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# Antioxidant Potential and Emulsifying Properties of Neem (*Azadirachita indica*, Family Meliaceae) Gum Polysaccharide

**Rishabha Malviya**[1,2*], **Pramod Kumar Sharma**[1] **and Susheel Kumar Dubey**[3]

[1]*Polymer Science Laboratory, Department of Pharmacy, School of Medical & Allied Sciences, Galgotias University, Greator Noida U.P., India*

[2]*Department of Pharmacy, Uttarkhand Technical University, Dehradun, Uttarakhand, India*

[3]*Siddarth Institute of Pharmacy, Dehradun, Uttarakhand, India*

***Corresponding author:** Rishabha Malviya, Department of Pharmacy, School of Medical & Allied Sciences, Galgotias University, Plot No 2, Sector 17-A, Yamuna Expressway, Greator Noida, Gautam Buddh Nagar, U.P., India, Tel: +919450352185; E-mail: rishabha.malviya@galgotiasuniversity.edu.in

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## Abstract

Neem Gum (NG) is exudates of plant. Crude NG was purified using water as solvent and ethyl alcohol as precipitating agent. Effect of temperature and concentration on the surface tension of neem gum was determined. Emulsifying properties of gum was evaluated using sunflower as oil phase and purified water as continuous phase further prepared emulsions were evaluated in terms of globular size, flow rate, emulsion capacity and emulsion stability, foam capacity and foam stability and creaming (%). Free radical scavenging properties of polymer was also studied using ascorbic acid as standard against DPPH and $H_2O_2$ as initiator. At higher concentration of NG better emulsifying and foaming properties were observed due to reduction in rate of coalescence and creaming. Globular size of prepared emulsions does not changed significantly after 45 days. The effective concentration (EC50) sufficient to scavenge 50% of free radical generated using DPPH was found to be 4.55 µg/ml ± 0.98 and 29 µg/ml ± 1.21 for ascorbic acid and NG respectively. The effective concentration (EC50) sufficient to scavenge 50% of hydroxyl ions generated using $H_2O_2$ was calculated and found to be 55.27 µg/ml ± 0.67 and 71.36 µg/ml ± 0.87 for ascorbic acid and NG respectively. So it can be concluded from the findings of the results that Ng can be used as emulsifying agents in food, cosmetic and pharmaceutical industry with significant antioxidant potential.

**Keywords:** *Azadirachita indica* gum; Emulsion; Surface tension; Antioxidant; Globules

## Introduction

Emulsion is a form to present water insoluble components for longer period of time. To improve the stability of emulsion, use of suitable emulsifying agent is prerequisite. Emulsifying agents are used to reduce surface tension at the interface of globular dispersed phase and continuous phase. In another term emulsifying agents are employed to prevent coalescence of dispersed globules. Previous studies show that this type of systems formed globular emulsions [1-4].

Food industry has placed significant attention for the development of emulsion using gum as emulsifying agents. Industrial manufacturers are keen to develop stable emulsion using natural gum and mucilages because of their low cast, ease of availability and biodegradability. As natural polysaccharides reduces surface free energy and increase viscosity of aqueous phase hence can be used as emulsifying agent in pharmaceutical, food and cosmetic industry [5]. Food hydrocolloids have been widely used in food, pharmaceutical and cosmetic industries to provide stability, appearance and texture. They are mainly composed of various monosaccharide units. These monosaccharide units are responsible of rheological behaviour i.e. viscosity of system [6]. Mainly xanthan gum and starch are used in food industry as emulsifier because they increase viscosity and provide stability to emulsion. The ability of polymer to enhance emulsion ability depends upon structure and concentration of polymer [4]. Natural gums may contain –COOH and $–CH_2OH$ groups in their molecular structure. They easily donate H+ ions in aqueous phase to neutralize free radicals and can be act as antioxidant. Studies also elicit the fact that protein content also responsible for antioxidant activity of polysaccharides [7-10].

Neem gum is a plant exudates obtained from the trunk of *Azadirachita indica* family Meliaceae. NG contains D-galactose, L-arabinose, D-glucuronic acid and some traces of D-xylose. Arabinose is present as furanose form and can be proved by acidic hydrolysis which removes arabinose and fucose in primary stage. Generally gum is found as methyl derivative of acid [11].

This manuscript showcase the emulsifying and antioxidant potential of NG to attract attention of food, drug and cosmetic manufacturer for its possible commercialization. The present study is divided in three parts. In the first step, process of purification of crude NG is presented, and then emulsions are prepared using various concentrations of NG and evaluated the effect of concentrations using various parameters. Finally antioxidant potential of NG is evaluated against both DPPH radicals and hydroxyl radicals.

## Material and Methods

Crude gum was purchased from local shop of New Delhi India. Gum was authenticated by Prof. D.K. Chauhan, Department of Botany University of Allahabad; Allahabad U.P. Crude gum was dissolved in sufficient amount of purified water and heated up to 40°C. After 2 h gum solution was filtered through double folds muslin cloth to remove un-dissolved portion. Gum was precipitated by using ethyl alcohol and dried in oven at 40°C. Further gum was powdered, passed through 60 # sieve and stored in airtight polypropylene jars under desiccated condition.

## Physicochemical characterization of gum

**Identification tests for proteins, carbohydrates and tannins:** Chemical characterization was done by using aqueous extract of tamarind gum. Test for proteins, alkaloids, carbohydrate, tannins, glycosides and amino acids were performed according to standard procedure [12,13].

**Tests for carbohydrates:** Molisch's test: 1% of TSP solution was prepared in distilled water. 2 drop of α-naphthol in alcohol was added and mixed well followed by adding concentrated sulphuric acid drop wise from the sides. Purple ring was observed at the junction of two liquids indicates the presence of carbohydrates.

**Tests for reducing sugars:** Fehling's test: 1 ml from each of Fehling's solution A and B were added to the 1% of TSP solution, mixed and heated for 5 min to 10 min on a water bath. Initially yellow followed by brick red color precipitate was formed indicates the presence of reducing sugars.

**Benedict's test:** Equal volumes of Benedict's reagent and TSP solution were heated in boiling water bath for 5 min. Green/yellow or red color was observed detects presence of reducing sugars.

**Test for proteins:** Biuret test: 4% of sodium hydroxide along with 1% of copper sulphate was added to the methanolic extract of sample, there was no formation of violet /pink color indicates the absence of protein.

**Million's test:** To the methanolic extract, mercury in nitric acid (Million's reagent) was added. No white precipitate was formed. It indicates the absence of protein.

**Test for lipids:** Sudan red III test: Sudan red III reagent was added into 1% of prepared sample solution. No red color is observed indicates the absence of lipids in polymer.

**Test for tannins:** Small amount of methanolic extract of gum was taken and treated with following reagents and appearance of endpoints of each test indicates the absence/presence of tannins:

***Methanolic extract of gum with 10% lead acetate solution:*** No white precipitate was formed.

***Methanolic extract of gum with 5% of ferric chloride solution:*** No deep blue color was observed.

***Methanolic extract of gum with 10% of potassium dichromate solution:*** No red color precipitate was formed. It indicates that tannin was absent in gum.

**Tests for aminoacids:** Ninhydrin test: Extract of gum was heated with 5% of ninhydrin reagent in boiling water bath for 10 min. Absence of purple/bluish color indicates the absence of aminoacids in gum.

**Tests for alkaloids:** Following reagents were used to detect presence/absence of alkaloids in polymer:

**Dragendroff's test:** 1% of TSP solution was treated with Dragendroff's reagent (solution of bismuth iodide), orange brown precipitate was not formed.

**Mayer's test:** Treated with Mayer's reagent (potassium mercuric iodide solution), Creamy precipitate was not formed.

**Hager's test:** Hager's reagent (saturated picric acid) was added to the TSP solution, yellow precipitate was not observed.

**Wagner's test:** With Wagner's reagent (solution of iodine in potassium iodide), reddish brown color precipitate was not formed indicates absence of alkaloid.

**Determination of surface tension:** 1% w/v solution of gum was prepared using distilled water and surface tension was determined. Effect of temperature on the surface tension was also determined. Surface tension was also studied at different concentration of polymer.

**Preparation of emulsion:** Emulsifying properties of NG was evaluated by formulation of O/W emulsion using sunflower oil as oil phase and NG as emulsifying agent. Initially 90 ml of gum solutions were prepared using 0.5% w/v, 0.75%, 1% w/v, 1.25% w/v and 1.5% w/v NG (named F1, F2, F3, F4 and F5 respectively). Gum solutions were prepared by triturating NG with 20 ml of double distilled water using mortal-postal followed by dilution up to 90 ml with continuous trituration using double distilled water. Further 10 ml of sunflower oil was added in each solution and homogenized at 5000 rpm for 30 min. Prepared emulsions were stored in airtight closed container at 30C for further evaluation.

**Characterization of emulsions:** Prepared emulsions were characterized for following parameters:

**Globular size and types of emulsion:** Globular size of prepared emulsions was analysed using optical microscopy (Globus, Ph/L/16/02). Initially magnification value was calculated using coincides point of stage and eye piece micrometre. In this measurement 50 globules in 5 different fields were examined.

Emulsion types were also evaluated using scarlet red as coloring agent.

**Viscosity and pH measurement:** Viscosity of emulsions was measured using Brookfield viscometer (Brookfield Laboratory Viscometer, model: DV1) and pH was determined using digital laboratory pH meter (Systronics, type 335).

**Flow rate:** Time required for each emulsion to flow through a 10 ml pipette was determined and flow rate was calculated using eqn. 1:

Flow rate=volume of pipette (ml)/ flow time (s) (1)

**Emulsion capacity and emulsion stability:** Freshly prepared emulsions were transferred into graduated Stoppard measuring cylinder and the emulsifying capacity was calculated using eqn. 2:

Emulsion capacity (%)=Flow time (s)/ total volume × 100 (2)

Emulsion stability was determined to evaluate effectiveness of preparation method [14]. After 6 h emulsion stability was determined using following eqn. 3:

Emulsion stability (%)=Final emulsion volume/ initial emulsion volume × 100 (3)

**Foam capacity and foam stability:** The foaming ability of emulsions was evaluated [15]. To determine foaming capacity emulsions were whipped at 10000 rpm for 5 min with a homogenizer (Ultra Turrax T-25, IKA, Germany). Foam volumes were noted after 10 s and foam capacity was calculated using eqn. 4:

Foam capacity (%)=Initial foam volume/ total volume of emulsion × 100 (4)

The reduction of foam volume after 30 min was expressed as foam stability and determined using following eqn. 5:

Foam capacity (%)=Foam volume after 30 min/ total volume of emulsion × (5)

**Determination of creaming:** Creaming rate is determined to evaluate stability of prepared sunflower emulsion [5]. In this study emulsions were transferred to graduate Stoppard measuring cylinder and stored at room temperature (30C). Height of creamed layer was measured at every 24 h for 21 days. Creaming rate was determined using following eqn. 6:

Creaming (%)=Height of creamed layer/ original height of emulsion × 100 (6)

**Antioxidant activity:** Polysaccharide (NG) backbone contain free -OH group which can easily donate proton when ionize in appropriate medium. So it becomes necessary to evaluate free radical scavenging properties of polymer. Radical scavenging characteristics of polymer were be evaluated for both DPPH radicals and hydroxyl radicals [16,17].

**DPPH radical scavenging assay:** DPPH (1, 1-Diphenyl-2-picryl hydrazyl) radical scavenging assay is a standard method to evaluate antioxidant potential of phytochemicals. Scavenging ability of phytochemical was compared with standard antioxidant ascorbic acid. To evaluate antioxidant activity of compound 0.1 mM solution of DPPH was prepared by adding 1.9 mg of DPPH in 100 ml volumetric flask and volume was made up to the mark. For the formation of free radicals solution was kept under dark for 30 min. Sample solutions were prepared in various concentrations viz. 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml. Same concentrations of ascorbic acids were also prepared as standard. 1 ml of each sample solution was added with same volume of DPPH solution, mixed vigorously and kept aside in dark place for 30 min. Absorbance's were measured at 517 nm. Same procedure was repeated with standard ascorbic acid solutions. % scavenging or % inhibition was calculated using following eqn. 7. Tests were performed in triplicate and the graph was plotted with the average of three observations.

% inhibition (or % scavenging)=Absorbance of control sample-Absorbance of test sample/ Absorbance of control sample × 100 (7)

$H_2O_2$ radical scavenging activity: In this study hydrogen peroxide was used to form hydroxyl radicals. Standard and sample solutions were prepared as above and concentrations of hydroxyl radicals were evaluated at 230 nm. % inhibition (or % scavenging) activity were calculated as per eqn. 7. Tests were performed in triplicate and the graph was plotted with the average of three observations.

## Results and Discussions

Water based method was feasible and effective to purify NG. Phytochemical screening of gum shows presence of carbohydrates while lipid, protein, volatile oils and alkaloids are absent in purified polysaccharide. Results of physicochemical test were summarized below in Table 1.

| S.no | Constituents | Chemical test | Observation | Inference # |
|---|---|---|---|---|
| 1 | Carbohydrates | Molisch's test | Purple ring at the junction of two liquids | + |
| 2 | Reducing sugar | Fehling's test, Benedict's test | Brick red color | + |
| | | | Green color | Confirmed |
| 3 | Protein | Biuret test | Red color | - |
| 4 | Tannins | Fecl2 test, Lead acetate test, and potassium dichromate test | Deep blue with Fecl2,white ppt with lead acetate solution and red ppt with potassium dichromate | - |
| 5 | Lipids | Sudan red III | No red color | - |
| 6 | Aminoacids | Ninhydrin test | Purple color | - |
| 7 | Alkaloids | Dragandroff's test, Mayer's test, Wagner's test, Hager's test | Orange brown ppt | - |
| | | | Creamy ppt | - |
| | | | Reddish brown ppt | - |
| | | | Yellow ppt | - |

**Table 1:** Physicochemical analysis of NG: # "+" Present; "-" Absent.

Surface tension is an important parameter to characterize as natural gums and mucilages are widely using as emulsifying agent in food and pharmaceutical industry. Results showed that as concentration of polymer increases, surface tension decreases (Figure 1).

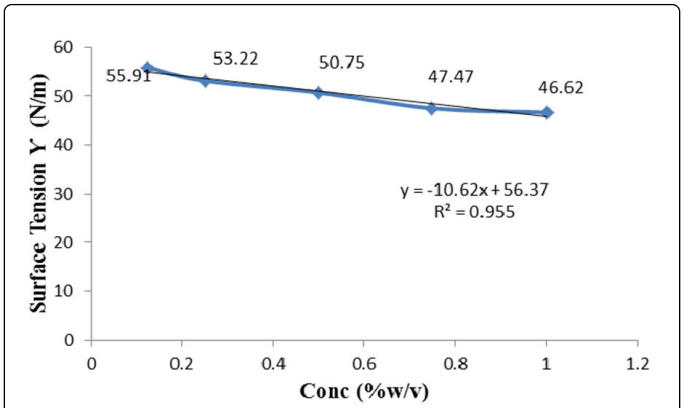


**Figure 1:** Effect of NG concentration on surface tension.

Results clearly demonstrate that NG shows significant surface activity. Surface activity of NG is due to its hydrophilic nature and absence of any lipophilic substance in purified form such as protein and fat. Thermodynamically emulsions are unstable due to high surface energy (tension) at the contact surface of oil and water molecules and it may leads to complete separation of two immiscible layers by coalescence and creaming. Hydrocolloids reduce surface tension and thickened the aqueous phase.

As shown in Figure 2 temperature has negative effect on surface tension. Surface tension of liquid is dependent on intermolecular forces between molecules. As the temperature increases, surface tension decreases because cohesive forces between liquid molecules decrease with increase in temperature. Cohesive forces reduce due to increase in the kinetic energy of liquid molecules [18].

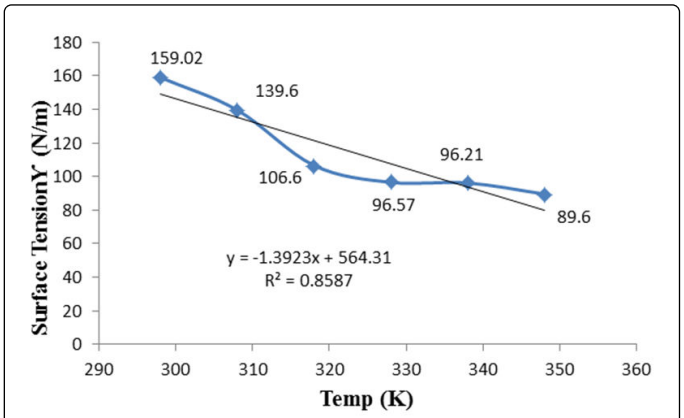


**Figure 2:** Effect of temperature on surface tension.

As shown in table globular size of prepared emulsions ranged from 1.4 µ to 2.6 µ. It was found from during globular size study that as the size increases with increase in polymer concentration (NG). pH of emulsions were found in the range of 5.50 to 5.80 shows slightly acidic nature of formulations. Flow rate of prepared emulsions were decreases as the concentration of emulsifying agent (NG) increases because of higher viscosity of continuous phase with polymer concentration. It is well known that higher the value of emulsifying agent better and more stable will be emulsions. This can be proved by data of emulsion capacity and stability shown in Table 2.

| Evaluation parameters | Formulation | | | | |
|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 |
| Globular size (µ) | 1.4 ± 11.06 | 1.6 ± 14.32 | 1.9 ± 18.33 | 2 ± 15.67 | 2.6 ± 22.38 |
| pH | 5.50 ± 0.08 | 5.5 ± 0.06 | 5.6 ± 0.05 | 5.62 ± 0.08 | 5.8 ± 0.07 |
| Viscosity (cPoise) | 0.8 ± 1.67 | 0.97 ± 2.07 | 1.67 ± 0.67 | 1.89 ± 1.47 | 2.52 ± 1.26 |
| Flow rate (ml/sec) | 4.1 ± 0.02 | 3.3 ± 0.02 | 2.9 ± 0.01 | 2.5 ± 0.01 | 2.2 ± 0.02 |
| Emulsion capacity (%) | 98 ± 1.56 | 97 ± 1.48 | 99 ± 1.84 | 98 ± 2.06 | 99 ± 1.89 |
| Emulsion stability | 98 ± 0.67 | 97 ± 1.53 | 100 ± 1.83 | 98 ± 1.74 | 96 ±2.42 |
| Foaming capacity (%) | 3.9 ± 0.36 | 4 ± 0.89 | 4.7 ± 1.02 | 5.1 ± 0.96 | 5.2 ±1.54 |
| Foam stability | 2.04 ± 0.67 | 2.04 ±1.47 | 2.04 ± 1.07 | 2.78 ± 0.98 | 3 ± 1.68 |

**Table 2:** Characterization parameters of prepared emulsions.

Natural polysaccharides have good foaming properties and these properties are directly proportional to concentration of polysaccharide in different formulations (solution, suspension and emulsion). Foaming capacity and stability data shown in Table 2 easily predict the concentration dependant foaming properties of prepared emulsions. At higher concentration of NG better foaming properties were observed.

Types of emulsion were identified using scarlet red as coloring agent. Scarlet red is oil soluble dye and red in color. As shown in Figure 3 droplets were appeared as red, so it can be concluded that oil was present as globules (dispersed phase) while water as continuous phase. Hence prepared emulsions were o/w in nature.

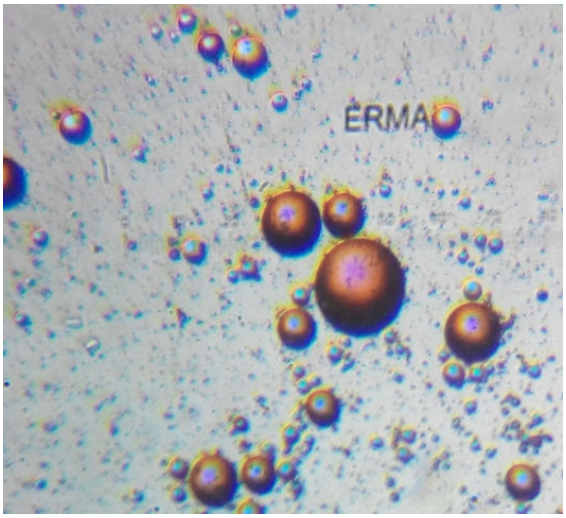


**Figure 3:** Evaluation of types of emulsion using scarlet red.

Emulsifying properties of NG may be due to entrapment of sunflower oil globules into polymeric chain. As the polymer concentration increases, globules become more tightly entrapped into polymeric conformation. Higher concentrations of polymer also cause more viscosity which further prevent mobility of globules and delayed their coalescence. As described in different studies emulsifying properties of gums either due to their interface activity [19,20] or due to viscosity modifying properties which further prevent interaction of globules [21]. Polymer imparts good viscosity in aqueous phase which further provide more time to polymer to absorb at the interface. Reduction among the droplets interactions at higher concentration was responsible for slow creaming.

As stated in Stoke's Law, rate of creaming is directly proportional to globular size. Homogenization at high speed leads to formation of smaller droplets hence improved emulsion stability. To prepare stable emulsion, it is advisable to use high speed homogenizer to produce relatively smaller globules.

| Formulation | Creaming (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | After 1 day | After 2 days | After 3 days | After 4 days | After 5 days | After 15 days | After 21 days | After 45 days |
| F1 | 0 | 0 | 0 | 0 | 2 | 2 | 4 | 8 |
| F2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 8 |
| F3 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 6 |
| F4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| F5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |

**Table 3:** Creaming (%) of prepared emulsions.

As described by Stoke's Law, rate of creaming is inversely proportional to viscosity of polymer. This characteristic can be proved by creaming studies of formulations. As shown in Table 3 formulations containing higher concentration of NG shown less creaming as compared to formulation having low NG concentration. Data obtained from different studies also support the gum concentration dependant creaming behavior of emulsions [22,23]. Emulsions prepared with high concentration of gum were found to be more stable and remarkable variation in droplet size were not observed. Similar results were also reported by Arash et al [24].

Droplet size measurement is important parameters to characterize an emulsion average droplet diameter of globules were presented in Table 1 with standard deviation. At low polymeric concentration (0.5% to 1% w/v), size distribution of droplets does not change significantly during storage and it was revealed from the globular morphology (size and shape) analysis after 45 days (Figure 4). Increase in globular size at higher concentration (1.25% w/v and 1.5% w/v) is mainly due to coalescence and aggregation. It was also observed that on storage number of larger droplets increases on storage.

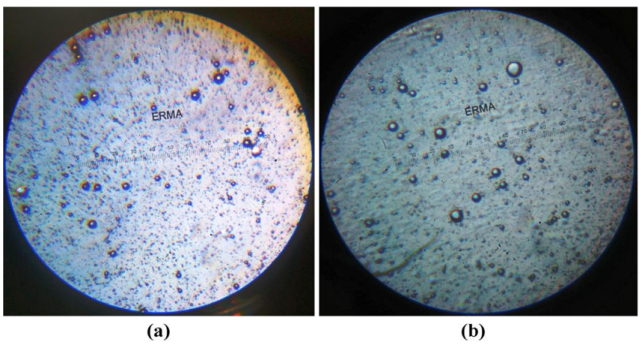


**Figure 4:** Particle size analysis of prepared emulsion (F4) (a) at t=0 (b) at t= 45 days.

DPPH has been widely used to evaluate antioxidant activity of various materials obtained from plant and microbial sources. DPPH is a chemical that create free radicals and these radicals are further scavenged by antioxidant. This experiment easily shows that NG has compounds that easily scavenged free radicals by donating free

hydrogen (proton) to a free radical in order to remove odd electron, which is responsible for the radical's reactivity. Hydrogen donating properties of polysaccharide can be supported by IR spectra analysis (shows presence of –OH groups). Figure 5 shows that as the concentration of NG increases scavenging properties of NG also increases. The effective concentration (EC50) sufficient to scavenge 50% of free radical was calculated and found to be 4.55 µg/ml ± 0.98 and 29 µg/ml ± 1.21 for ascorbic acid and NG respectively.

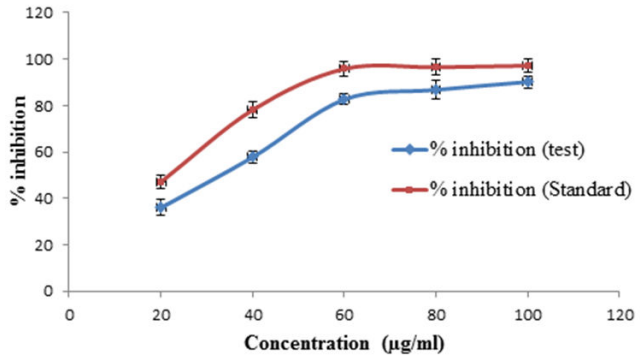


**Figure 5:** DPPH free radical scavenging activity of NG.

Figure 6 shows that NG has hydroxyl group scavenging activity in concentration dependant manner. As the concentration of polymer increases hydroxyl radical neutralizing power of polymer increases. The effective concentration (EC50) sufficient to scavenge 50% of hydroxyl was calculated and found to be 55.27 µg/ml ± 0.67 and 71.36 µg/ml ± 0.87 for ascorbic acid and NG respectively.

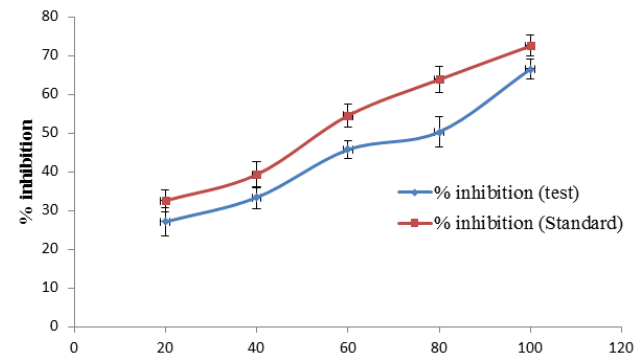


**Figure 6:** Hydroxyl radicals scavenging activity of NG.

## Conclusion

Natural polysaccharides have been widely used as emulsifying agent in food and pharmaceutical industry. They increases viscosity of continuous phase and significantly reduce surface tension. NG was used as emulsifying agent to stabilize o/w emulsions. Findings of studies elicit the fact that NG can be used as emulsifying agent in biphasic preparations and stabilize sunflower emulsions for longer period of time. It also be concluded that NG have significant antioxidant potential but less than ascorbic acid when used against DPPH and hydroxyl free radicals. So NG can attract worldwide manufacturer as substitute of other emulsifying agent. These studies also open new area of research regarding the use of emulsifying agent with antioxidant potential.

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