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Characterization of nanorosemary and encapsulated rosemary nanoparticles and their effect on lead induced toxicity in Wistar rats.

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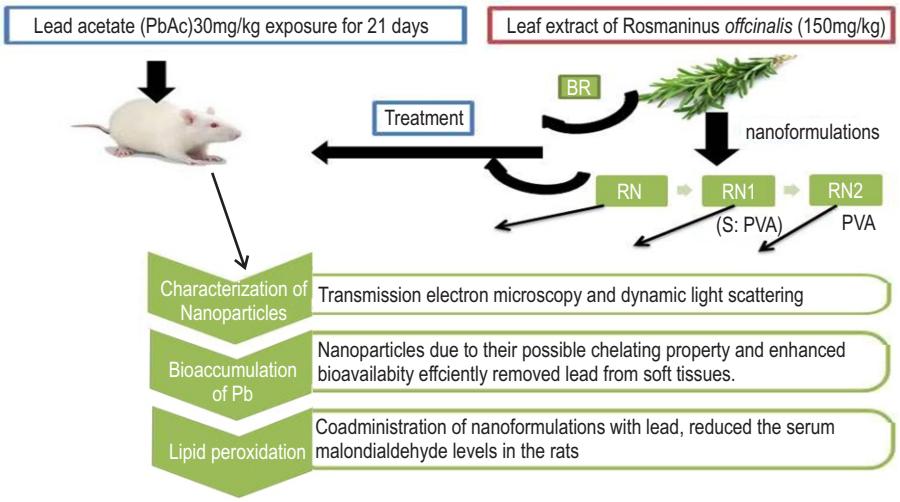
Abstract

Aim : Lead-induced toxicity is responsible for physiological disarray in humans and animals. The present study was aimed to synthesize'green'nanoparticles of rosemary *(Rosmarinus officinalis)* leaf extract and investigate the comparative ameliorative efficacy of rosemary extract and the nanoformulations against the lead toxicity in male Wistar rats.

Methodology : Non-capsulated rosemary particles were synthesized with dichloromethane. Encapsulated nanoparticles of rosemary were synthesized with two polymers, starch and a combination of starch with polyvinyl alcohol.The nanoparticles were characterized by transmission electron microscopy and dynamic light scattering.Parallel to this, Wistar rats were exposed to 30mgKg^{-1} lead acetate, four experimental groups were treated with rosemary extract (150mgKg^{-1}) as bulk (BR), nanorosemary (RN), encapsulated nanoparticles RN1 and RN2, respectively, for a period of 21 days.

Results : Green synthesis of water soluble nanorosemary (RN) and encapsulated nanoparticles RN1 and RN2, with composite and single polymer, was successfully achieved. The micrographs showed spherical and irregular morphology of RN, while RN1 and RN2 were more spherical.The average size of nanorosemary (RN) was 75.45 nm, while that of encapsulated rosemary nanoparticles (RN1) and RN2 was 2.665 nm and 3.026 nm. Treatment with nanoformulations reduced the level of serum malondialdehyde in the rats. Also, due to their enhanced bioavailability, the nanoparticles efficiently reduced the lead concentrationin tissues compared to the bulk rosemary.

Interpretation : The results demonstrate the enhanced protective efficacy of encapsulated nanoparticles, in particular RN1. Thus, the formulation could be a potential ecofriendly, safe and novel therapeutic approach against lead toxicity.



Introduction

Lead is a potent heavy element ubiquitous in the environment. Although lead toxicity has been extensively researched and reported, it remains a widespread occupational and environmental problem throughout the world. Owing to its unique physical and chemical properties, it is extensively used in industrial processes. It is well documented that Pb can cause neurotoxicity, nephrotoxicity and deleterious effects on the hematological and cardiovascular systems (ATSDR,2007). Lead exposure occurs mainly through the respiratory and gastrointestinal systems. Lead-induced hepatic damage is primarily caused by oxidative stress, a disturbance of the prooxidant–antioxidant balance by generation of reactive oxygen species manifested as lipid per oxidation (Gurer and Ercal, 2000; Flora *et al*., 2012). Although chelation therapy is preferred for metal intoxication , the associated side effects of chelating agents has favored the use of natural antioxidants for alleviating metal induced oxidative tissue damage (Flora and Pachauri, 2010). Plants, including herbs and spices, are endowed with phytochemicals which are potential source of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz *et al*., 2006; Erkan *et al*., 2008). Rosemary (*Rosmarinus officinalis*) is a native to the Mediterranean and is widely used in cosmetics as culinary herb.It is commonly used as a spice and flavoring agent in food processing (Saito *et al*., 2004; Panda, 2009; Ibarra *et al*., 2010). Rosemary leaf extract has also been proposed by the European Community Reference Laboratory as a feed additive (Dossier no. FAD -2004-0003) in the classof antioxidants.The most active antioxidative constituents of rosemary are phenolic diterpenes (carnosic, carnosol, rosmanol, rosmadial, 12-methoxycarnosic acid, epi-, and iso-rosmanol) and phenolic acids (rosmarinic and caffeic) (Brewer, 2011). Carnosic acid has several times the antioxidative activity as BHT and BHA (Richheimer *et al*.,1996). Rosemary leaf extract in particular, has been demonstrated to possess strong antioxidant activity, predominantly due to the carnosic acid which is present in the alcoholic extract (10%).This effectiveness of rosemary extract as an antioxidant has led to its commercial exploitation. The major drawback in using the herbal antioxidants as nutratherpeutics is their low bioavailability. However, the efficacy of such natural antioxidants can be improved by enhancing their bioavailability. Thus, current strategies in targeted drug delivery can be an alternative and promising approach in administration of these compounds. These approaches include improved formulations for better delivery such as liposomes, micelles, phospholipid complexes and nanoparticles (Anand *et al*., 2007). Further, nano formulations of the antioxidants offer enhanced drug delivery system and has proved to be more effective (Yadav *et al*., 2012; Flora *et al*., 2012).

Green synthesis of nanoparticles and nano encapsulation is a novel approach to enhance the bioavailability of phytocompounds. Starch is a natural biopolymer and provides a stable nanocomposite along with polyvinyl alcohol. The present study is an attempt to synthesize and characterize nanorosemary and nano encapsulated rosemary using starch and polyvinyl alcohol by a simple and cost effective method. The study alsocompares the efficacy of bulk rosemary leaf extract to nanorosemary and nano encapsulated rosemary incombating lead-induced oxidative stress and reversing the bioaccumulation of metal in rat model.

Materials and Methods

Synthesis of Nanorosemary (RN) : Rosemary nanoparticles were prepared from 150 mg of rosemary leaf extract powder (10% carnosic acid, Hunan Geneham Biomedical Technology Ltd. China) dissolved in 25 ml dichloromethane (99.5–99.8%; Merck, Singapore) and mixed with a magnetic stirrer. Further, 3 ml rosemary solution was sprayed into 60 ml boiling water dropwise at a flow rate of 0.2 ml min-1 in 5 min under ultrasonic conditions (ultrasonic power of 100 W and a frequency of 30 kHz). After sonication for 20 min, the contents were stirred at 200-800 rpm at room temperature for about 30 min and then dried.

Synthesis of Rosemary nanoparticles encapsulated in (S and PVA) composite polymers (RN1) : Nanoparticles encapsulated in composite polymers were prepared with rosemary extract: starch: polyvinyl acohol (1:5:5 w/w/w) by nano precipitation technique (Bilati *et al*., 2005; Zili *et al*., 2005). Rosemary powder(150mg) and appropriate amount of starch (20 wt% of helical amylase, 80 wt% of branched amylopectin, and 16 wt% of moisture,Aldrich, Germany) were dissolved in 90 ml of ethanol. The internal organic phase solutions were quickly injected into 200 ml external aqueous solution containing appropriate amount of polyvinyl alcohol (BDH Chemicals, England) and the solutions were then homogenized at 35,000 rpm for 35 min. Ethanol was completely removed and freeze-dried. The nanoparticles powder were collected and stored until further use.

Synthesis of Rosemary nanoparticles encapsulated in PVA polymer (RN2) : The rosemary nanoparticles encapsulated in PVA polymer was prepared with rosemary extract: polyvinyl alcohol (1:10: w/w/w) by the nano precipitation technique (Bilati *et al*., 2005; Zili *et al*., 2005). Rosemary powder (150mg) and half amount of PVA were dissolved in 90 ml of ethanol. The internal organic phase solutions were quickly injected into the 200 ml external aqueous solution containing the residual amount of PVA, and then the solutions were homogenized at 35,000 rpm for 35 min. The ethanol was completely removed, and then freeze-dried. The nanoparticles powder were collected and stored until further use.

Experimental design : Forty two male Wistar rats (150-200 g) were procured from the Animal House Facility at King Saud University, Riyadh in the month of April, 2015. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Zoology Department, College of Science, King Saud University. The animals were acclimatized to laboratory conditions for two weeks prior to the experimental period. The animals were divided into six groups each comprising seven rats. Group I was considered as negative control and the rats were

administered only normal saline. Rats of group II were to treated with 30mg kg^{-1} lead acetate. Rats of group III were treated with 30 mg kg^{-1} lead acetate and 150 mg kg^{-1} of rosemary leaf extract. Rats of group IV were adminestrened 30 mg kg^{-1} and lead acetate with 150 mg kg^{-1} nanorosemary. Rats of group V were given 30 mg Kg^{-1} lead acetate with 150 mg kg^{-1} nano encapsulated rosemary (RN1). Rats of group VI were given mg kg^{-1} lead acetate 30 with 150 mg kg^{-1} nano encapsulated rosemary (RN2).

The doses were given daily at 8:00 am. During the experimental period, the rats were fed with laboratory chow. Blood was drawn by the orbital sinus venipuncture from the rats of each experimental group and serum was prepared. The serum was prepared by drawing the blood into red stoppered tubes and left at room temperature for clotting followed by centrifugation at 3500 rpm for 15-20min.The animals were sacrificed and dissected to excise the liver, kidney and spleen. The tissues and serum were storedat -80° Cuntil further analysis. The serum was used for analysis of MDA level.

Characterization of nanoparticles : The average particle size of nanoparticles of rosemary were measured by dynamic light scattering performed on Zetasizer (Nano series, HT Laser, ZEN3600 from Molvern Instrument, UK). The intensity of scattered light was detected at 90° to the incident beam. Aqueous solutions of nanoparticles of rosemary (RN, RN1, RN2) were placed in a quartz cuvettes for estumation. Transmission electron microscopy (TEM) was performed (JEM-1011, JEOL, Japan) to assess the morphology of prepared nanoparticles. The samples were prepared by placing a drop of aqueous dispersion of nano formulations on the copper grid and allowing it to air dry.

Determination of lead in tissue : Tissue samples (0.5 g each) were digested in a Teflon vessel with 3 ml concentrated HNo_3 and kept overnight at 85°C. Thereafter, 1 ml 30% H_2O_2 was added and the mixture was heated to 85°C for 1 hr. The clear supernatant was diluted to 10 ml with deionized water. An aliquout of 5 µl was mixed with 5 µl modifier (EDTA). The lead concentration in the sample was determined with Atomic Absorption Spectrometer (Model AA240 Z, Agilent Technologies, Santa Clara, USA) at 217 nm wavelength and detection limit of 4.4 μgl^{-1}. The lead concentration in the tissues was expressed as $\mu g\ g^{-1}$ wet wt .

Determination of serum MDA level : MDA level in serum was determined with the Alliance Waters High performance Liquid Chromatography (HPLC) 2695 system and a multi fluorescence detector, Model 2475. This system was operated by a Dell Optiplex GX1 computer and Empower software. The reversed-phase analytical HPLC column was a ODS Hypersil from Thermo Scientific (4.6mm x 25.0cm x 5µm). A guard column, Waters Symmetry TM C18 (4.6 mm x 2 cm, 5-µm particle size) with the same packing materials was placed in front of the analytical column for protection. Elution was carried out at a flow rate of 1.0 ml min^{-1}. The column effluent was quantified at an excitation and emission wavelengths of 515 and 553 nm respectively. Run time per sample was 4.0 min. Aliquots of 25µl of serum were mixed thoroughly with 1ml of TBA reagent and 10µl of 5% BHT solutions, vortexed for 2 min and then heated it for 60 min in a water bath at 95°C. Thereafter, the sample was cooled for 20 min and then centrifuged at 4,000 rpm for 15 min. Supernatants were transferred to a glass vial, and a 5-µl aliquot was injected into column. The stock solution of TEP (10 µmol ml-1) was prepared by dissolving 240µl of 4.176M TEP in 100 ml ethanol and stored at 4°C. The intermediate working standards were prepared by diluting the MDA stock solution with water to concentrations of 0.5,1.0,2.5,5.0,7.5 and 15 nmol ml^{-1}. The MDA level in the samples was expressed as nmol ml^{-1} of serum.

Statistical analysis : All the data presented expressed as mean values ± SE. One-way analysis of variance (ANOVA) was performed followed by unpaired student's t-test to analyze group differences. Numerical data was analyzed using SPSS correlated with SPSS 16.0 statistical software (Chicago, IL, USA). The significance level was set to $p \leq 0.05$.

Results and Discussion

Chelation therapy is the most accepted and recommended strategy to combat metal toxicity, including lead poisoning. However, chelation therapy is always coupled with drawbacks such as toxic effects of the chemicals, low specificity and poor bioavailability of lipophilic compounds (Flora *et al*., 2013). Natural antioxidants in the form of phytocompounds offer a more safer and novel approach and can be used as chelates against metal toxicity. An increased bioavailability of phytocompounds is be possible through nanosizing these compounds. Novel methods of phytosynthesis of nanoparticles has attracted considerable attention lately owing to their biocompatibility, low toxicity, cost-effectiveness and eco-friendly nature (Rajagopal *et al*., 2015). Nano formulations attempted in the present study emphasize on the 'green synthesis' of nanoparticles which is ecofriendly and safe. The nanorosemary are natural nanoparticles as they have no chemical stabilizers and metals. Eencapsulation also makes use of a natural polymer, starch along with PVA. The synthesis of nanoparticles of rosemary, as nanorosemary (RN) and encapsulated nanoparticles (RN1 and RN2), were confirmed by DLS and TEM images. The average size of nanorosemary (RN) was 75.45 nm (Pdi 0.244), rosemary nanoparticles encapsulated with starch and polyvinye alcohol composite polymers (RN1) was 2.665 nm (Pdi 0.113) and rosemary nanoparticles encapsulated with PVA polymer (RN2) was 3.026 nm (Pdi 0.084) respectively. All nanoparticles, RN, RN1 and RN2 showed mono-dispersity (Fig.1A, B and C) which can be observed clearly from a single peak in the DLS graphs. The TEM images of nanoparticles (RN, RN1 and RN2) in Fig.2 also demonstrated mono dispersity of the particles, which corresponds to the DLS results.The electron micrographs showed spherical and irregular morphologies of RN, while RN1 and RN2 represented a more spherical morphology as these particles were encapsulated by polymers. Encapsulation with

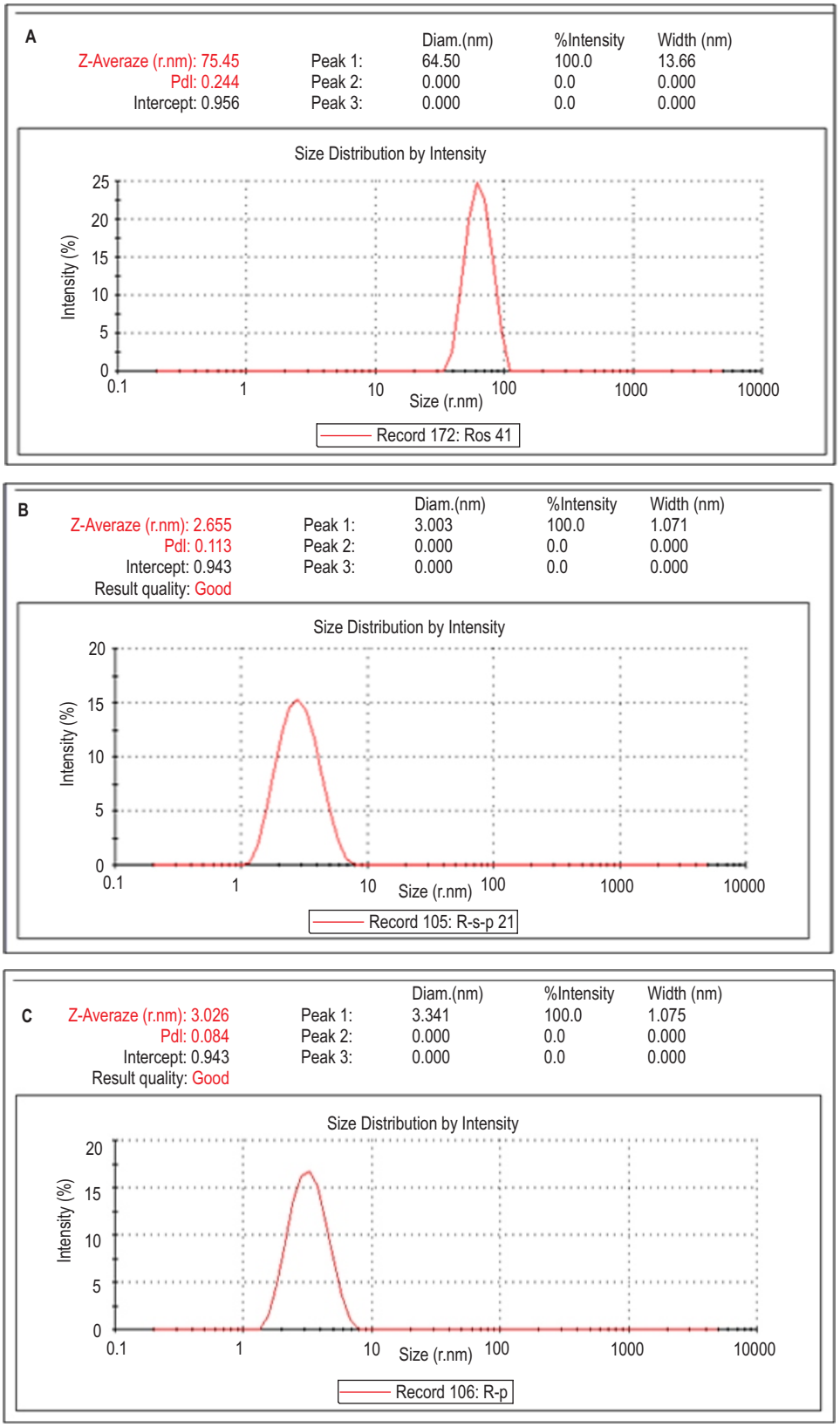


Fig. 1 : Nanoparticle size characterization using dynamic laser light scattering; (A) Rosemary nanoparticles (RN); (B) Rosemary nanoparticles encapsulated in starch and PVA polymers (RN1) and (C) Rosemary nanoparticles encapsulated in PVA polymer (RN2)

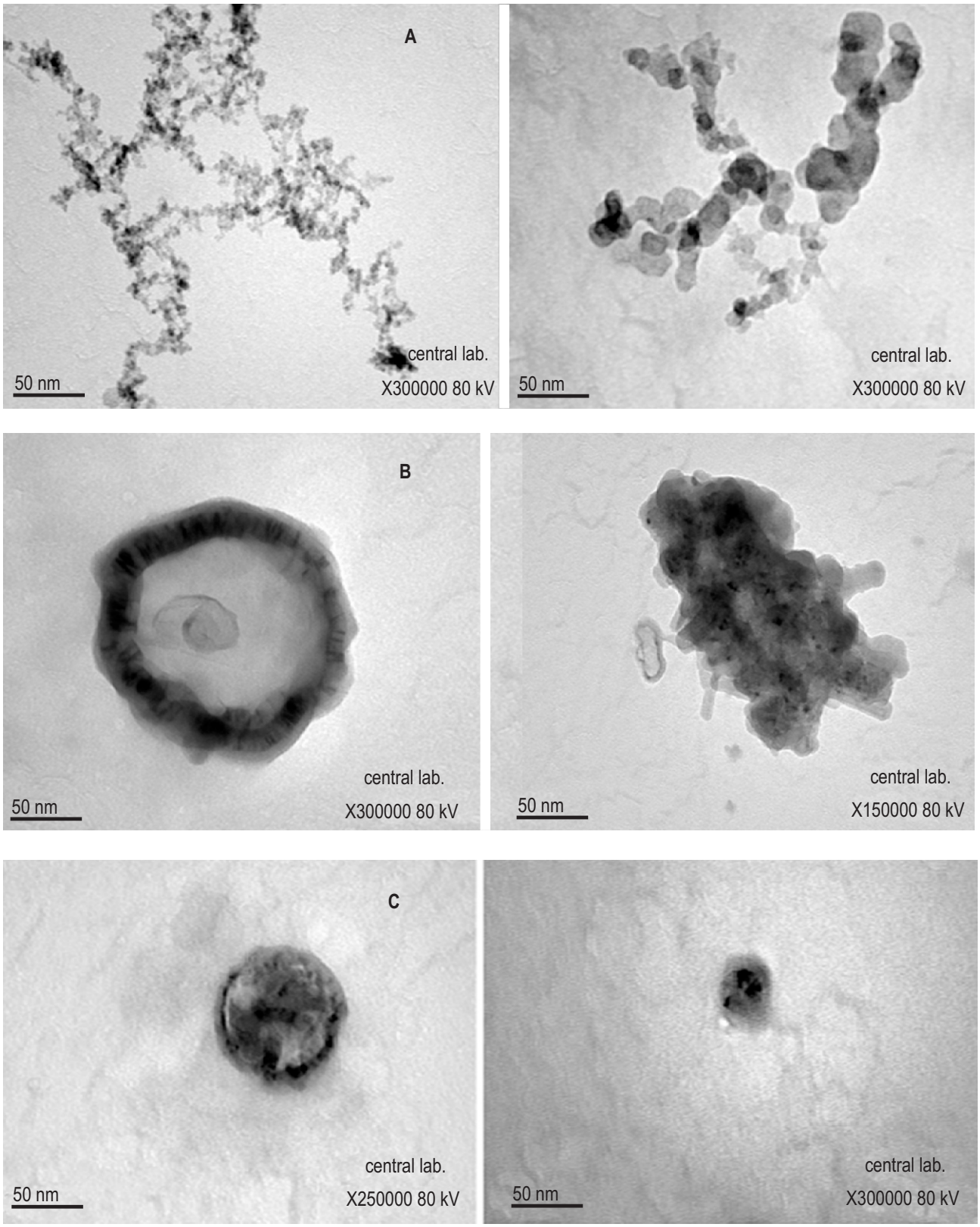


Fig. 2 : Electron micrographs showing morphology of the nanoformulations: (A) Rosemary nanoparticles (RN); (B) Rosemary nanoparticles encapsulated in starch and PVA polymers(RN1); (C) Rosemary nanoparticles encapsulated in PVA polymer(RN2)

Table 1 : Lead concentrations (µg g^{-1}) in brain, liver, kidneys and muscle of rats from different experimental groups

Experimental Groups	Brain	Liver	Kidneys	Muscle
Grp I	0.0086±0.00529[a]	0.0362±0.00174[a]	0.0020±0.00200[a]	0.0812±0.00338[a]
Grp II	0.4300±0.001761[b]	1.6040±0.02182[b]	3.2760±0.07679[b]	0.2820±0.01393[b]
Grp III	0.2100±0.01304[c]	1.6400±0.07483[b]	2.4860±0.02993[c]	0.1260±0.00678[c]
Grp IV	0.1400±0.01140[d]	1.0620±0.01241[c]	3.0880±0.01158[b]	0.1380±0.00374[c]
Grp V	0.0520±0.00374[a]	1.3460±0.00748[d]	2.5400±0.09274[c]	0.0880±0.00663[a]
Grp VI	0.2280±0.01020[c]	1.3340±0.01364[d]	2.5380±0.02010[c]	0.1380±0.00374[c]

Values with different superscript letter within each column are significantly different at 5% probability level. Values are mean of five replicates ± SE

composite polymer produced a better nanoparticle in terms of nanoscale that enhanced it efficacy as a therapeutic agent.

The results of the biological assays demonstrate that nano formulation retained the antioxidant and metal-chelating properties of rosemary leaf extract. All the nano formulations formed were physically and chemically stable with monodispersity, readily dispersible in water, and could be stored at room temperature over a period of time without any decomposition or aggregation. The enhanced aqueous solubility of nanoparticles could be attributed to their larger surface area, which promotes dissolution (McNeil, 2005).

A significant (p≤ 0.05) increase was observed in the Pb concentration in tissues after an exposure period of 21 days. Heavy metals such as lead have been reported to accumulate in the tissues of vertebrates from diverse taxa and environments (Mehrotra *et al*., 2008).Treatment with bulk rosemary extract and its nano formulations (RN, RN1, RN2) significantly reduced lead bioaccumulation in the tissues. Lead concentration in the brain of the group II rats (0.4300 µgg^{-1}) was significantly (p≤0.05) higher in comparison to that of group I rats (0.0086µg g^{-1}). In comparison to group II rats, all lead exposed rats treated with rosemary and their nanoparticles (BR, RN, RN1, RN2) showed a significant (p≤0.05) reduction in the lead concentration ,being most effective in group V treated with RN1. (0.0520 µg g^{-1}). Within the treated groups, lead concentration in group III rats (0.210µg g^{-1}) was significantly (p≤ 0.05) higher than groups V and VI rats but was comparable to the group IV rats (Table 1). There was also a significant (p≤ 0.05) increase observed in the lead concentration in the kidneys from group II (3.2760 µg g^{-1}) in comparison to group II (0.0020 µg g^{-1}). Treatment with rosemary and its encapsulated nano formulations significantly (p≤ 0.05) reduce the lead burden in the kidneys. However, no significant effect on the lead concentration was observed in group IV rats. The effect of RN1 and RN2 was to comparable eachother (Table 1). Further, the lead concentration in the liver of rats exposed to lead only (1.6040µg g^{-1}) showed a significant (p≤0.05) increase when compared to the control group (0.0362µg g^{-1}). Treatment with bulk rosemary (BR) did not have a significant effect on the lead concentration. Howeve, treatment with nanoparticles of rosemary (NR, NR1, NR2) significantly (p≤ 0.05) reduced the lead concentration in comparison to lead control group. Treatment with NR was significantly effective in reducing the lead concentration in liver than NR1 and NR2 (Table 1). A significant (p≤ 0.05) increase in lead concentration also was observed in the muscles of rats exposed to lead only (0.2820µg g^{-1}) in comparison to control group (0.0812µg g^{-1}). Treatment with rosemary leaf extract, both as bulk and nano formulations significantly (p≤ 0.05) reduced the lead concentration in comparison to group II rats. Among the treated groups, group V (0.0880µg g^{-1}) rats showed least lead concentration in the muscles and was comparable to the control (Table 1).

Table 2 : Serum MDA levels (nmol ml^{-1}) of rats from different experimental groups

Experimental Groups	Serum MDA levels
Grp I	2.0942±0.00684[a]
Grp II	3.5302±0.6596[b]
Grp III	2.3816±0.02561[c]
Grp IV	2.5512±0.02560[d]
Grp V	1.6869±0.00400[e]
Grp VI	1.6215±0.04687[e]

Values with different superscript letter within each column are significantly different at 5% probability level. Values are mean of five replicates ± SE

Thus, the results on the bioaccumulation of lead in the present study showed a marked ameliorative effect of rosemary leaf extract, which was evident from a reduction in bioaccumulation of metal in target tissues. Futhermore, an assessment of lipid peroxidation showed that the serum MDA levels were significantly(p≤ 0.05) elevated in group II rats, (3.5302nmolml^{-1}) in comparison to the control group (2.0942 nmol ml^{-1}). Treatment with rosemary, both as bulk (BR) and nanoparticle (RN,RN1,RN2) significantly (p≤ 0.05) reduced the serum MDA levels. However, within the treated groups ? treatment with encapsulated rosemary nanoparticles (RN1 And RN2) was more effective in reversing the effect of lead on lipid peroxidation (Table 2), this could be attributed to the antioxidant activity of rosemary extract primarily due to the presence of carnosic acid and carnosol (Kadri *et al*., 2011; Machado *et al*.,

2013). Similar findings on the ameliorative potential of rosemary leaf extract against metal toxicity and associated oxidative stress has previously been reported (Virk *et al*., 2013; Sakr *et al*., 2015; Al-Anazi *et al*., 2015; Rašković *et al*.,2015). The results on lead bioaccumulation in tissues clearly showed the mitigating effect of rosemary extract, both as bulk and nanoparticles. An overall assessment of the results showed that nano formulations of rosemary were more effective in chelating the metal from the tissues in comparison to the bulk extract used. This is in consensus with a similar experimental study on rat model by Flora *et al*. (2013) where nanocurcumin due to its possible chelating property and enhanced bioavailability efficiently removed lead from blood and soft tissues compared to bulk curcumin. Within three different types of nano formulations used in the present study, the encapsulated nanoparticles (NR1 and NR2) were more effective in comparison to nanorosemary, owing to the increased stability and bioavailability due to encapsulation. Further, the encapsulated nanoparticles with composite polymer was most effective in enhancing the chelating activity of rosemary extract, which could be attributed to the fact that encapsulation efficiency was enhanced with a composite polymer, a combination of starch and PVA, as suggested in earlier studies on nano encapsulation with composite polymers (Sehra *et al*., 2005; Mu and Zhong, 2006).The lead -induced oxidative stress was evident by an enhanced level of lipid peroxidation in exposed rats. Treatment with rosemary, bulk and nanoparticles markedly reduced the serum MDA levels and were effective in combating the oxidative stress. The antioxidative effect of rosemary leaf extract against metal induced oxidative stress has been previously reported (Virk *et al*., 2013; Al-Anazi *et al*., 2015; Sakr *et al*., 2015). This could be attributed to the presence of a major a constituent, carnosic acid in the leaf extract used in the present study. The nanoformulation proved to be more effective in reducing the lipid peroxidation, and the effect was more profound in encapsulated nanoparticles.

Thus, a broad assessment of the biological end points evaluated to investigate the therapeutic efficacy of rosemary and its nano formulations showed that the nanoparticles enhanced the protective effect of rosemary leaf extract against lead-induced toxicity in rats. Which can be explained on the fact that nanosizing the particles enhances bio availability making the drug /compound more efficacious. In addition, encapsulation of rosemary extract was more effective as an antioxidant and chelator as encapsulation with polymers made the nanoparticles more stable, and further enhanced their bioavailability. This was evident from the smaller size of encapsulated nanoparticles in comparison to nanorosemary.The success of application of nanoparticles as therapeutic agents depends on their ability to evade the reticulo endothelial system that ensures a prolonged circulation time in the blood (Guo and Huang, 2011). The results of the study show that polymer encapsulated nanoparticles have distinct properties, which aided in evading the RES uptake, and hence had a longer circulation time in blood and provided a sustained release of rosemary extract. Similar results were reported in a study on arsenic-induced toxicity in rats, where curcumin encapsulated in chitosan nanoparticles was more effective than free cucurmin (Yadav *et al*., 2012).

The key findings of the study suggest that rosemary nanoparticles with encapsulation can be an effective nutritional intervention against lead toxicity, as rosemary leaf extract is an established dietary antioxidant. This alternative drug delivery approach would possibly bring out rosemary leaf extract as an effective and promising agent to treat metal-induced toxicity.

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