Research Paper

Evaluation of Crude and Modified *Cordia myxa* Gum for its Nutraceutical Benefits

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Plants are a rich source of safe and valuable bioactive compounds and since ancient times they have been used as medicines. Medicinal herbs are used as traditional means of treating many human diseases in many parts of the world. In current study, the green synthesis pathway was investigated for modification, reducing and crown potential of *Cordia myxa* gum for the synthesis of silver nanoparticles. The seeding process and the synthesis of silver nanoparticles were tracked by taking ultraviolet/visible absorption spectra during the reaction. Nutritional as well as medicinal potential of raw and modified gums were analyzed. Synthesized spherical nanoparticles were up to 65 nm in size. The ultraviolet/visible spectroscopic results revealed an intense peak at 428 nm together with other small peaks in the region of 390-450 nm due to the presence of nanoparticles of polydispersed silver. The purified gum showed the highest phenolic contents (i.e. 14.71±0.04 mg) and percentage scavenging activity (i.e. 87.92±2.25). The maximum bactericidal activity was showed by nanoformulated gum against *Escherichia coli* with inhibition zone of 42 mm while minimum was found to be against *Staphylococcus aureus* with inhibition zone of 14 mm. Nano formulated gum samples also exhibited optimal fungicidal effects against *Fusarium solani* with inhibition zone of 11.09 mm and *Aspergillus niger* with inhibition zone of 15.53 mm. The results of current study portrayed that modifications in *Cordia myxa* gum would be beneficial to food products as an additive.

Key words: *Cordia myxa*, silver nanoparticles, phenolics, antimicrobial activities, gum based nanoformulation

Today, plant research is the center of attraction because it has the potential to be used in various areas of the world's industry. A wide range of natural materials is used to maintain the health of all living beings[1]. About 80 % of the world's population still depends on traditional medicine. *Cordia myxa* (*C. myxa*), commonly known as 'lasura' belonging to family Braginaceae, possessed analgesic, anti-inflammatory, immunomodulatory, antimicrobial, antiparasitic, insecticidal, cardiovascular, respiratory, gastrointestinal and protective effects while traditionally *C. myxa* is used in Ayurveda, Unani and Siddha systems of medicine[2]. Plant materials of *C. myxa* include various phytoconstituents including gum. Gums have been known since ancient times as plant products[3]. Gum *C. myxa* is obtained from its fruits and its modified forms are also used for the drug delivery system. Chemical and physical modifications are used to improve the functional properties of the gum as a biopolymer[4].

Using silver nanoparticles (AgNPs) for different purposes has been gaining attention in recent years. AgNps formulated gums are progressively played large role in different disciplines such as food, health care, medical and industrial purposes because of their particular chemical and physical properties[5]. Nanoparticles can be synthesized using various biological, chemical and physical methods. Biological methods, on contrary to chemical methods, not only result in the productions of non-hazardous products but also are energy efficient. For that reason, chemical synthesis of nanoparticles is expensive[5]. As compared to other biological systems, biosynthesis of nanoparticles by means of plants and plant based

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extracts tends to be harmless, less time consuming and have a lesser farming cost. Moreover, plant based biosynthesis is a somewhat simple method that can be expanded to produce nanoparticles of large proportions[6].

Gums being nontoxic, biologically active, biodegradable, biologically compatible and capable to modify chemically, more over economic and easily available have made their attraction towards pharmacological applications. Various research studies have revealed that gums have advantage over synthetic polymer or materials and can be used potentially or can lead fascinating discoveries in pharmaceutics and food industry. Therefore, the current study was designed to purify and modify *C. myxa* gum by acrylamide grafting. Bioanalytical techniques were employed to investigate and characterize natural and modified *C. myxa* gum for the synthesis of nanoformulations. The study will add more benefits of this modified/formulated gum to our foods and/or other edible formulations as functional additives in future.

MATERIALS AND METHODS

Chemicals and reagents:

The chemicals used were at least of analytical grades and of companies such as Sigma-Aldrich (U.S.A), Fluka (U.S.A), BB1 (UK), Oxoid (UK), Merck (Germany), Pharmacia and ICN.

Target sample for research:

Gum *C. myxa* was purchased commercially from herbal medicine store 'Bada Dawakhana' Karkhana Bazar, Faisalabad. The collected gum was solid and reddish brown in color. Gum sample was subjected to identification and authentication by Department of Botany, Government College University, Faisalabad, Pakistan. This mucilaginous substance has strong adhesion as well as good emulsifying and binding properties. The *Cordia* gum has pseudoplastic behavior and high viscosity.

Decontamination of gum:

Sample was washed with double distilled water in order to expel dust and other surface impurities and then dried. Physical wash and spotlessness of gum was proceeded by following the procedure of Munir *et al.*[7] with some modifications. Gum was soaked overnight in deionized water. A clingy thick arrangement/solution was gotten that was separated using a nankeen material (muslin cloth). This sifted arrangement/solution was introduced to 70 % ethanol which resulted in smoggy white precipitates. The obtained precipitates were dried in oven at about 40-45°[8].

Modification of gum:

By grafting: The purified gum sample (i.e. 1.00 g) was dissolved in 25 ml of distilled water. To the solution, acrylamide (16×10^{-2} M), silver nitrate ($AgNO_3$) ($8.\times10^{-5}$ M) and ascorbic acid (22×10^{-3} M) were added. The solution was regulated in a water bath at 35°. After 30 min, potassium persulfate ($K_2S_2O_8$) (8.0×10^{-3} M) was added and reaction was allowed for 1 h. Ethanol was used to separate the modified gum from mixture. The precipitates (modified gum) were washed with ethanol and dried in hot air oven for further use[9].

By nanoparticles formulation: Pure gum was taken as green matrix to form AgNps following the method as reported by Munir *et al.*[7]. $AgNO_3$ solution (1 mM) was added drop wise in the solution of pure gum (0.5 % w/v). This solution was then subjected to heat treatment at 121° for 15 min that resulted in the evolution of AgNps. Nanoparticles were spectroscopically analyzed.

Nano particles analysis by Zetasizer:

Nanoparticl***es*** synthesized in the present research work were analyzed by particle size analyzer (Malvern zetasizer 2000, Malvern Instruments Ltd., U.K) at 24.9° with 90° detection angle[10].

Characterization of *C. myxa* samples:

Ultraviolet/visible (UV/VIS) spectrophotometric analysis: Crude as well as nanoformulated samples of *C. myxa* were characterized by UV/Vis spectrophotometer (T60-UV-Visible/ Deuterium lamp/tungsten/halogen lamp). Detector used was the photodiode with scanning wavelength range of 190-1100 nm. Resolution of spectra was 1 nm as reported by Kora *et al.*[11].

Biochemical analysis of crude gum:

Proximate analysis: Proximate analysis (protein, moisture, ash, fats and fiber contents) of untreated gum was performed according to the protocol as reported in Association of Official Analytical Chemists (AOAC)[12] and Galla and Dubasi[13].

Antioxidative potential:

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity: Potential of crude,

purified as well as modified gum samples to scavenge free radicals was evaluated[14].

Analysis of total phenolics: Total phenolics of samples under investigation were analyzed and the protocol with slight modification was adopted as reported by Ainsworth and Gillespie[15].

Antimicrobial activity: Well diffusion method with different fungal strains was adopted for the evaluation of antiseptic behavior of experimental samples.

Antibacterial activity: Experimental samples of *Cordia* gum (i.e. crude, purified, acrylamide grafted and AgNps) were subjected to antibacterial activity. *Escherichia coli* (*E. coli*) (ATCC 35218) and *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) strains were used according to the method of Balouiri *et al.*[16]. Ampicillin was used as a positive control.

Antifungal activity: The activity of *Cordia* gum samples (i.e. crude, purified, acrylamide grafted and AgNps) to inhibit growth of As*pergillus niger* (*A. niger*) and *Fusarium solani* (*F. solani*) was analyzed[17]. Terbinafine was used as a positive control.

Toxicological analysis:

Assay for hemolysis: Hemolytic activity of experimental samples (i.e. crude, purified, acrylamide grafted and AgNps) was evaluated by running the sample according to the protocol reported by Irshad *et al.*[18]. Human blood samples (3 ml each) from volunteers were centrifuged (850 xg) for 5 min and transferred to sterilized polystyrene tubes (15 ml capacity). Phosphate buffer saline (PBS) (pH 7.4) was used to isolate red blood cells (RBC) from plasma at 4° and retained in 20 ml chilled PBS. The diluted blood cell suspension (180 µl) was added in erythrocyte cells (108 erythrocytes cells/ml) in a tube of 2 ml capacity and incubated for 30 min at 37° with agitation for 10 min. Afterwards, kept in ice for 5 min and centrifuged at 1310 xg. The supernatant (100 µl) was withdrawn from the tubes and diluted with 900 µl chilled (4°) PBS.

All tubes were made stable on ice then introduced into 96 well plates (200 µl). Triton X-100 (0.1 %) was taken as a positive control with maximum lysis whereas PBS was taken as a negative control with least lysis. Micro Quant ELISA plate reader (BioTek, Winooski, VT, USA) was used to measure intensity of absorption at 576 nm.

The percentage lysis of RBCs was calculated by the following formula:

Lysis of RBCs (%) =Absorbance of sample-Absorbance of negative control/Absorbance of positive control×100

Ames test for mutagenicity: For the evaluation of mutagenicity of the experimental samples, Ames test was performed using microbial strains such as *Salmonella typhimurium* (*S. typhimurium*) TA98 and *S. typhimurium* TA100. Nutrient agar was used to culture these bacteria at 4°±1° and inoculation was done in nutrient broth and incubated at 37° for 18-24 h prior to the analysis[19].

Procedure used for Ames: Reagent mixture of Davis-Mingioli salt, Dextro-glucose, bromocresol purple, Dextro-biotin and Levo-histidine was prepared in a sterile bottle. Reagent mixture, gum, distilled water and standard mutagen were homogenized in number of bottles. Micro well plates were prepared according to the method of Razak and Aidoo[20] and sealed in plastic bags and incubated at 37° for 4 d. The reference plate was noted first followed by the experimental plates when all wells in blank were colored purple indicating the non-contaminated assay. The background, standard and test plates were scored visually. All yellow, partial yellow and turbid wells were counted as positive wells while purple wells were counted as negative. The extract was considered toxic to the test strain if all wells in the plate showed purple coloration. For an extract to be mutagenic, the number of positive wells had to be more than twice the number of positive wells in the background plate.

To perform Ames test, test sample (0.005 ml) was run containing reagent mixture (2.5/17.5 ml deionized water) and *Salmonella* strain (0.005 ml). Blank was run containing only reagent mixture whereas background contained reagent mixture along with microbial strain. Standard was run under the same conditions but without test sample.

RESULTS AND DISCUSSION

The selected gum was purified using ethanol precipitation method. The percentage of purified gum obtained and percent yield were estimated by comparing the gum used before and after the purification. A significant yield (i.e. 74.65 %) was obtained in the current research work (fig. 1a and fig. 1b).

Gum *C. myxa* was processed for biochemical analysis and results are given in Table 1. It is clear from the table that research sample contained contents of moisture (9.68 %), crude fats (2.04 %), ash (2.58 %), proteins

(2.49 %), fibers (2.21 %) and carbohydrates (88.41 %) and total calculated energy was 349.84 %.

C. myxa as a green matrix was subjected to AgNps formulation that was confirmed by the development of dark yellow color (fig. 1c). Nano particles were further analyzed using UV/VIS spectrophotometry by running the crude and nanoformulated samples. Sharp peak at 428 nm was the confirmation of the formation of AgNps along with other smaller peaks in the region of 390-450 nm whereas in crude gum no sharp peak was present in this region but in the region of 423 nm (fig. 2).

In the samples under study, free radical scavenging activity was found maximum (87.92 %) shown by purified gum followed by polyacrylamide grafted gum (85.86 %), nanoformulated (76.23 %) and crude gum (66.79 %) as shown in Table 2.

Total phenolic contents were analyzed in the experimental samples and results are reported in Table 3. It is clear from the table that the crude sample contained phenolics up to 13.40±0.01 mg whereas purified gum contained somewhat higher amount of phenolics (14.71±0.03 mg). Minimum amount of phenolics was detected in grafted gum sample (8.21±0.03 mg). Nano formulated gum sample also contained significant amount of phenolics (10.73±0.03 mg).

Antibacterial activities of experimental samples were determined and results are given in Table 4. Antibacterial activity was found higher in nanoformulated sample. The calculated zones of inhibition against *Bacillus subtilis* (*B. subtilus*) and *E. coli* were 16 mm against both strains followed by crude (14 and 16 mm), purified (14 mm each) and modified (12 mm each), respectively.

In the present research work, the antifungal activity was also evaluated against *A. niger* and *F. solani* (Table 5).

TABLE 1: PROXIMATE ANALYSIS OF CRUDE GUM *C. myxa*

S. No.	*C. myxa* gum	Results
1	Crude fat	2.04±0.05
2	Moisture content	9.68±0.08
3	Crude protein	2.49±0.01
4	Ash content	2.58±0.05
5	Crude fiber	2.21±0.06
6	Total carbohydrates	88.41±0.36
7	Nitrogen free extract	91.01±0.04
8	Total energy (kcal/g)	349.83±0.74

TABLE 2: ACTIVITY OF *C. myxa* TO SCAVENGE FREE RADICAL

S. No.	Sample ID	DPPH scavenging %
1	Crude	66.79±3.53
2	Purified	87.92±2.25
3	Acrylamide grafting	85.86±1.71
4	AgNps	76.23±1.48

Fig. 1: (a) Crude gum; (b) Purified gum; (c) Nanoformulated gum

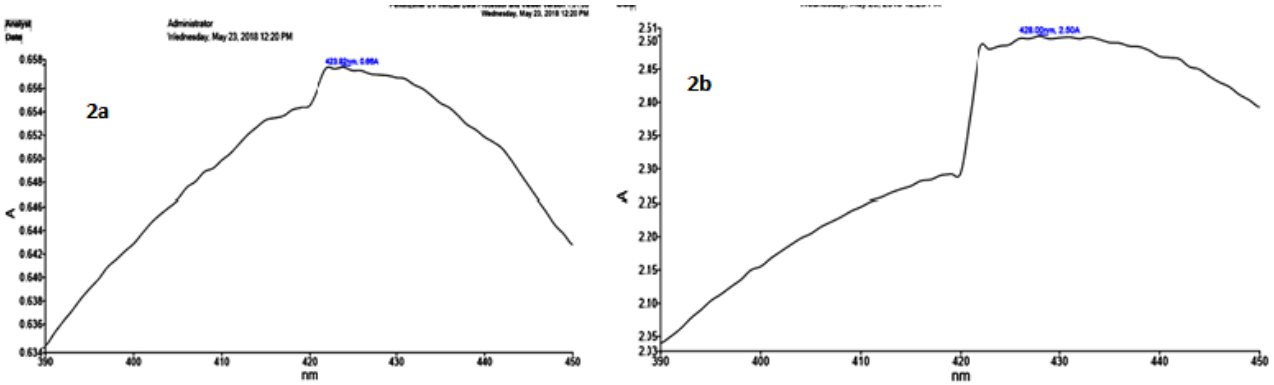

Fig. 2: (a) UV/Visible spectra of crude *C. myxa*; (b) UV/Visible spectra of nanoformulated *C. myxa*

The crude gum sample as well as purified, modified and grafted samples exhibited excellent antifungal behavior. Maximum antifungal activity was demonstrated by the grafted sample and zone of inhibition was found to be 10 mm for *A. niger* and *F. solani* whereas minimum zone of inhibition of 5 mm against both strains was calculated of crude sample. Nano formulated and purified samples also showed significant values of antifungal activity and calculated zones of inhibition were 9.0 against *A. niger* and 11 mm against *F. solani* and 7 mm against both strains, respectively.

Experimental samples under investigation were subjected to hemolysis by taking human blood sample to examine toxicity (Table 6). Non-significant toxicity was observed by all the experimental samples. Toxicity of crude gum sample detected was found to be 8.19 % and those of purified, modified and nanoformulated samples were found to be 8.53 %, 6.01 % and 9.91 %, respectively.

Mutagenicity of all experimental samples was analyzed by taking two bacterial strains *S. typhimurium* strain TA98 and *S. typhimurium* strain TA100 for the possible mutagenic activity and results are demonstrated in Table 7. It is clear from the table that all the experimental samples were non-mutagenic and not contaminated with mutagenic agents.

Ethanol was used as a solvent for the purification of selected gum. A change in color from brown to yellowish brown (due to removal of impurities) was detected in purified gum with the percentage yield of 74.65 % (fig. 1a and fig. 1b). This purified gum might be utilized as additive in food products. The present findings were in accordance to the results reported by

TABLE 3: VALUES OF TOTAL PHENOLICS IN *C. myxa* GUM SAMPLES

S. No.	Sample ID	Total phenolic (mg GAE/100 g dw)
1	Crude	13.4±0.01
2	Purified	14.71±0.03
3	Acrylamide grafting	8.21±0.03
4	AgNps	10.73±0.03

TABLE 4: ANTIBACTERIAL ACTIVITY OF *C. myxa* GUM SAMPLES AGAINST *B. subtilus* AND *E. coli*

S. No	Sample ID	*B. subtilus* (mm)	*E. coli* (mm)
1	Crude	14	16
2	Purified	14	14
3	Acrylamide grafting	12	12
4	AgNps	16	16
5	Positive control (ampicillin)	20	24

TABLE 5: ANTIFUNGAL ACTIVITY OF *C. myxa* AGAINST *F. solani* AND *A. niger*

S. No.	Sample ID	*F. solani*	*A. niger*
1	Crude	5 mm	5 mm
2	Purified	7 mm	7 mm
3	Acrylamide grafting	10 mm	10 mm
4	AgNps	11 mm	9 mm
5	Positive control (Terbinafine)	20 mm	15 mm

TABLE 6: HEMOLYTIC ACTIVITY OF *C. myxa* SAMPLES

S. No.	Sample ID	Hemolysis %
1	Crude	8.19±0.12
2	Purified	8.53±0.05
3	Acrylamide grafting	6.01±0.07
4	AgNps	9.91±0.10
5	Positive control	68.94 %

Vinod and Sashidar[21] and Kwakye *et al.*[22]. Vidyasagar *et al.*[23] conducted their study to isolate and purify the *C. dichotroma* gum and their results are also in accordance with the results of current study.

After biochemical analysis, it is clear that this gum might be a rich source of basic nutrients such as proteins, carbohydrates, minerals, fibers and fats (Table 1). Values of these nutrients are somewhat higher as reported by other scientists in other gums. Murwan *et al.*[24] performed proximate analysis of guar gum endosperms and detected moisture (4.8-8.7 %), protein (3.5-5.0 %), ash (0.5-0.8 %), fiber (1.4-2.0 %) and carbohydrate (83.3-87.5 %) whereas Gupta *et al.*[25] reported contents of moisture, protein, fats and ash in the range of (7.4-12.4 %), (0.5 %-0.9 %), (0.29-0.34 %) and (0.80-0.86 %), respectively in three samples of guar gum obtained from different resources. Similarly, Osman *et al.*[26] analyzed fiber contents (2.3 %) in guar gum. In consistent to our results, Jamil *et al.*[27] have made the profile of proximate and mineral composition of uncharted date palm varieties collected from different geographical regions of Pakistan. According to them, moisture contents were 1.6 to 9.8 % whereas soluble minerals were 1.82-2.87 %. They reported highly significant contents of proteins (32.5-41.25 %) as compared to results of present study and crude fiber was noticed in the range of 62.11 to 86.08 % that is significantly lower as compared to present findings. It is assumed that gums and mucilages have higher amounts of crude fibers than seeds or fruits. These findings are in close agreement with the present research outcomes.

In AgNps formulation in current study, the gum was found to be expanded to have more surface area

TABLE 7: MUTAGENIC ACTIVITY OF *C. myxa* SAMPLES AGAINST *S. typhimurium* STRAIN TA98 AND TA100

S. No.	Sample Name	*S. typhimurium* TA98		*S. typhimurium* TA100	
		No. of positive wells/total wells	Results	No. of positive wells/ total wells	Results
1	Blank	-	Not contaminated	-	Not contaminated
2	Background	10/96	-	10/96	-
3	Standard	72/96	Mutagenic	72/96	Mutagenic
4	Crude	1/96	Non-mutagenic	2/96	Non-mutagenic
5	Purified	2/96	Non-mutagenic	1/96	Non-mutagenic
6	Grafted	3/96	Non-mutagenic	6/96	Non-mutagenic
7	AgNps	2/96	Non-mutagenic	2/96	Non-mutagenic

for silver ions to react with functional group present on the gum matrix. The hydroxyl groups oxidized to carbonyl groups caused by the presence of silver ions thus resulted in the production of reduced elemental silver[28] that were confirmed by the development of dark yellow color (fig. 1c). UV/VIS spectrophotometric analysis confirmed the formation of AgNps along with other smaller peaks in the region of 390-450 nm due to Surface plasmon resonance (SPR) whereas in the crude gum no sharp peak was present in this region but found at 423 nm (fig. 1b).

In purified as well as modified gum samples, optimal values of free radical scavenging activity were detected (Table 2). It is assumed that activity of flavoniods might be modified by the introduction of polyacrylamide that enhanced the free radical scavenging activity. Results of the current study are in accordance with previously reported results. Xu *et al.*[29] modified the polysaccharides extracted from *Ganoderma lucidum* that resulted in the drastic boost in antioxidant behavior. These results are in strong agreement with the outcomes of Haq *et al.*[30] who investigated gum *C. myxa* for extraction and characterization. These modified polymers exhibited excellent hydroxyl free radical scavenging activity (83.4 %) while the simple polysaccharide showed hydroxyl scavenging activity up to 42.9 %. Similarly these polymers also have the maximum potential to scavenge superoxide anion and hydrogen peroxide in comparison to the native polymer. The structure of the polysaccharide gets modified by the incorporation of carboxyl group that could be the cause of scavenging of free radicals. Munir *et al.*[7] also studied DPPH scavenging effect of *Dalbergia sissoo* and *Acacia modesta*. They reported that crude gum contains high DPPH scavenging activity as compared to modified forms. The percentage scavenging effect was decreased in the hydrolyzed and modified samples as compared to the crude gum. This pattern of decreasing DPPH activity was shown by both selected gums.

Number of metabolites of phenolics and derivatives of phenolics are present in plants. These phenolic compounds have the ability to scavenge reactive oxygen species without causing further oxidative reactions[15]. In present research work, significant amount of phenolics were detected in the purified and modified gum samples (Table 3). In contrast to our results, Saleh *et al.*[31] reported significantly higher amount of total phenolics in fruits of khalas variety of date palm (i.e. 238.54 mg/100 g dry weight (DW)). Louaileche *et al.*[32] also detected phenolics (169.18 to 381.76 mg gallic acid equivalent (GAE)/100 g) in aqueous extract of date fruits. These studies reported higher amounts of phenolics that could be due to the difference in varieties collected from different geographical regions. Munir *et al.*[7] concluded that *Acacia modesta* exhibits more antioxidant activity. Crude gum samples were rich in phenolic compounds. But the amount of phenolics was decreased after purification, hydrolysis and modification. The consumption of these gums can be useful in degenerative diseases by preventing the oxidative stress. The analyzed gums can also be used as natural antioxidant in pharmaceutical and cosmetic industry.

Antibacterial activity was found higher in nanoformulated gum sample followed by crude, purified and modified gum samples (Table 4). Antibacterial activity of gum was affected by different steps of purification and modification. Crude gum sample exhibited maximum antiseptic behavior as it was not treated by any of the chemical reagents. One of the main purposes of modification given in literature is to minimize the microbial contamination possessed by crude gum and to increase their food and pharmaceutical applications. As the gum was subjected to purification as well as alteration, some of the functional groups that were responsible for antiseptic behavior might be lost and resulted in the change in the decrease in the antimicrobial behavior of the gum. According to the

result of conducted antimicrobial assay the activity shown by modified and hydrolyzed samples was lower as compared to the crude gums. Similarly, Ravishanker and Raut *et al*.[33] reported that date palm bark methanol fraction exhibited excellent activity against microbial strains with zones of inhibition of 22 mm against *S. aureus* and 20 mm against *E. coli*. Jayaprakash *et al.*[34] examined outstanding bactericidal effects of AgNps of fruit extract against eight bacterial strains. Fruit extract also showed excellent activity against microbes due to the presence of polyphenols.

In the present research work, the antifungal activity of crude gum sample as well as purified, modified and grafted samples exhibited excellent antifungal behavior. Maximum antifungal activity was demonstrated by the grafted gum sample and minimum was found by the crude gum sample. Nano formulated and purified samples also showed significant values of antifungal activities. Similarly, Boulenouar *et al.*[35] assessed the antifungal potential of different date palm cultivars extracts against *F. oxysporum* and calculated zone of inhibition that was found to be 6.50 mm.

Toxicity assay was performed to evaluate that modification in gum samples are beneficial or not (Table 6). It is clear from the table that non-significant toxicity was observed by all the experimental samples and values are in normal limits and therefore could be used as additives in nutraceuticals. In a study, Shahid *et al.*[36] reported a little toxicity of grafted guar gum sample as compared to unprocessed gum. Similarly, Munir *et al.*[7] found no toxicity of aqueous extract of gums to human erythrocytes. Dhiman *et al.*[37] reported that the grafted *C. myxa* gum has enhanced mucoadhesion and hence the grafted *Cordia* gum would be a promising excipient to develop mucoadhesive drug delivery systems.

To evaluate the DNA damaging activity of crude as well as modified gum samples, mutagenicity test was performed and results are depicted in Table 7. It is clear from the table that all the experimental samples were non-mutagenic and not contaminated with mutagenic agents. Das *et al.*[38] also reported similar results of the samples under investigations.

In conclusion, the *C. myxa* gum is a medicinal gum and has been used in medicines as an excipient. The purification, modification and nanoformulation of *Cordia* gum were done in this study through green pathways. From present research findings it has been revealed that the purified gum has the highest free radical scavenging activity while nanoformulated gum has the highest antibacterial activity. Therefore, the modified gum has more nutritional as well as medicinal potential as compared to its conventional or crude form and has portrayed more benefits to food or other edible formulations as functional additives.

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Conflict of interests:

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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