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Pharmacological comparison of four biopolymeric natural gums as hemostatic agents for management of bleeding wounds: preliminary *in vitro* and *in vivo* results

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Abstract

Background: Uncontrolled bleeding is one of the primary reasons for preventable death in both civilian trauma and military battle field. This study evaluates *in vitro* and *in vivo* hemostatic potential of four biopolymeric natural gums, namely, gum tragacanth, guar gum, xanthan gum, and gum acacia. *In vitro* evaluation of whole blood clotting time and erythrocyte agglutination assay were carried out. *In vitro* cytotoxicity studies with respect to each gum were done in human lymphocytes to ascertain percent cell viability. *In vivo* hemostatic potential of each gum (as sponge dressing and powder form) was evaluated in Sprague Dawley rats using tail bleeding assay and compared with commercially available hemostatic sponge. Other important parameters like (a) time taken for complete hemostasis, (b) amount of blood absorbed, (c) adherence strength of developed hemostatic dressing(s), (d) incidence of re-bleeding, and (e) survival of animals were also studied.

Results: Of the four test gums studied, xanthan gum (@3mg/ml of blood) and gum tragacanth (@35mg/ml of blood) were able to clot blood in least time (58.75 ± 6.408 s and 59.00 ± 2.082 s, respectively) and exhibited very good hemostatic potential *in vitro*. Except for xanthan gum, all other test gums did not exhibit any significant cytotoxicity at different time points till 24 h. In rat tail bleeding experiments, gum tragacanth sponge dressing and powder achieved hemostasis in least time (156.2 ± 12.86 s and 76 ± 12.55 s, respectively) and much earlier than commercially available product (333.3 ± 38.84 s; $p < 0.01$).

Conclusion: Results indicate potential of gum tragacanth to be developed into a suitable hemostatic product.

Keywords: Biopolymeric natural gums, Gum tragacanth, Hemostatic potential, Hemagglutination, Cytotoxicity

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Background

In spite of advances in medical care in recent years, uncontrolled bleeding or hemorrhage still remains one of the primary causes of preventable death in civilian and combat trauma situations [1, 2]. Although during the last couple of decades, a number of topical hemostatic products like HemCon[®], Celox[®] Gauze, Chito-SAM[®], and QuickClot[®] have been developed [3, 4], pre-clinical studies and case reports have revealed some limitations for most of these agents [5, 6]. Few recently developed dressings are engineered with human coagulation factors, resulting in expensive formulations with low stability [7].

In India, there are no affordable *in situ* hemostatic agents, which have been developed indigenously. The hemostatic dressings, especially for military and paramilitary personnel engaged in combat roles, are presently imported at an exorbitant cost and consequently much of the demand remains largely unfulfilled. Thus, there is an urgent need to develop an indigenous, efficacious and cost-effective hemostatic product for managing combat-related hemorrhage in Indian troops.

Hemostatic agents are known to exert their effect through one of the three mechanisms: as mucoadhesive agents (e.g., chitosan-based products), coagulation factor concentrators (e.g., zeolite based QuickClot[®]) or coagulation factor supplementors (e.g., fibrin-based products). Recently, there has been some interest among pharmaceutical experts in exploring hemostatic behavior of biopolymeric natural gums, mainly owing to their absorbent nature, high viscosities at relatively lower concentrations, easy availability and cost-effectiveness [8, 9]. In the present study, we have compared hemostatic effect of four biopolymeric gums, namely, gum tragacanth (GT), xanthan gum (XG), guar gum (GG), and gum acacia (GA) under *in vitro* and *in vivo* conditions. Following parameters were evaluated with respect to each of the four gums: *in vitro* whole blood clotting time, *in vitro* cytotoxicity, and *in vivo* hemostatic potential in experimental animals. Concentration of calcium ions, being an integral part of the natural blood coagulation cascade, was also optimized and added into the test compositions in the form of calcium gluconate, so as to accelerate the activation of blood clotting process. We hypothesize that these gums can potentially act as factor concentrators in exerting their pro-coagulant effect.

Materials and methods

Materials

The four test gums were purchased from Merck Pvt. Ltd., Mumbai, India. Roswell Park Memorial Institute Medium (RPMI)—1640 medium, Histopaque[®]-1077 and Thiazolyl-blue-tetrazolium-bromide (MTT), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were

obtained from Sigma-Aldrich, St. Louis, USA. Surgispon[®], used as comparative positive control dressing, was purchased from local market. All other chemicals were of analytical grade.

Ethics statement

All animal procedures and care protocols were followed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations and the experimental protocol was approved by the Institutional Animal Ethics Committee vide approval no. INM/IAEC/2018/23.

Experimental animals

Sprague Dawley (SD) male rats weighing 200-250g were obtained from Experimental Animal Facility of the institute. All animals were housed in standard environmental conditions of temperature (21-24 °C), humidity (45-50%), and a 12 h light and 12 h dark cycle in polypropylene cages and were provided with standard feed (Golden Feed Ltd., Delhi, India) and water *ad libitum*.

In vitro studies

Determination of whole blood clotting time

In vitro blood clotting time was measured using the Lee and White method [10]. One milliliter of anticoagulated fresh whole blood was collected in glass tubes from the retro-orbital plexus of healthy Sprague Dawley rats and treated with varying concentrations of each of the four test gums (range, 7-35 mg/ml for GT; 1-4 mg/ml for XG; 1-8 mg/ml for GG; and 1-20 mg/ml for GA) in combination with a fixed amount of optimized calcium gluconate (10mg/ml) for determining concentration of gum required for clotting of blood in least time. Tube contained blood without any anticoagulant taken as control. The tubes were tilted at 45° angle every 30 s to check for blood clot formation and the time taken for clotting was noted. The experiment was repeated five times for each of the test material.

Erythrocyte hemagglutination assay

Erythrocyte hemagglutination assay was carried out by the method reported by Pogorielov et al. [11]. Five milliliters of blood from the retro-orbital plexus of healthy Sprague Dawley rats was pooled in 10% potassium ethylene diamine tetracetic acid (EDTA)-coated tubes. The tubes were centrifuged to separate erythrocytes. Separated erythrocytes were washed 3 times with phosphate-buffered saline (PBS, pH 7.4) to remove any leucocytes, plasma, platelets, and other cell debris. One milliliter of erythrocytes mass was then suspended in 19 ml PBS to make 5% erythrocyte dilution. Fifty microliters of this dilution was transferred to “U” bottom 96-well microplates and treated with sample gum solutions

in a ratio of 1:1. The sample solutions contained varying concentration of each of the four test gum; viz., 0.5, 0.1, 0.01, and 0.001%, while control wells contained only PBS. The microplates were incubated at 37 °C for 1 h and visual and photographic evaluation was done. The experiments were performed in triplicates.

***In vitro* cytotoxicity study**

Any potential cytotoxicity of the four biopolymeric gums was evaluated in isolated human lymphocytes using the standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay [12]. Lymphocytes were isolated from whole blood by density gradient centrifugation method using Histopaque®-1077 and cultured in RPMI 1640 media containing 10% fetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin and buffered with HEPES. The cells were seeded into 96-well plates at a density of 1×10^4 per well and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% carbon dioxide. After incubation, the cells were treated with autoclaved solution of the four test gums separately in varying concentrations (0.5, 0.1, 0.01, and 0.001%) for different time intervals, i.e., 1 h, 4 h, 16 h, and 24 h. The samples were treated with 10 µl of 5% MTT solution in PBS at the respective time intervals and incubated for 4 h at 37 °C for the formazan crystals to be formed. After incubation, the precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and again incubated for 20 min for the colored product to be formed. The readings were recorded using enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 570 nm and percent cell viability was calculated for all the samples.

***In vivo* experiment: evaluation of hemostatic potential in Sprague Dawley rats**

In vivo hemostatic potential of the four gums, GT, GG, GA, and XG was evaluated in Sprague Dawley (SD) rats using tail bleeding model as reported earlier by Sogut et al. [13]. Sixty healthy SD rats (200-250g) were randomly selected by using simple randomization method and assigned in ten groups of 6 rats each as mentioned below. The assessment was carried out in a non-blinded manner.

Group I: Treated with plain sponge without any coating; negative control

Group II: Treated with Surgispon®, a marketed hemostatic sponge; positive control

Group III: Treated with gum tragacanth (420 mg)-CG coated sponge

Group IV: Treated with gum tragacanth (420 mg)-CG in powder form

Group V: Treated with xanthan gum (36mg)-CG coated sponge

Group VI: Treated with xanthan gum (36mg)-CG in powder form

Group VII: Treated with guar gum (96 mg)-CG coated sponge

Group VIII: Treated with guar gum (96 mg)-CG in powder form

Group IX: Treated with gum acacia (60 mg)-CG coated sponge

Group X: Treated with gum acacia (60 mg)-CG in powder form

For preparing gum-coated sponge dressings, commercially available sponge material was taken and cut in circular shapes of 4 cm diameter. Each test gum in powder form was individually mixed with 120 mg of calcium gluconate (CG) using a mortar and pestle. The optimized amount of each gum and CG was calculated from the *in vitro* blood clotting experiment results described in earlier section. Water (q.s.) was added to these mixtures to make them into a thick paste. Using dip coating method, each test gum was individually coated onto different circular sponges. After coating, all sponge dressings were dried in a tray dryer at 37 °C for 2 h. Finally, gum-coated sponge dressings as well as each gum in simple powder form were evaluated for its hemostatic potential in subsequent rat tail bleeding experiments.

Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) [14]. Tails of all rats were disinfected using 98% alcohol. Four centimeters length of tail from the tail tip was amputated longitudinally with the help of a surgical blade. The bleeding tails were immediately covered with respective gum-coated sponge dressing/gum powder. The test material was initially applied along with direct pressure for 30 s. Pressure was then withdrawn and the wound was observed for any reduction in bleeding. Following parameters were studied for the evaluation of hemostatic potential as per previously published literature [15]:

- a) Adherence strength of the dressing(s)
- b) Time for complete hemostasis
- c) Amount of blood absorbed by the dressing(s)
- d) Incidence of re-bleeding, if any
- e) Survival of animal

At the end of the experiment, animal wounds were cleaned with normal saline and povidone iodine was applied. Antibiotic (Amikacin sulfate @ 80 mg/kg body weight given through intraperitoneal injection) and analgesic (Diclofenac sodium @ 40 mg/kg body weight given through subcutaneous injection) treatments were given to the animals for 5 consecutive days followed by dressing with povidone iodine to prevent the occurrence of any infection and pain. Animals were kept under

observation for 1 week. Thereafter, animals were rehabilitated at the Experimental Animal Facility of the institute for their use in future experiments.

Statistical analysis

The results were expressed as mean ± S.D. The data were analyzed statistically using GraphPad Prism 5.00. Statistical analysis was performed by one way ANOVA followed by Tukey’s post hoc test. For *in vitro* cytotoxicity study, the results were analyzed by two way ANOVA followed by Bonferroni test. A value of $p < 0.01$ was considered significant in all cases.

Results

***In vitro* studies**

Determination of whole blood clotting time

Figure 1 shows whole blood clotting time results with respect to each of the four test gums and their comparison with control. *In vitro* whole blood clotting time was significantly reduced when whole blood was treated with xanthan gum (@ 3 mg/ml) or gum tragacanth (@ 35 mg/ml) in comparison to untreated control ($p < 0.01$). While, xanthan gum took 58.75 ± 6.41 s for complete blood clotting, gum tragacanth also showed efficient blood clotting ability in almost similar time (59.00 ± 2.08 s). The other two gums, guar gum and gum acacia, were comparatively less efficient in their blood clotting ability.

Erythrocyte hemagglutination assay

Erythrocyte agglutination assay was performed to check the interaction between blood cells and the four test gums. Degree of hemagglutination was described using the following scale devised by Stavitsky and as reported by Pogorielov et al. [11]:

- ++++ Compact granular agglutinate
- +++ Smooth mat on bottom of well with folded edges
- ++ Smooth mat on bottom of mat with ragged edges
- + Narrow ring of red around edge of smooth mat
- Discrete red button in center of bottom well

Visual determination indicated a complete hemagglutination index (++++) in 0.5% solutions of gum tragacanth and xanthan gum. While considerable hemagglutination (+++) was seen in 0.1% solution of gum tragacanth, a relatively weak agglutination index (++) was observed in 0.1% solution of xanthan gum. Mild agglutination was also seen in 0.5% and 0.1% solutions of guar gum. There was no indication of any hemagglutinate formed (-) in solutions of gum acacia at any of the concentrations studied. Erythrocytes suspended in PBS served as negative control (Table 1).

***In vitro* cytotoxicity study**

The results of *in vitro* cell toxicity in lymphocytes indicated no significant cytotoxicity of guar gum, gum tragacanth, and gum acacia at all the concentrations studied (0.5, 0.1, 0.01, and 0.001%) at different time points till 24

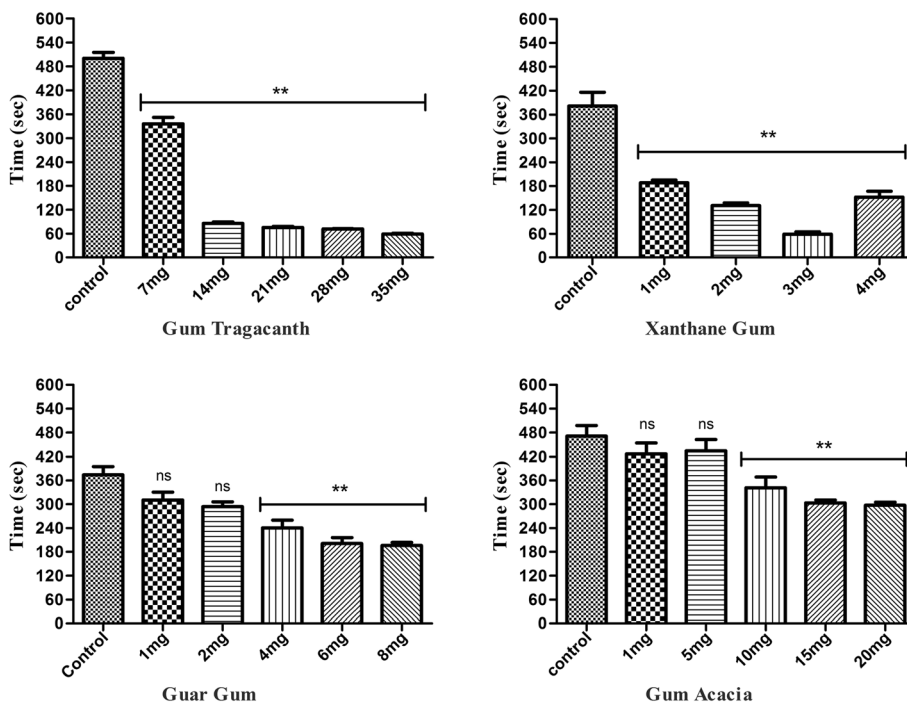











Fig. 1 Effect of individual test gums on *in vitro* whole blood clotting time. Data is expressed as Mean±SD, n=5. ** $p < 0.01$ when each test gum-treated group compared with untreated control

Table 1 Results of *in vitro* erythrocyte hemagglutination assay in different test gum solutions

Samples	PBS	Gum Tragacanth		Xanthan Gum		Guar Gum		Gum Acacia	
		(0.5%)	(0.1%)	(0.5%)	(0.1%)	(0.5%)	(0.1%)	(0.5%)	(0.1%)
Results	-	++++	+++	++++	++	+	+	-	-
Image									

*Erythrocytes suspended in PBS—negative control

**There was no indication of any hemagglutinate formed (-) in solutions of GT, XG, and GG at lower concentrations (0.01 and 0.001%)

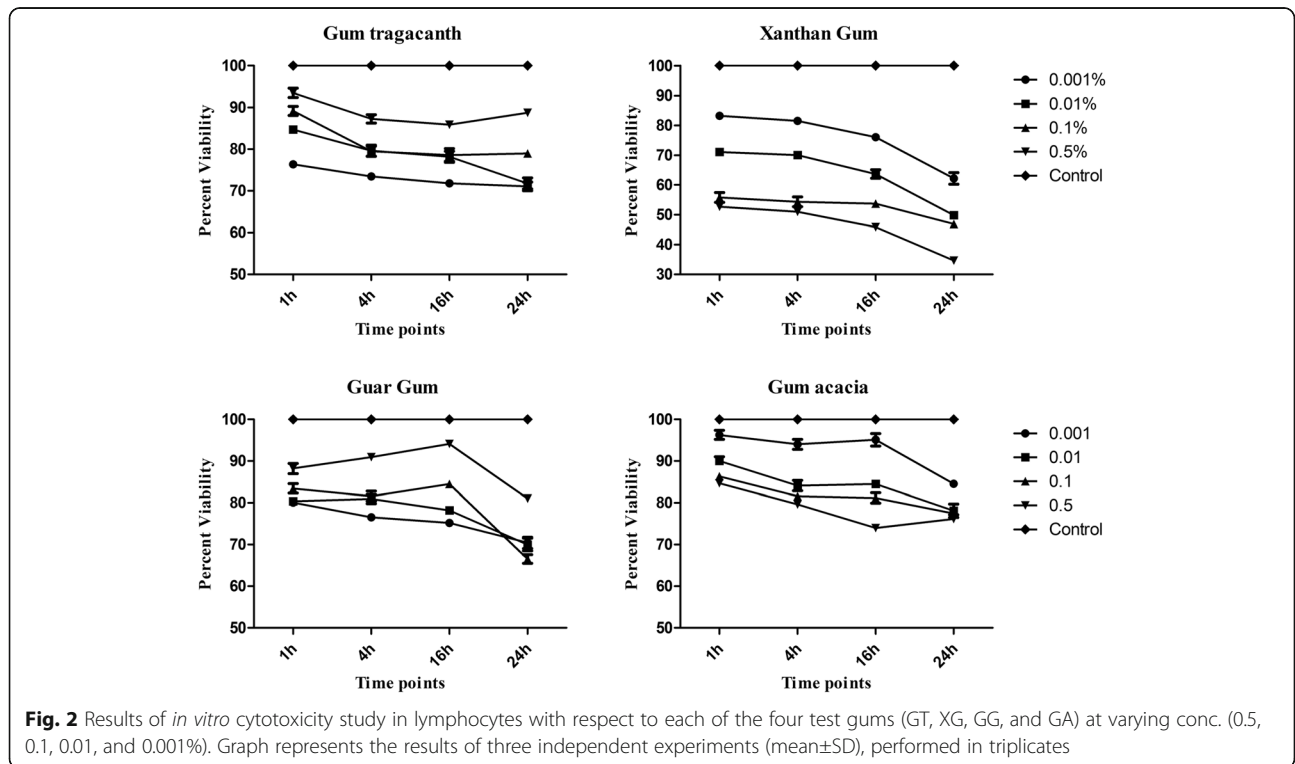
h, suggesting that these natural gums were not likely to cause any cellular damage on coming in contact with the blood cells. However, some cytotoxicity in terms of cell viability was observed in case of xanthan gum in a concentration and time dependent manner (Fig. 2).

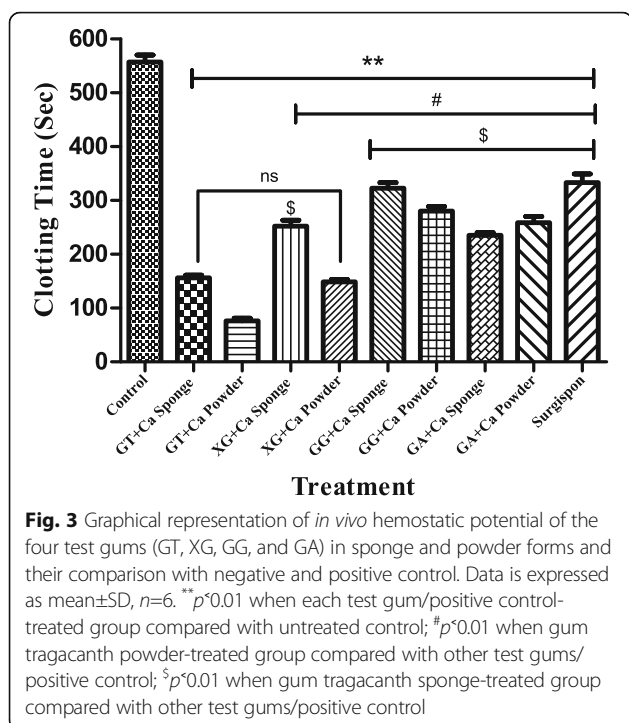
In vivo experiment: evaluation of hemostatic potential in rats

The results of this study are summarized in Fig. 3. Our data indicates that sponge dressings of xanthan gum and guar gum were comparable to marketed gelatine-based

sponge dressing used as positive control in terms of time taken for achieving hemostasis. However, the fastest hemostatic action was exhibited by gum tragacanth powder (76±12.55 s) and gum tragacanth sponge dressing (156.2±12.86 s), which was significantly better ($p < 0.01$) than the marketed product (333.3±38.84 s).

Gum tragacanth demonstrated fairly good clot retention when the dressing was removed for checking incidence of re-bleeding in the animals at the site of injury, indicating the likelihood of clot being stable within the dressing. Although gum tragacanth in powder form took





the least time for achieving hemostasis, it had one major drawback that it adhered to the wound strongly and hence it was difficult to remove it without rupturing the wound. Among the sponge dressings of all the test gums, the adherence strength of gum tragacanth dressing was found to be the best. Other parameters, included in the study like incidence of re-bleeding and survival of animals, are summarized in Table 2. Animals were observed for 1-week post experiment and no occurrence of any infection was seen in the animals.

Discussion

Use of biopolymeric natural gums as hemostatic materials is being explored with some interest in recent years due to several potential advantages [9]. With this study, we aimed to screen the behavior of four natural gums, viz., gum tragacanth, xanthan gum, guar gum, and gum acacia and compare their hemostatic potential using *in vitro* and *in vivo* efficacy parameters.

In vitro whole blood clotting time experiments indicated that of the four natural gums tested, xanthan gum and gum tragacanth showed efficient blood clotting abilities in almost similar times (58.75 ± 6.41 s and 59.00 ± 2.08 s, respectively), while guar gum and gum acacia were less efficient in clotting the blood. In these experiments, the test gums were used in combination with fixed amount of calcium gluconate (10 mg/ml), conc. of which was optimized in a separate set of experiments (in the range of 2-12 mg/ml of blood; results not shown). Normal hemostatic process requires calcium ions for

most events in the blood coagulation process [16]. Gum tragacanth and xanthan gum probably helped in assimilating the cationic Ca^{2+} ions and together they were able to clot blood in a significantly lesser time as compared to other groups.

In vitro hemagglutination assay also confirmed the ability of these two gums in agglutinating blood proteins. Results of erythrocyte hemagglutination assay indicating complete agglutination of rat blood cells at higher concentrations of gum tragacanth and xanthan gum, and relatively weak or no agglutination at lower concentrations is consistent with previously reported literature, which suggests that agglutination process is affected by conc. of the test sample [11, 17]. Reason for appearance of hemagglutination in gum tragacanth and xanthan gum could be the presence of more number of protonated groups in these gums, thereby increasing their chances of binding with the erythrocyte wall.

In vitro cytotoxicity experiments were performed since it is important to evaluate the biological behavior of a test material intended to be used in any physiological system. We carried out basic *in vitro* cell culture study to assess the effect of test gums on cell viability when lymphocytes come in contact with them. So while xanthan gum had exhibited excellent *in vitro* blood clotting ability during *in vitro* blood clotting time experiments, it also proved to be cytotoxic to the cells, indicating a major limitation with its use. On the other hand, gum tragacanth was able to clot blood in comparable time as xanthan gum, without inducing any significant cytotoxicity. This observation could be an important advantage while developing gum tragacanth-based hemostatic products. The probable reason for cytotoxicity observed in case of xanthan gum could be attributed to the fact that among all the four gums screened, xanthan gum showed the most viscous behavior in solution, resulting in formation of a thick layer on top of the wells containing lymphocyte cells. This might have hindered oxygen supply to the cells, affecting their viability.

In vivo experiments in experimental animals clearly demonstrated efficacy of gum tragacanth in achieving hemostasis in least time as compared to other gums as well as the marketed product ($p < 0.01$). Sorption properties are thought to be important for a hemostatic agent for aggregating blood cells, especially the platelets, at the wound site. However, in our experiments, gum tragacanth-based dressing absorbed significantly lesser amount of blood (0.14 ± 0.09 g) as compared to other gums (guar gum, 0.65 ± 0.05 g; xanthan gum, 0.56 ± 0.05 g; gum acacia, 0.29 ± 0.08 g) and the positive control (0.60 ± 0.09 g), but nonetheless exhibited best hemostatic action. Apparently, sponge dressing of gum tragacanth absorbed less amount of blood as compared to other test gums because it was able to clot blood faster. Therefore in our

opinion, the amount of blood absorbed by a dressing cannot be taken as a measure for its hemostatic potential. Our observation is supported by similar studies carried out on other hemostatic test materials elsewhere [1, 18]. The critical parameters therefore should be the time taken for achieving hemostasis and the amount of blood loss during treatment. On both these critical parameters, gum tragacanth was found significantly better than the other three exudates gums as well as the marketed product.

This observed hemostatic behavior of test gums vis-à-vis standard plain sponge dressing (negative control) could be due to the fact that while plain sponge dressing after application at the site of injury sorbed plasma and blood cells, the cellular components were not able to adhere to the dressing. Therefore, while the sponge dressing was able to absorb significant amount of blood but no clot formation occurred at the injury site. On the other hand, all the four test gums were probably able to adhere erythrocytes, platelets, and other components of the coagulation cascade on the surface of their respective dressings, while addition of calcium ions helped in the activation of those coagulation factors, thereby facilitating clot formation.

Earlier reports have indicated that efficacy of hemostatic products can be augmented by increasing their viscosity [19, 20]. Although the exact hemostatic mechanism of these natural exudates gums needs to be elucidated further, we feel that the viscous nature of these gums and the ability to agglutinate blood proteins, especially seen in case of gum tragacanth and xanthan gum, may have also contributed in formation of plugs and the resulting hemostatic efficacy.

Finally, although powder form of gum tragacanth was able to achieve hemostasis in least amount of time; in our opinion, use of gum tragacanth based sponge dressing would still be a better option than the powder form. This is because of the following few basic difficulties we observed during the course of our study, (a) high adherence of the powder form is liable to rupture the wound again if not carefully removed, (b) wastage of drug is less if we use its sponge dressing in place of powder, and (c) in actual field scenario/clinical settings; the ease of applicability would be more for a sponge-based dressing than the powder form because the powder after being applied on to the bleeding wound might still be needed to be covered with a cotton gauze to provide mechanical pressure.

Conclusion

Results of this study indicate a novel use of gum tragacanth and its potential to be developed into a suitable hemostatic product. Further evaluation including physico-chemical characterization of gum tragacanth coated sponge dressing and its efficacy in large animal bleeding models is needed, the work on which is currently underway in our laboratory.

Abbreviations

CG: Calcium gluconate; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; DMSO: Dimethyl sulfoxide; EDTA: Ethylene diamine tetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; GA: Gum acacia; GG: Guar gum; GT: Gum tragacanth; HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate-buffered saline; SD rats: Sprague Dawley rats; XG: Xanthan gum

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Not applicable.

Authors' contributions

HK performed the experiments. NS and MC helped in planning of *in vitro* and *in vivo* experiments. GM analyzed and interpreted the data, besides writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

No human studies were involved. All animal procedures and care protocols were followed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations and the experimental protocol was approved by the Institutional Animal Ethics Committee vide approval no. INM/IAEC/2018/23.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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