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In Vivo Application of *Jania Rubens* Silver Nanoparticles As A Chemopreventive Agent

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ABSTRACT

The present study focused on the synthesis of silver nanoparticles using the ethanolic extract of *Jania rubens (Linnaeus) J.V. Lamouroux*. The synthesized silver nanoparticles were characterized by UV-Vis spectra, TEM and FT-IR analysis. The formation of silver nanoparticles by *Jania rubens* showed the absorbance peak values at 350. The FT-IR spectrum exhibit a presence of bands at about 2418.3, 1994.03, 1909.18, 1841.6, 1769.37, 1719.44, 1641.13 and 1559.17cm1. TEM analysis exhibited a large and small spherical and rod particles. The chemical constituents, of *Jania rubens* extracts, were identified by GC-MS. In vivo experiment was conducted to test anti- nephrotoxicity of silver nanoparticles prepared by *Jania rubens* caused by gentamicin (GM). Additionally, a histopathological assessment in liver and kidney of the treated rats indicated the potentiality of *Jania rubens* nanoparticles to prevent the nephrotoxicity.

INTRODUCTION

The green chemistry was integrated into nanotechnologies especially when nanoparticles are to be used in medical applications (Albrecht *et al.*, 2006). The greenish biosynthesis of nanoparticles can be achieved via the selection of an environmentally acceptable solvent with eco-friendly reducing and stabilizing agents (Jegadeeswaran *et al.*, 2012). Reduction of silver nitrate with red alga *Jania rubens* is an effective method for the synthesis of silver a This alga is a quite plentiful biomass in nature, easy performance at chamber temperature used dead biomass and environmentally friendly compared to other chemical methods that use toxic chemicals. Recent findings evidenced that marine algae own active against bacteria, virus, fungi and tumor potentials (Ibraheem *et al.*, 2012, 2016, 2017; Al-Saif *et al.*, 2014). Marine algae have recently received significant attention for their potential as natural antioxidants (Abdel-Raouf *et al.*, 2015). Antioxidant activity of marine algae may arise from carotenoids, tocopherols, and polyphenols. These compounds directly or indirectly contribute to inhibition or suppression of free radical generation (Abirwami and Kowsalya, 2012). Today, marine algae research has been increased considerably for the search of new and effective medicines of natural origin. Synthesis of silver nanoparticles by algal extract is more advantageous than other biological processes. It is cost effective, eco-safe and suitable for human therapeutic use. In the present study, we synthesized AgNPs by using of the macro marine red alga *Jania rubens* and characterize these nanoparticles by UV-Vis spectra, TEM, and FT-IR analysis. Finally, we investigate in vivo studies the potentiality of the formed AgNPs against the gentamicin induced nephrotoxicity in albino rate.

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MATERIALS AND METHODS

The study area:

The fresh algal species were collected from the intertidal region of Alexandria city on the Mediterranean Sea along the 32 km. and situated about 225 km from Cairo (South-West) specifically, at a latitude of 31° 11' 53" N and longitude 29° 55' 9" E **(Map1**).

Sites of algal collections

Map. 1: Map of study area.

Aggregate and preparation of algal specimen:

The algal sample was manually collected were collected from the inter-tidal region of Alexandria maximum. This area has a unique feature which is highly rich in flora and fauna. Collected algal sample was immediately brought to the laboratory in new plastic bags containing pond water to prevent evaporation. The algal material was washed thoroughly with tap water and distilled water to remove extraneous materials and shade-dried for 5 days and oven dried at 60^{o}C until constant weight was obtained, then was grind into a fine powder using an electric mixer and stored at 4^{o}C for future use. Algal species were identified according to Aleem (1993) and Coppejans, *et al.* (2009).

Synthesis of silver NPs by algal ethanolic extract:

The synthesis of silver nanoparticles from marine alga *Jania rubens*, were carried out according to Singaravelu *et al.* (2007).

Characterization of AgNPs:

UV–Visible spectroscopy analysis:

The color change in a reaction mixture (metal ion solution + alga extract) was recorded through optical observation. The bioreduction of silver ion in aquatic solution was monitored by periodic sampling of aliquots 3–5 ml and subsequently measuring UV–Vis spectra of the solution at 300–400 nm using a 3–5 mm quartz cuvette. UV–Vis spectrum of these aliquots was observed as a function of time of their action on a UV–V is spectrophotometer (Japan). All the measurements were carried out at room temperature.

TEM analysis of AgNPs:

The morphological analysis of the nanoparticles was done with transmission electron microscopy (TEM). A drop of aqueous silver nanoparticle sample was loaded on a carbon-coated copper grid and it was allowed to dry completely for an hour at room temperature. The TEM micrograph images were recorded on a JEOL JEM 2100 high-resolution transmission electron microscope (Japan). The clear microscopic views were observed and documented in different ranges of magnifications.

FTIR measurement:

Fourier Transforms Infrared Spectroscopy (FTIR) was used to identify the possible biomolecules responsible for the reduction of the Ag ions and capping of the bioreduced silver nanoparticles synthesized by extract *Jania rubens*. In order to determine the functional groups and their possible involvement in the synthesis of silver nanoparticles, liquid algal silver nanoparticles analyzed on a Jasco 430 (Japan), in the diffuse reflectance mode operating at a resolution of 4 cm^{1}.

GC-MS Data analysis:

The crude ethanolic extracts of *Jania rubens* was analyzed by Gas chromatography-Mass spectrometry (GC-MS) for determination of active substances in extracts.

Biochemical analyses:

Chemicals and drugs:

Gentamicin was purchased from Sigma Company (United Kingdom), urea kit from Biomed (Egypt), creatinine kit from Diamond Diagnostics (Egypt) and uric acid kit from the spectrum (Egypt).

Experimental animals:

White male albino rats (*Rattus norvegicus*) weighing about 110 -150gm were used as experimental animals in the present achievement. They were gained from the animal home of research Institute of ophthalmology, El-Giza, Egypt. They were protected down notice for about 10 days before the onset of the experiment to exclude any intercurrent infection. The selection animals were lived in soft cages with well aerated covers at the normal atmospheric temperature ($25\pm5^{o}C$) as well as 12 hours daily normal light time. furthermore, they were given access to water and supplied daily with standard diet of known composition and consisting of not less than 20% proteins, 5.5% fibers, 3.5% fats and 6.5% ash and were also supplied with vitamins and mineral mixtures.

Experimental design:

The considered rats were divided into 3 groups containing six animals for each. These groups were:

Group 1:

It was regarded as normal animals which were kept without treatments under the same laboratory conditions.

Group 2:

The animals in this group were received an intraperitoneal nephrotoxic dose of gentamicin for 14 days (80 mg/kg body weight) (Hozayen *et al*., 2011). This group was considered as the control for the remained groups.

Group 3:

Toxic treated with nanoparticles *Jania ruben* aqueous extract): The rats in this group were administrated nanoparticles aqueous extract of *Jania ruben* by gastric intubation after gentamicin at injection at a dose level of 200mg/kg dry wt for 14 days (Ahmed *et al*., 2011). All the treatments were performed orally and daily between10.00 and 11.00 a.m. By the end of the experimental periods, normal, control groups and treated rats were sacrificed under diethyl ether numbness. Blood specimens were taken and centrifuged at 3000 r.p.m. for 30 minutes. The clear non- haemolyzed supernatant sera were taken away, divided into three section for each body animal, and kept at $-20^{0}C$ till used.

Assay of kidney functions:

Urea concentration in serum was determined according to the method of Vassault, (1986). Uric acid concentration in serum was determined according to the method of Tiffany *et al*., (1972). Creatinine level in serum was determined according to the method of Henry and Bernard, (2001).

Histopathological assessment:

After scarification and anatomy, kidneys were instantly excised, washed and rinsed in an ice-cold normal saline solution (0.9% NaCl, pH 7.4) until bleached of all the blood and blotted dry on filter paper sheets to remove blood, then kept in 10% neutral buffered formalin (pH 7.4) for 24h to histopathological investigation at the histology unit, National Cancer Institute, Cairo University, Egypt for preparation. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for desiccation. Samples were cleared in xylene and embedded in paraffin at 56 degrees in hot air oven for 24 hours. Paraffin beeswax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The acquired tissue portions were collected on glass slides, deparaffinized, and soiled by hematoxylin & eosin stain for routine examination through the light electric microscope (Banchroft *et al*., 1996).

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Statistical analysis:

The data were analyzed using the one-way analysis of variance (ANOVA) (PC-STAT, University of Georgia, 1985) followed by LSD test to compare various groups with each other. Results were expressed as the mean ± standard deviation (SD) and values of $P > 0.05$ were believed non-significantly various, while those of $P < 0.05$, $P < 0.01$ and $P < 0.001$ were believed significantly, highly and very highly significantly various, respectively.

RESULTS AND DISCUSSION

Synthesis of AgNPs by J. rubens:

The present wok describes the simple synthesis of AgNPs utilizing extracts from the marine macroalga *J. rubens*, which proceeded with the addition of 1mM $AgNO^{-3}$ at room temperature, while the solutions with $AgNO^{-3}$ changed color to dark brown without stirring, high temperature or static condition (see Plate 3.1). This result agree with Abdel-Raouf *et al*., (2013) who reported the color change to dark brown in a few min, but Princy and Gopinath, (2013) who reported the biosynthesis of Ag nanoparticles need to stir well for 1 minute, kept in a water bath at 60^{o}C for 1 hour and then incubated in dark at room temperature under static condition. Mulvaney, (1996) reported that the color change was due to excitation of surface Plasmon vibrations in the metal nanoparticles, indicating the transition of silver nitrate to AgNPs. According to the report of Medina *et al*., (2009) the formation of AgNPs is attributed to hydrophilic- hydrophobic interaction resulting in intermolecular force. The use of this simple and eco-friendly synthetic procedure can help promote interest in the synthesis and application of metallic nanoparticles.

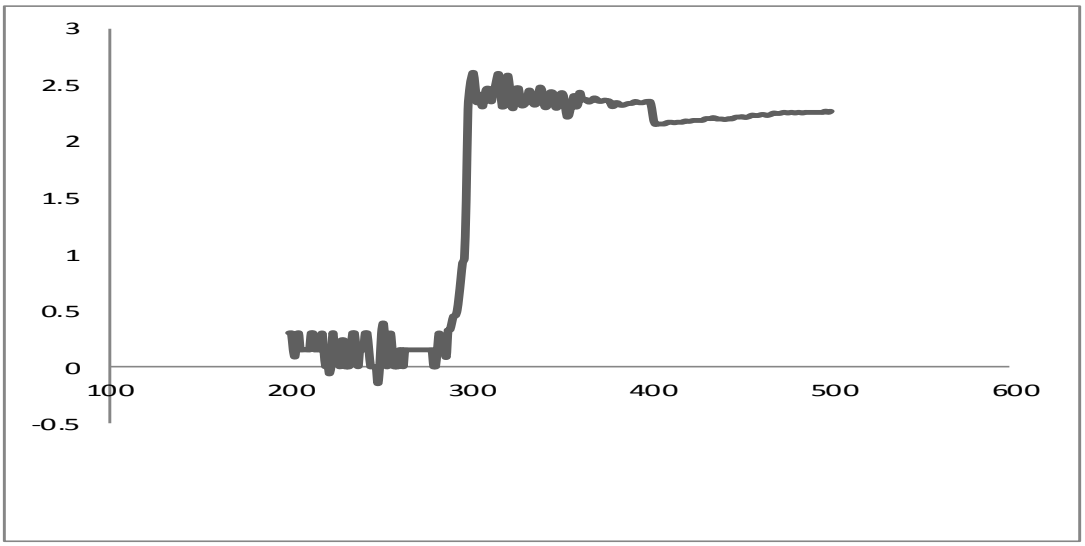
(A) **(B)** **(C)** **(D)**

Plate 3.1: Tubes containing the aqueous solution of 10^{-3} M of silver nitrate with clear color at the beginning of reaction (A); after adding *Jania rubens* ethanolic extract with yellow color after 2 minutes (B) ;after 10 min with brown (C) and after 20 min with dark brown color (D).

Characterization of AgNPs synthesized by J. rubens:

UV-visible spectroscopy analysis of AgNPS synthesize by J. rubens:

The absorption spectrum of AgNPs showed a well observable peak in visible range at 350 nm (graph 3.1), which is the characteristic wavelength range of AgNPs with the resolution of 1 nm between 200 to 800 nm. According to Kreibig and Vollmer (1995), UV- visual spectroscopy is an important technique for confirmation of formation and stability of metal nanoparticles in aqueous solution. A powerful absorption peak produces from the surface Plasmon absorption of the nano size silver particles (Stepanov *et al*., 2002).



Graph 3.1: UV-visible range spectra of AgNPS synthesized from AgNPS synthesized from *J. rubens* ethanolic extract.

TEM of AgNPs synthesized by J. rubens:

The TEM images of *Jania rubens* synthesized silver nanoparticles exhibited large and small spherical particles and small percentages of a rod, triangular. TEM analysis of particle size also showed maximum particles in size 0.2 µm, 100nm (see plate 3.2).

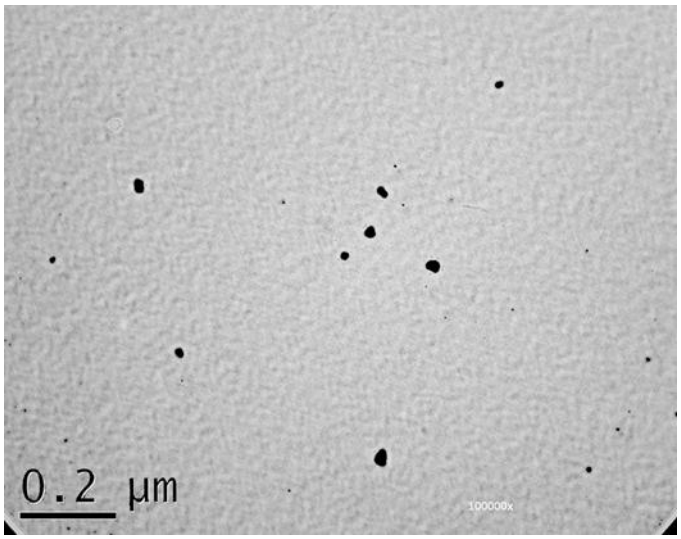
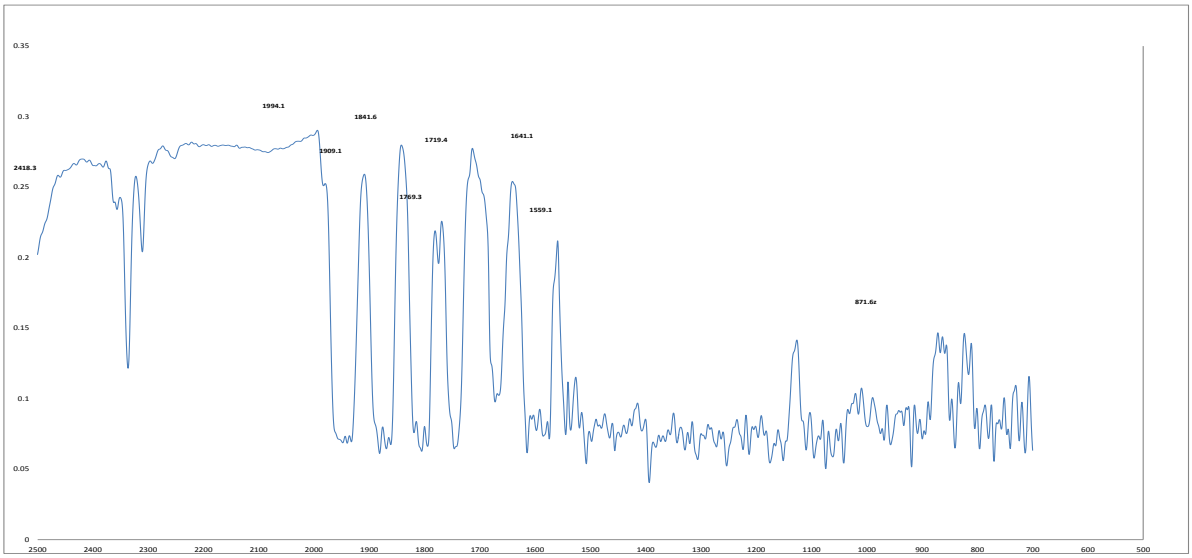


Plate 3.2: Representative TEM micrograph silver nanoplates synthesize by the reduction of $AgNo^{-3}$ ions in a *Jania rubens* ethanolic extract.

FT-IR analysis of AgNPs synthesized by J. rubens:

The FT-IR spectrum of the silver nanoparticles of on *Jania rubens* ethanolic extract is shown in graph 3. The presence of bands at about 2418.3, 1994.o3, 1909.18, 1841.6, 1769.37, 1719.44, 1641.13, 1559.17, 871.66 bands at about 2418.3,1994.o3, 1909.18, 1841.6 cm-1, corresponding to alkynes –C≡C– stretch, at 1769.37 C=O stretches Carboxylic acid. Since a member of C=O group within the cage of the cage of cyclic peptides was involved in stabilizing the nanoparticles, the peptides could play a major role in the reduction of silver ions. On the other hand, the shift of band from 1641.13 was attributed to the binding N–H bend I^{o} amines and the 871.6 peak showed Stretching mode S-O.



Graph 3.1: FT- RI of silver nanoparticles synthesis by *Jania rubens* extract ethanol

Gc-Ms analysis of the ethanolic extract of J. rubens:

The GC–MS analysis of ethanolic extracts of *J. rubens* revealed many components, phytochemical screening of the algae showed the presence of fatty acid e.g Butyric, Stearic, Myristic acid, Palmitoleic acid, Palmitic acid and Loliolide as a good antioxidant. Glycerin is used in medicine and food sweetener and preservative**,** Phytol used as Antimicrobial, anticancer, anti-inflammatory, antidiuretic, Immuno stimulatory and anti-diabetic,(4-Fluorophenyl) cyclopropane, 2-Methyl-5H-dibenz[b,f] azepine, Ethylic acid, 2-methoxy-4-aminophenol, 2-Pentadecanone,6,10, 14-trimethyl.

Table 3-1: Gc-Ms analysis of the ethanolic extract of *Jania rubens*

S	Compound (IUPAC-Name)	Common name	Peak area%	Activity
1	Fatty acids Tetradecanoic acid Hexadecanoic acid 9- Octadecenoic acid, (E) Octadecenoic acid Cis9- Hexadecanoic acid	 Myristic Palmitic acid Elaidic acid Stearic acid Palmitolic acid	 0.97 22.41 1.84 0. 36 0.97	Antioxidant Muscle weakness, Tetany, Anemia, Diarrhea, Pulmonary edema, Respiratory failure. Antiviral, antibacterial, antioxidant activities antiviral, antibacterial ,antioxidant activities
2	Esters -Hexadecanoic acid, ethyl ester	 Palmitic acid, ethyl ester	 0.77	antiviral, antibacterial, antioxidant activities
3	Terpenese -(-)Loliolide (2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol 3-Buten-2-one,4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-I-yl) Dihydroactinidiolide	 -(-)Loliolide Phytol B-Ionon-5,6-epoxide	 3.93 0.93 3.01 2.23	 Antioxidant Antimicrobial, anticancer, anti-inflammatory, antidiuretic, immunostimulatory and anti-diabetic Flavoring agents
4	Ketones 2-Pentadecanone,6,10,14-trimethyl-	 Hexahydrofarnesyl acetone	 0.65	
5	Sulfur compounds Dimethyl Sulfoxide	DMSO	13.1	Antibacterial, antifungal, anticancer, asdietary supplement
6	Sterols Cholest-5-en-3-ol(3.beta.)		 1.89	anti-inflammatory, antipyretic drug
7	Alcohols 1,2,3-Propanetriol	 Glycerin	 7.63	medicine, food sweetener and preservative
8	Phenolics 2-methoxy-4-aminophenol		 1.29	The manufacturing of drugs such as paracetamol and clofibrate as well as being used as developer and antioxidants, pharmaceutical intermediates, dyes
9	Organic acid Acetic acid n-Butyric	 Ethylic acid Butanoic	 20.56 1.97	Pharmaceutical preparations e.g Vaginal gels anti-inflammatory, antimicrobic For disinfectants, and as an Asthlab, and in pharmaceuticals.
10	2-Butyloxirane	Butyloxirane	1.90	
11	1,2,3,4-Butanetet	Threitol, L	1.77	antibacterial

12	Cyclotrisilxane, hexamethyl		3.80	Antifungal, antibacterial and antimicrobial
13	2-Methyl-5H-dibenz[b,f]azepine		2.22	
14	Indole-3-Carboxylic acid,5-hydroxy		3.79	
15	(4-Fluorophenyl) cyclopropane		2.14	Anticancer andanti-inflammatory
16	1,1,1,3,3,5,5,7,7-Nonamethyltetrasiloxane		0.80	

Effect of Jania rubens nanoparticles on Urea, Uric acid, Creatinine levels in rats:

The data describing the effect of *Jania rubens* nanoparticles on serum uric acid, urea and creatinine levels in rats are presented in the table 3-1 and illustrated in figures 3-1, 3-2 and 3-3.Data in the table (3-1) indicated that blood creatinine, uric acid, and urea nitrogen activities were significantly (P < 0. 001) elevated in gentamicin intoxicated rats in comparison to control group. Administration of nanoparticles of *Jania rubens* at daily doses of (200 mg/kg b.wt, orally) showed significantly (P < 0. 001) decrease of these levels as compared with gentamicin intoxicated rats, the percentages of change were (-0.408%) for uric acid, (-0.398%) for urea and (-0.536%) for creatinine respectively.

Table 3-2: Effect of *Jania rubens* nanoparticles on uric acid, urea and creatinine level in rats.

	Uric acid (mg/dl)	% change	Urea (mg/dl)	% change	Creatinine (mg/dl)	% change
Normal	0.68 ± 0.1^{b}	-	35.4 ± 0.9^{b}	-	0.45 ± 0.04^{b}	-
Gentamycin	1.15 ± 0.04^{a}	0.408	60.2 ± 3.0^{a}	0.700	1.10 ± 0.09^{a}	1.44
Gentamycin + *Jania*	0.68 ± 0.03^{b}	-0.408	36.2 ± 1.8^{b}	-0.398	0.51 ± 0.04^{b}	-0.536
F- Probability	< 0.001		< 0.001		< 0.001	
LSD at 5 % level	0.102		6.622		0.175	
LSD at 1 % level	0.139		9.031		0.239	

- Data are expressed as mean ± standard error.
- Number of rats in each group is 6.
- Means, shared only superscript symbol (s) are not significantly different.
- Percentage changes (%) were calculated by contrast gentamicin group with normal, gentamicin treated group (gentamicin + *Jania* and gentamicin) were compared with gentamycin group.

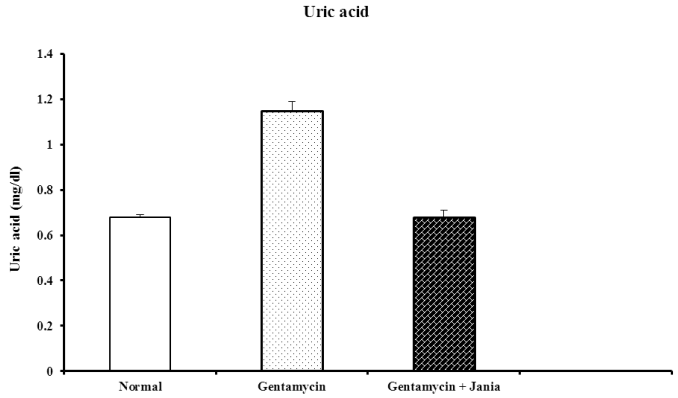


Fig. 3-1: Effect of *Jania rubens* and nanoparticles on serum Uric acid level.

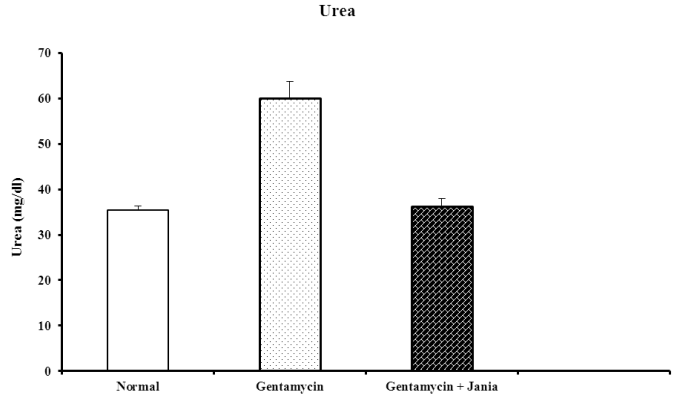


Fig. 3-2: Effect of *Jania rubens and* nanoparticles on serum Urea level.

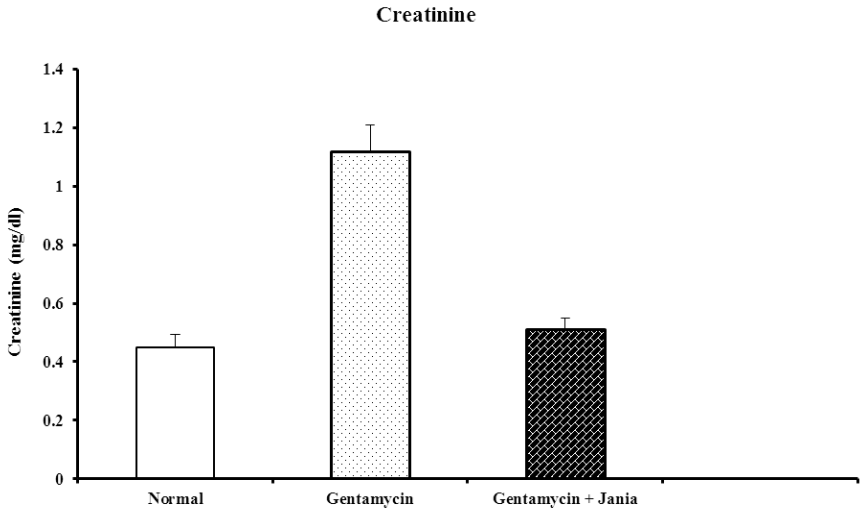


Fig. 3-3: Effect of *Jania rubens* and nanoparticles on serum creatinine level.

Gentamicin is one antibiotic that is used a lot. This Drug is used for the treatment of gram-negative bacterial infection in humans and animals. The present study aimed to evaluate the protective effect of nanoparticles *Jania rubens* against gentamicin-induced nephrotoxicity in rats. Gentamicin-induced toxic effects in the kidney. The renal dysfunction due to gentamicin treatment was manifested by a very highly significant increase in serum urea, uric acid, and creatinine levels as compared to the normal group of the rat. This is in agreement with the results of Hozayen *et al*., (2011). It was reported that treatments with gentamicin produce nephrotoxicity as a result of the reduction in renal functions which was characterized by an increase in serum creatinine and serum urea level accompanied by impairment in glomerular functions. Serum creatinine level was more significant than the urea levels in the earlier phase of the renal damage. In the present study, it was shown that treatment with gentamicin alone to rats caused nephrotoxicity, which was correlated with heighten urea, and creatinine levels in plasma. Our result showed that the treatment of gentamicin intoxicated rats with nanoparticles algae cause decreasing in serum creatinine, serum uric acid, and serum urea level due to its ability to treatment nephrotoxicity. This is in agreement with the results of Hozayen *et al*., (2011). Urea is the end product of protein catabolism, and the presence of some toxic compounds might increase blood urea and decrease plasma protein (Varely *et al*., 1987). Increased catabolic proteins and accelerated amino acid deamination for gluconeogenesis is possibly an acceptable postulate to interpret the elevate urea levels (Bishop *et al*., 2005). Uric acid is the end product of nucleic acid catabolism in tissues, and the raise in its concentration may be due to degradation of purines or by either overproduction or inability of excretion.

Elevated serum creatinine is indicative of renal injury and associated with the abnormal renal function, especially as it connect to glomerular function (Bennett, 1996; Bishop *et al*., 2005). Upon treatment of intoxicated rats with nanoparticles, *Jania rubens* improve and normalize the levels of urea, uric acid, and creatinine. These results indicated that these extracts may protect against gentamicine-induced renal toxicity due to these extract contain on Sulfur compounds and active antioxidant constituents. It was reported that treatments with gentamicin produce nephrotoxicity (Atessahin *et al*., 2003) as a result of the reduction in renal functions which was characterized by an increase in serum creatinine and serum urea level accompanied by impairment in glomerular functions. Serum creatinine level was more significant than the urea levels in the earlier phase of the renal damage. Our result showed that the treatment of gentamicin intoxicated rats with nanoparticles algae caused decreasing in serum creatinine and serum urea level. The effects of the algal extracts on kidney function activities (Uric acid, Urea, and Creatinine) under the aforementioned conditions are documented in Table 3-2. Serum Uric acid, Urea, and Creatinine levels were slightly affected by the oral administration of the selected nanoparticles algal extracts in comparison with the toxic group. Marine algae appear to be a potential source of unsaponifiable sterols. Different steroidal compounds were reported to have antitumor effects (Zhang, 2006). Also, it was reported that the cytotoxic activity of some red and brown algal species could be attributed to the presence of a mixture of organic acids such as oleic, myristic, capric, lauric, palmitic, linoleic, and stearic acids, respectively (Kamenarska *et al*., 2009). *J. rubins* consist of rich components, Carotenoids, Halides, Polysaccharides, Proteins, Lipoidal matters, mixture of acids concentrated nitric, perchloric, and sulfuric, element (Na, Ca, Mg, P, and K), *Jania rubens* contained the highest protein, total lipid, fatty acid, carobyhates, amino acids (Khairy and El-Shafay, 2013). In the current study, *Jania rubens* nanoparticles extracts possess strong nephroprotective activity which could be attributed to their active antioxidant constituents, bioactive phytoconstituents such as fatty acids, esters, terpenes, sterols. Additionally, FT-IR spectrum of *Jania rubens* nanoparticles extracts showed indicated the presence of alkynes, carboxylic acid, amino, amines, and sulfonate.

Uric acid, Urea, and Creatinine levels decreased significantly in the groups 3 with each of the tested nanoparticles algal extracts in comparison with the group 2.

Nephrotoxicity induced by gentamicin is evident from the abnormal histological findings. Histologic alterations provide a clear evidence of nephrotoxicity following gentamicin administration, including the renal tubules showed coagulative necrosis in the lining epithelial cells. This is in agreement with Ramhariya *et al*., (2015). On the other hand, treatment with nanoparticles of *Jania rubens* had shown better improvement in the Congestion in the cortical blood vessels this show that antioxidants have the special role in the prevention and treatment of diseases.

Histopathological assessment of kidney of normal control rat confirmed healthy anatomical features (Fig.3.4). Gentamicin treated rat's kidney illustrated the presence of inflammatory depositions and the renal tubules showed coagulative necrosis in the lining epithelial cells (Fig. 3.5) cell necrosis. Kidney of rats treated with nanoparticles *Jania rubens* showed minimal necrosis and least inflammatory accumulations with normal kidney anatomy in fig.3.6, which revealed nephroprotective effect.

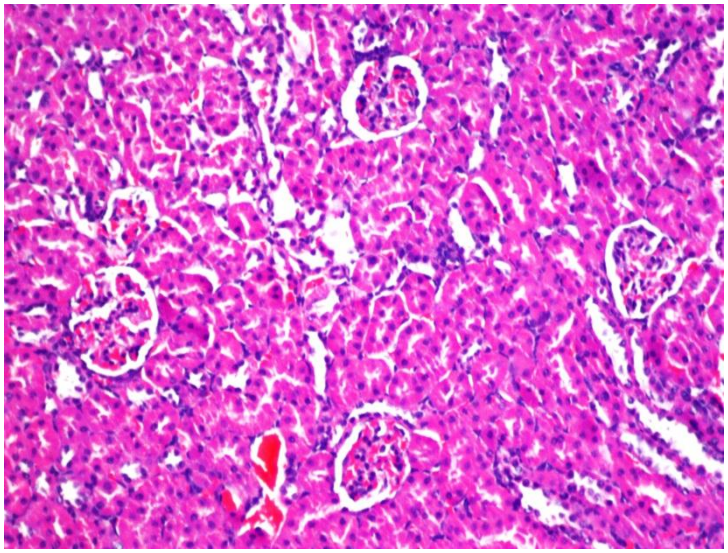


Fig. 3.4: Kidney of rat showing normal histological structure of the gloimeruli and tubules at the cortex

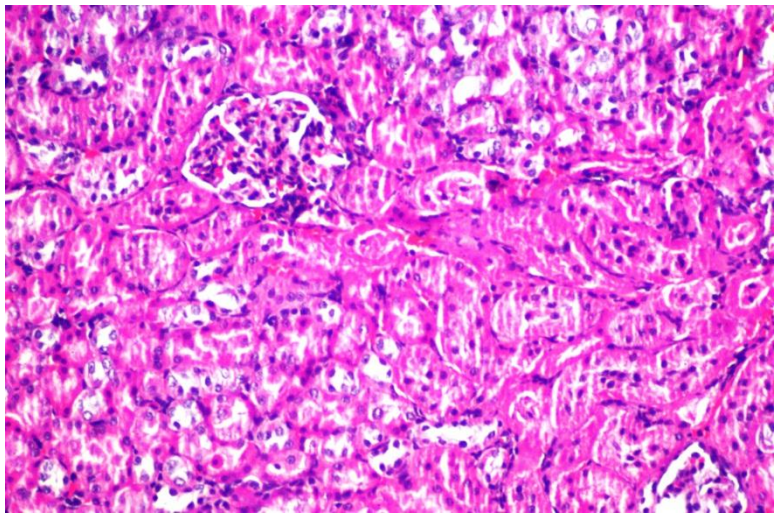


Fig. 3.5: Showing coagulation necrosis in lining tubular epithelium of the cortical portion .

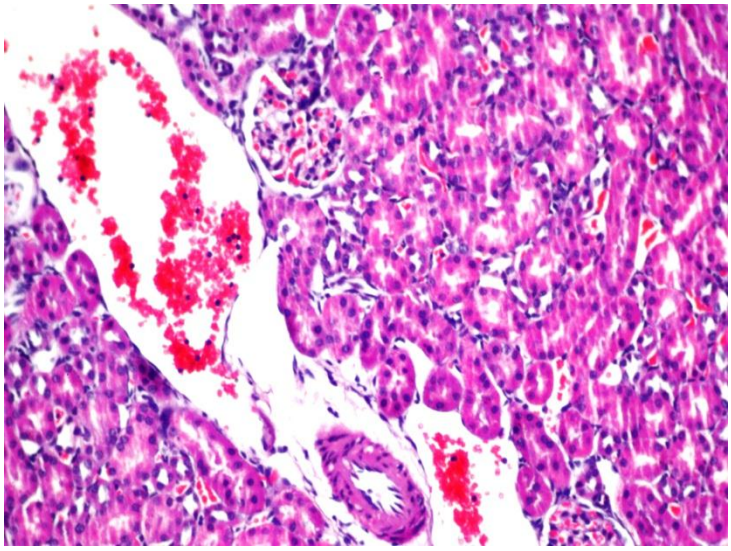


Fig. 3-6: Kidney of rate showing congestion in cortical blood vessels

Conclusion:

In conclusion, the bioreduction of aqueous silver ions to silver nanoparticles by using of the marine red alga *Jania rubens* has been demonstrated by easy, ecofriendly, economical, fast and low-cost approach. The characteristics of the obtained silver nanoparticles were studied using UV-V, FTIR, and TEM techniques. The formed silver nanoparticles with high stability and without any impurity. The chemical constituents, of *Jania rubens* extracts, were identified by GC-MS. In vivo experiment was conducted to test anti- nephrotoxicity of silver nanoparticles prepared by *Jania rubens* caused by gentamicin (GM). Additionally, a histopathological assessment in liver and kidney of the treated rats indicated the potentiality of *Jania rubens* nanoparticles to prevent the nephrotoxicity.

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