried sample were ground and incubated with 2 l of 0.4 M sodium sulphate ($Na_2SO_4$) at 55 °C for 1.5 h. The pH was maintained at 11.5 using sodium hydroxide (NaOH). After incubation, aluminium sulphate ($Al_2SO_4$) was added to bring down the pH to 7.7 and heated to 95 °C for 1 h. The sample was then filtered through a cheese cloth and treated with CPC. To the collected filtrate, 135 ml of 3% CPC in 0.8 M sodium chloride (NaCl) was added. The suspension was incubated at 37 °C for 18 h, and a white precipitate was formed. It was then centrifuged at 3,000×*g* for 30 min at 4 °C in a refrigerated centrifuge to collect the crude sulphated polysaccharide complex. The precipitate was redissolved in 135 ml of 2 M NaCl (40 °C) to remove pyridinium salts from the compound. The mixture was filtered through a Whatman no. 1 filter paper and three volumes of 95% ethanol to precipitate the crude sulphated polysaccharides followed by centrifugation at 3,000 rpm at 4 °C for 30 min. The precipitate was washed twice with 99.9% methanol and diethyl ether and dried by keeping in vacuum desiccators.

Acetone fractionation was used for further purification. Crude polysaccharide extract (50 mg) was dissolved in 1 ml of 0.15 M NaCl. After centrifugation, acetone (400 μl) was added to the supernatant. The resulting solution was kept in 5 °C for 24 h, and the precipitate formed was collected and dried [19].

### Gel Electrophoresis

The sulphated polysaccharide from *T. procumbens* was analysed by agarose gel electrophoresis as described by Santos et al. [20]. The samples (50 μg) were applied to a 0.5% agarose gel in 0.05 M 1, 3-diaminopropane/acetate buffer (pH 9) and run for 1 h at 110 V. After electrophoresis, the sulphated polysaccharides in the gel were fixed with 0.1% cetyltrimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, *v*/*v*) and destained with the same solution without toluidine blue.

## Sulphate Estimation

Total sulphate content was measured by the $BaCl_2$/gelatine method [21].

## Fourier Transform-Infrared Spectroscopy

The Fourier transform-infrared spectrum (FT-IR) is recorded with a RX1, Perkin Elmer Spectrometer between 400 and 4,000 $cm^{-1}$. The samples (10 mg) are analysed as a KBr pellet [22].

## Pharmacological Properties

### *Anticoagulant Activity*

Anticoagulant activity was determined by measuring the activated partial thromboplastin time of normal human plasma supplemented with standard heparin and sulphated polysaccharide from *T. procumbens*. The aPTT was measured by incubating 100 μl of test plasma (normal human plasma+*T. procumbens* extract) with 100 μl of platelin L aPTT reagent for 5 min at 37 °C. Clotting time was measured after addition of 100 μl of 0.025 M calcium chloride [23].

### *Cytotoxicity Assay*

Vero cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% ($CC_{50}$) was calculated as the compound concentration required reducing cell viability by 50%.

### *Antiviral Assay*

Different non-toxic concentrations of test drugs, i.e. lower than $CTC_{50}$, were checked for antiviral property by cytopathic effect (CPE) inhibition assay [24] against different virus challenge doses. In CPE inhibition assay, cells were seeded in a 24-well microtitre plate with 10,000 cells per well, incubated at 37 °C in a humidified incubator with 5% $CO_2$ for a period of 48 h. The plates were washed with fresh MEM and challenged with different virus challenge doses and incubated at 37 °C for 90 min for adsorption of the virus. The cultures were treated with different dilutions of sulphated polysaccharide from *T. procumbens* in fresh maintenance medium and incubated at 37 °C for 5 days. Every 24 h the observation was made and cytopathic effects were recorded. Anti-HSV-1 activity was determined by the inhibition of cytopathic effect compared with control, i.e. the protection offered by the test samples to the cells was scored. In virus yield assay, reduction in the yield of virus when cells were treated with the sulphated polysaccharide extracts was determined.

*Bacterial Isolation and Identification*

Bacterial strains (*Vibrio alginolyticus* and *Vibrio harveyi*) were isolated from the gills of oil sardine (*Sardinella longiceps*) purchased from the Royapuram fishing harbour (latitude 13°06′ N, longitude 80°18′ E) north of Chennai, India. Gills were crushed and plated on thiosulphate citrate bile salt sucrose agar (TCBS; Difco), selective to *Vibrionaceae*. Plates were incubated for 24 h at 30 °C and the colonies were isolated on tryptone soy agar to obtain a pure culture for identification, and biochemical tests were performed by the methods of Bergey's manual of systematic bacteriology [25].

*Antibacterial Susceptibility Test*

The antibacterial susceptibility test was performed to check antibacterial effect of sulphated polysaccharide isolated from *T. procumbens* against *V. alginolyticus* and *V. harveyi.* Antibacterial assay was performed by the disc diffusion method [26], and fresh overnight cultures of inoculum (0.1 ml) of each culture were spread on Muller–Hinton agar medium plate. One hundred micrograms of fractions was impregnated onto a small disc of sterile filter paper (diameter 5 mm) and placed on top of the seeded medium. In order to compare the activity of the test material, 10 μg norfloxacin disc was used. After overnight incubation at 37 °C in the incubator, the zones of inhibition were measured.

Statistical Analysis

Results are presented as mean±standard deviation ($n$=3). Student's $t$ test was used to determine the level of significance ($p<0.05$).

## Results and Discussion

Gel Electrophoresis

The electrophoretic migration of sulphated polysaccharide in the 1,3 diaminopropane/ acetate buffer depends on the structure of the polysaccharide, which forms the complex with the diamino buffer. Therefore, different electrophoretic mobilities of sulphated polysaccharides indicate differences in their structure. The sulphated polysaccharide from *T. procumbens* was purified with acetone fractionation and subjected to agarose gel electrophoresis which showed only one band migrated towards heparin standard, which confirmed the homogeneity of the active compound and presence of heparin-like sulphated polysaccharide (Fig. 1). The same results were reported in *Ecklonia cava* [27] where only one spot confirmed the presence of sulphated polysaccharide. Therefore, presence of single bands in electrophoretic system revealed their purity and homogeneity, which was supported by other results found in other species.

Sulphate Estimation

The sulphate content was found to be 2% which is significantly ($p<0.05$) less when compared to brown alga *Laminaria cichorioides* (2.19%) [28] and more than the *E. cava* (0.95%) [18]. The results confirm the presence of sulphated polysaccharides with high sulphate content. This may explain the high anticoagulant activity by the earlier reports as

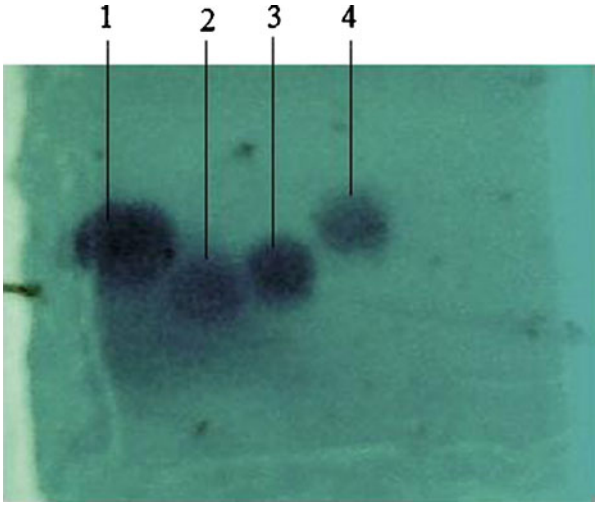


**Fig. 1** The electrophoretic migration of *T. procumbens*. *1* heparin, *2* chondroitin sulphate, *3* dermatan sulphate, *4 T. procumbens* sulphated polysaccharide

fucan sulphate with high sulphates and low uronic acid content are well documented for high anticoagulant activity [29]. This proves that the sulphate content vary with species and is responsible for anticoagulant activity.

## Fourier Transform-Infrared Spectroscopy

The FT-IR spectroscopic analysis was performed for the characterisation of *T. procumbens* for the presence of different groups (Fig. 2). Infrared spectroscopy provides useful information on the position of sulphate groups of polysaccharides. The IR spectrum of sulphated polysaccharide from *T. procumbens* showed an absorption band at 1,100–1,200 $cm^{-1}$, indicating the presence of sulphate esters. *T. procumbens* showed absorbance ranging from 3,160 to 3,640 $cm^{-1}$ that corresponds to (O–H) hydroxyl groups and peaks between 600 and 1,700 $cm^{-1}$ in the spectrum confirmed the presence of amide I group. The results were confirmed by the reports of Silva et al. [30] in seaweed *Padina gymnospora*, where absorption bands were observed at 1,264, 822 and 1,720 $cm^{-1}$. Therefore, the confirmation of chemical properties by different absorption peaks proves the presence of sulphated polysaccharide.

## Anticoagulant Activity

The anticoagulant properties of sulphated polysaccharide from *T. procumbens* were assessed by aPTT using human plasma. The normal values of aPTT for healthy human plasma were 32.5 s. Sulphated polysaccharides from *T. procumbens* prolonged aPTT and reached 113 s at 100 μg/ml which was approximately 4.0-fold compared with the saline group (the control). Higher concentrations of sulphated polysaccharide from *T. procumbens* were required to achieve ($p<0.05$) significantly the same effect as with heparin in the aPTT assay (Fig. 3). Anticoagulant activity by aPTT assay in brown alga *L. cichorioides* and brown seaweeds *P. gymnospora* was reported 5-fold lesser than standard heparin [28, 30]. Therefore, sulphated polysaccharide had aPTT effect, this being expected because of the presence of sulphate groups necessary to provide anticoagulant effects, but also dependent on the position of the sulphate groups as proved by other investigations.

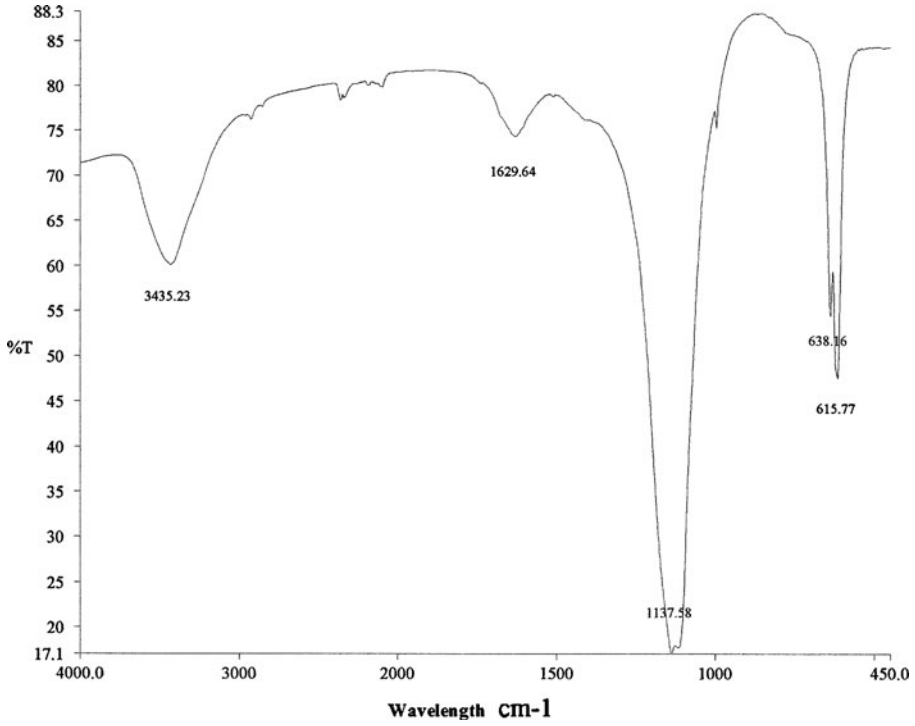


**Fig. 2** FT-IR spectra of sulphated polysaccharide extract of *T. procumbens* ranging from 4,000 to 450 $cm^{-1}$

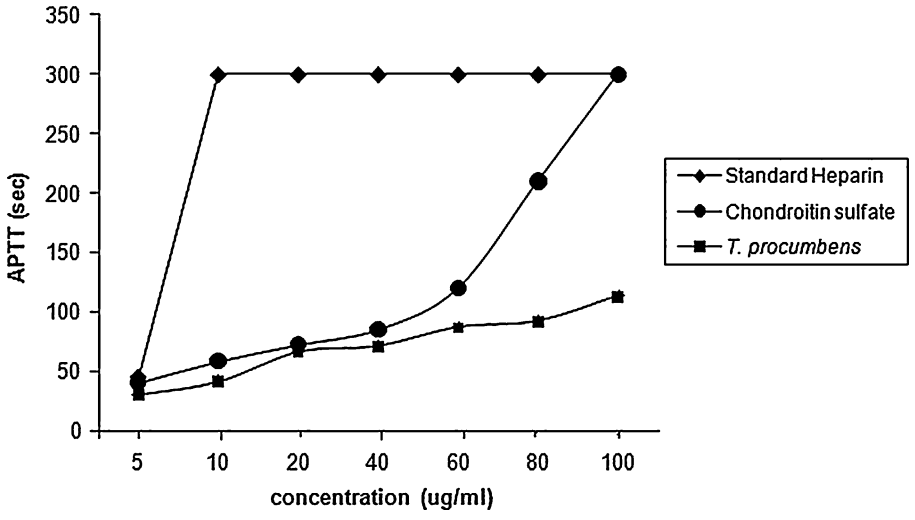


**Fig. 3** Anticoagulant activity of sulphated polysaccharide of *T. procumbens* measured by activated partial thromboplastin time (*aPTT*)

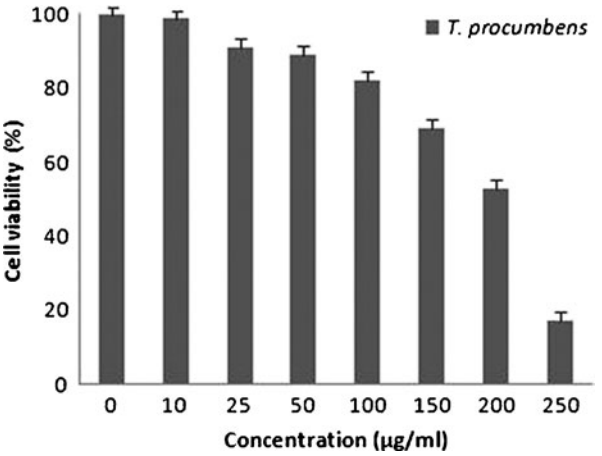


**Fig. 4** In vitro cytotoxicity assay of *T. procumbens* at different concentrations. All assays were performed in triplicates

## Cytotoxicity Assay

Sulphated polysaccharide from *T. procumbens* leaves was initially evaluated for cytotoxicity by assessing their effects on cell viability at concentrations ranging from 10 to 300 μg/ml and results revealed that sulphated polysaccharide was non-toxic up to concentrations 200 μg/ml with $CC_{50}$ value 200 μg/ml (Fig. 4). The results of cytotoxicity studies thus obtained indicate that the tested sample at different concentrations was cytotoxic only when the cultures are exposed to very high concentrations. High concentrations of any compound, under normal conditions, are cytotoxic to cell cultures [31]. Hence, the results showed weak cytotoxic properties of the sulphated polysaccharide of *T. procumbens* against Vero cell lines. Thus, it was found that sulphated polysaccharides were non-cytotoxic at lesser concentration.

## Antiviral Assay

The extract was evaluated for antiviral activity against HSV-1 strain by a virus plaque reduction assay at a concentration non-toxic to the cell line (Vero) used. There was a cytopathic effect between 10 to 50 μg/ml concentrations, however exhibited detectable antiviral effect towards HSV-1 with an inhibitory concentration for 50% ($IC_{50}$) at 100–150 μg/ml (Table 1). The similar studies were carried in red microalga *Porphyridium* sp. in which the polysaccharide was tested on HSV-1, the polysaccharide significantly inhibited viral infection at 100 μg/ml and the polysaccharide extract was able to inhibit the

**Table 1** Inhibition of HSV-I related CPE by using different concentrations of *T. procumbens*

| Concentration (μg/ml) | CPE inhibition (%) |
|---|---|
| 10 μg | 0 |
| 25 μg | 10 |
| 50 μg | 22 |
| 100 μg | 32 |
| 150 μg | 60 |
| 200 μg | 76 |
| 250 μg | 80 |

**Table 2** Biochemical tests for *V. alginolyticus* and *V. harveyi* isolates from oil sardine

| Biochemical tests | *Vibrio alginolyticus* | *Vibrio harveyi* |
|---|---|---|
| Gram staining | Gram-ve, short rods | Gram-ve, short rods |
| Growth on TCBS | Yellow colonies | Green colonies |
| Motility | + | + |
| Oxidase test | + | + |
| Catalase test | + | + |
| Amino acid decarboxylase test | | |
| Lysine | + | + |
| Arginine | − | − |
| Ornithine | + | + |
| Salt tolerance test (% NaCl) | | |
| 0% | − | − |
| 4% | + | + |
| 8% | + | + |
| 10% | + | − |
| 12% | + | − |
| Sugar fermentation test | | |
| Melibiose | − | + |
| Lactose | − | − |
| Sucrose | + | + |
| Dextrose | − | − |
| Xylose | − | − |
| Arabinose | − | − |
| Galactose | + | − |
| Mannose | + | + |
| Mannitol | + | + |

+ positive effect, − negative effect

development of the cytopathic effect in HSV-infected cells [32]. The potency of the sulphated polysaccharide in *T. procumbens* proved the inhibitory effect on the HSV-1 in the required concentration which was also strongly supported by earlier experiments carried out using sulphated polysaccharides.

## Isolation and Identification of Bacteria

The bacterial strains were isolated, maintained in pure culture and identified as *V. alginolyticus* and *V. harveyi* by using the biochemical tests like, arginine decarboxylase,

**Table 3** Results of antibacterial activity of sulphated polysaccharide of *T. procumbens*

| Bacteria | Concentration (μg/disc) | | Zone of inhibition (mm) | |
|---|---|---|---|---|
| | Norfloxacin | Extract | Norfloxacin | Extract |
| *Vibrio alginolyticus* | 10 | 100 | 33±0.41 | 19±0.57 |
| *Vibrio harveyi* | 10 | 100 | 31±0.32 | 16±0.51 |

Results are triplicates of mean±SD

ornithine decarboxylase, lysine decarboxylase, salt tolerance test and sugar fermentation test (Table 2). It has been previously reported that bacteria-like genus *Vibrio* are frequently isolated from marine fish such as *V. alginolyticus* in the gilthead seabream *Sparus aurata* [33], *V. harveyi* in seabass *Lates calcarifer* [34] and in the summer flounder *Paralichthys dentatus* [35]. Moreover, Alcaide et al. [36] reported *V. harveyi* from an outbreak in sea horses, *Hippocampus kuda*.

Antibacterial Susceptibility Test

In present study, the sulphated polysaccharide extract at concentration (100 μg/disc) was strongly inhibitory to *V. alginolyticus* and *V. harveyi* forming zone of inhibition with 19 and 16 mm, respectively (Table 3). Bacterial diseases in marine fishes caused by *Vibrio* sp. is one of the most important causes of economic losses. This bacterium is normally found in the marine environment, and the disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factors [37]. *Vibrio* sp. infections like gastroenteritis, wound infections and septicaemia are common, and transmission of *Vibrio* sp. infections is primarily through the consumption of raw or undercooked shellfish or exposure of wounds to warm seawater [38]. Many reports have explained antimicrobial activities of medicinal plants viz. solvent extracts of *B. variegata*, *T. cardifolia* and *P. longum* have strong antimicrobial activity against staphylococcal activities [9].

## Conclusions

The results present here revealed that the sulphated polysaccharides derived from *T. procumbens* showed excellent anticoagulant activity and can be useful in anticoagulant therapy. The extract does not have any toxic effect on the cell viability of Vero cell lines. The sulphated polysaccharide extract showed potent antiherpetic action against the HSV-I strain with $IC_{50}$ values between 150 and 170 μg/ml. Moreover, sulphated polysaccharide from *T. procumbens* was highly inhibitory to the bacterial against *V. alginolyticus* and *V. harveyi* isolated from oil sardine. Further in vivo studies relating to the antiviral efficacy of sulphated polysaccharide from *T. procumbens* in animals need to be initiated to determine their role as against HSV and *Vibrio* infections.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Mehta, S., Rai, P. K., Rai, D. K., Rai, N. K., Rai, A. K., Bicanic, D., Sharma, B., & Watal, G. (2010). *Food Biophysics*. doi:10.1007/s11483-010-9158-z.
2. Rai, P. K., Jaiswal, D., Rai, D. K., Sharma, B., & Watal, G. (2010). *Indian Journal of Clinical Biochemistry, 25*, 175–181.
3. Rai, P. K., Jaiswal, D., Rai, D. K., Sharma, B., & Watal, G. (2010). *Journal of Food Biochemistry, 34*, 78–92.

4. Rai, D. K., Sharma, R. K., Rai, P. K., Watal, G., & Sharma, B. (2011). *Cellular and Molecular Biology, 57*, 135–142.
5. Singh, S. K., Rai, P. K., Jaiswal, D., Rai, D. K., Sharma, B., & Watal, G. (2008). *Journal of Ecophysiology and Occupational Health, 8*, 195–199.
6. Rai, P. K., Jaiswal, D., Rai, D. K., Sharma, B., & Watal, G. (2008). *Indian Journal of Clinical Biochemistry, 23*, 387–390.
7. Rai, P. K., Rai, D. K., Mehta, S., Gupta, R., Sharma, B., & Watal, G. (2011). *Cellular and Molecular Biology, 57*, 31–39.
8. Sharma, R. K., Chatterji, S., Rai, D. K., Mehta, S., Rai, P. K., Singh, R. K., et al. (2009). *Journal of Medicinal Plants Research, 3*, 944–948.
9. Mishra, A. K., Kumar, S., Bhargava, A., Sharma, B., & Pandey, A. K. (2011). *Cellular and Molecular Biology, 57*, 16–25.
10. Mishra, A. K., Mishra, A., Kehri, H. K., Sharma, B., & Pandey, A. K. (2009). *Annals of Clinical Microbiology and Antimicrobials, 8*, 1–21.
11. Toida, T., Amornrut, C., & Linhardt, R. J. (2003). *Trends in Glycoscience and Glycotechnology, 81*, 29–46.
12. McLellan, D. S., & Jurd, K. M. (1992). *Blood Coagulation and Fibrinolysis, 3*, 9–77.
13. Cassaro, C. M., & Dietrich, C. P. (1977). *Journal of Biological Chemistry, 252*, 2254–2261.
14. Saraf, S., & Dixit, V. K. (1991). *Fitoterapia, 62*, 534–546.
15. Ali, M., Ravinder, E., & Ramachandran, R. A. (2001). *Fitoterapia, 72*, 313–315.
16. Ravi kumar, V., Shivashangari, K. S., & Devaki, T. (2005). *Molecular and Cellular Biology, 269*, 131–136.
17. Durgacharan, A., Bhagwat, S. G., & Killedar, R. S. A. (2008). *International Journal of Green Pharmacy, 2*, 26–128.
18. Holick, M. F., Judikiewicz, A., Walworth, N., & Wang, M. Y. (1985). *Biotechnology of marine polysaccharides* (pp. 389–397). New York: Hemisphere.
19. Dietrich, C. P., Paivao, J. F., Castro, R. A., Chavante, S. F., Jeske, W., Fareed, J., et al. (1999). *Biochimica et Biophyisca Acta, 1428*, 273–283.
20. Santos, J. C., Mesquita, J. M. F., Berlmiro, C. L. R., Da Silveira, C. B. M., Viskov, C., Mourier, P. A., et al. (2007). *Thrombosis Research, 121*, 213–223.
21. Dodgson, K. S. (1960). *Biochemical Journal, 78*, 312–319.
22. Garnjanagoonchorn, W., Wongekalak, L., & Engkagul, A. (2007). *Chemical Engineering and Processing, 46*, 465–471.
23. Jeske, W. P., McDonald, M. K., Hoppensteadt, D., Bau, E. C., Mendes, A., Dietrich, C. P., et al. (2007). *Clinical and Applied Thrombosis/Hemostasis, 13*, 137–145.
24. Hu, J. M., & Hsiung, G. D. (1989). *Antiviral Research, 11*, 217–232.
25. Elizabeth, K. M. (2005). *Indian Journal of Clinical Biochemistry, 20*, 150–153.
26. Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1996). *American Journal of Clinical Pathology, 45*, 493–496.
27. Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). *Carbohydrate Polymers, 66*, 184–191.
28. Yoon, S. J., Pyun, Y. R., Hwang, J. K., & Paulo, A. S. M. (2007). *Carbohydrate Research, 342*, 2326–2330.
29. Shanmugam, S., & Mody, K. H. (2000). *Current Science, 79*, 1672–1683.
30. Silva, T. M. A., Alves, L. G., De Queiroz, K. C. S., Santos, M. G. L., Marques, C. T., Chavante, S. F., et al. (2005). *Brazilian Journal of Medical and Biological Research, 38*, 523–533.
31. Badami, S., Vijayan, P., Mathew, N., Chandrashekhar, R., Godavarthi, A., Dhanaraj, S. A., et al. (2003). *Indian Journal of Pharmacology, 35*, 250–251.
32. Huheihel, M., Ishanu, V., Tal, J., & Arad, S. (2002). *Journal of Biochemical and Biophysical Methods, 50*, 189–200.
33. Akaayli, T., Timur, G., Aydemir, B., Kiziler, A. R., Coskun, O., Albayrak, G., et al. (2008). *Israeli Journal of Aquaculture-Bamidgeh, 60*, 89–94.
34. Tendencia, E. A. (2002). *Aquaculture Research, 33*, 455–458.
35. Gauger, E., Smolowitz, R., Uhlinger, K., Casey, J., & Gómez-Chiarri, M. (2006). *Aquaculture, 26*, 10–20.
36. Alcaide, E., Gil-Saz, C., Sanjuán, E., Esteve, D., Amaro, C., & Silveira, L. (2001). *Journal of Fish Disease, 24*, 311–313.
37. Austin, B., & Austin, D. (2007). *Bacterial fish pathogens: Diseases of farmed and wild fish* (4th ed., p. 594). Springer: Chichester.
38. Levine, W. C., & Griffin, P. M. (1993). *Journal of Infectious Diseases, 167*, 479–483.