

Original Article

Synthesis, characterization and protection effect of black rice anthocyanins nano-composite against hepatotoxicity induced by methotrexate in rats

Síntese, caracterização e efeito protetor de nanocompósito de antocianinas de arroz preto contra hepatotoxicidade induzida por metotrexato em ratos

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Abstract

The present study aimed to investigate the beneficial of prepared black rice anthocyanins nano-composite (An-AgNp₃) against hepatotoxicity induced by methotrexate (MTX) in rats. Anthocyanins nano-composite was prepared by silver as the metallic ion reduction and were characterized by IR and SEM. The rats in our experiment were divided into five groups. Serum lipid profile, serum transaminases (ALT and AST), ALP, LDH, TBA, GSH and SOD were examined. The results show that SEM of An-AgNp₃ has average particle size from 70 to 130nm. In the group treated with MTX; TC, TG, LDL-c, ALT, AST, ALP, LDH and TBA levels were significantly ($P \leq 0.05$) increased than NC, while, HDL-c, SOD and GSH levels were significantly ($P \leq 0.05$) decreased. On the other hand, An-AgNp₃ + MTX treated groups were reversed the levels of all biomarkers similar to NC. In conclusion, the results show that An-AgNp₃ has a protective effect on MTX-induced hepatotoxicity and oxidative stress.

Keywords: anthocyanins nano-composite, hepatotoxicity, methotrexate, black rice, infrared spectroscopy, scanning electron microscopy.

Resumo

O presente estudo teve como objetivo investigar o benefício de nanocompósito de antocianinas de arroz preto preparado (An-AgNps) contra a hepatotoxicidade induzida por metotrexato (MTX) em ratos. O nanocompósito de antocianinas foi preparado a partir da prata por meio da redução do íon metálico e caracterizado por IR e SEM. Os ratos em nosso experimento foram divididos em cinco grupos, e foram examinados o perfil lipídico sérico, as transaminases séricas (ALT e AST), ALP, LDH, TBA, GSH e SOD. Os resultados mostram que SEM de An-AgNps tem tamanho médio de partícula de 70 a 130 nm. No grupo tratado com MTX, os níveis de TC, TG, LDL-c, ALT, AST, ALP, LDH e TBA aumentaram significativamente ($P \leq 0,05$) do que NC, enquanto os níveis de HDL-c, SOD e GSH diminuíram significativamente ($P \leq 0,05$). Por outro lado, nos grupos tratados com An-AgNps + MTX, foram revertidos os níveis de todos os biomarcadores semelhantes ao NC. Em conclusão, os resultados mostram que o An-AgNps tem um efeito protetor contra a hepatotoxicidade induzida pelo MTX e o estresse oxidativo.

Palavras-chave: nanocompósito de antocianinas, hepatotoxicidade, metotrexato, arroz preto, espectroscopia de infravermelho, microscopia eletrônica de varredura.

1. Introduction

Methotrexate (MTX) (4-amino-N10-methyl folic acid) has been used in the various therapy types of diseases including psoriasis, cancers immunologic disorders, Crohn's disease, rheumatoid arthritis, for the multiple sclerosis treatment, sarcoidosis, dermatomyositis and stage of various cancerous (breast cancer, lung cancer, head and neck tumours, lymphoma, leukemia, osteosarcoma, etc.) (Ozogul et al., 2013; Vardi et al., 2010; Celik et al., 2013). Additionally, long-term administration or high doses of MTX

has toxic side effect on tissue of liver (Kumari, 2016) and seems to relate to reactive oxygen species (ROS) generation (Şener et al., 2006a). Decreased the count of white blood cell, hepatotoxicity and ulcerative stomatitis are the most common adverse effects of MTX (Şener et al., 2006b).

Nanoparticles are engineered materials produced within the nanoscale range of 1-100 nm in one or more dimensions (Barnes et al., 2008). In all the metals the pure silver has the highest thermal conductivity, and electrical, and has

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low resistance of contact (Thomas and Sayre, 2005). In various fields many applications of silver nanoparticles (AgNPs) are widely used including bacteria, sensors, virus, catalysis high proportion of surface atoms, bio labeling, antimicrobial agents, filters, microelectronics (Tavares et al., 2012), because they have specific biological and physico-chemical properties (Rai et al., 2009). AgNPs synthesis from the phytochemical by using fruit or plants extracts plays an important role in the field of nanomedicine and nanotechnology as it offers alternative therapeutic options which are free side effects, effective for a wide variety of diseases and safe (David et al., 2014).

Anthocyanins has polyphenols typical chemical structure and belong to flavonoids (Kong et al., 2003), and they have diverse healthy functions including anti-proliferative activities, anti-inflammatory, antioxidant and anti-cancerous and their most attractive biological feature might owe to their capacity to affect the growth of vulnerable micro-organisms (Bowen-Forbes et al., 2010), therefore, they considered health-promoting substances. Based on the anthocyanin's antioxidant capacity, they are exhibit many bioactivities such as Alzheimer's disease, prevention and preservation of vision etc. (Miyake et al., 2012). Many studies reported that the extracts of anthocyanin from plants and natural food have hepatoprotective effect and believed to be beneficial to liver health, because they have strong antioxidant activity (Sankhari et al., 2012). The anthocyanins phenolic hydroxyl groups causing a reduced in anthocyanins biological activity because they are oxidized easily into quinones, as a result of that, the anthocyanins get unstable and need to combine with macromolecules to increase their stability (Amin et al., 2017). Therefore, our present study aimed to increase the stability of anthocyanins by synthesis anthocyanins silver nanoparticles, characterize the prepared nanoparticles and investigate its protective effects on liver hepatotoxicity induced by Methotrexate.

2. Experimental Methods

2.1. Extraction of anthocyanin from black rice

The black rice was requested from Agriculture Research Center, Gize, Egypt. The extract of anthocyanin from soft black rice was produced by adding 100g from the black rice powder into 150 mL methanol and stirred for 24h. The final extracted was centrifuged to gain the black rice anthocyanin extract (Septiani et al., 2017).

2.2. Preparation of anthocyanin silver nanoparticles

Black rice anthocyanin with the metallic ion reduction were used to obtained anthocyanin silver nanoparticles. NaOH solution (1M) was used to brought black rice anthocyanin extract at value of pH=7.5. 0.06M AgNO₃ concentration was used to synthesis anthocyanin nanoparticles: added metallic ion solution (6.6 mL) and anthocyanin black rice extract (16.6 mL) to boiling distilled water (200 mL). Note the change in color immediately, indicated that the anthocyanin silver nanoparticles are

obtained. After that, stopped the heating and continues the stirring till to cool the solution (Olenic and Chiorean, 2015).

2.3. Biological methods

Fifty male waster albino rats (230±270g) were purchased from the Vaccination Centre, Helwan, Giza, Egypt. They were kept under the standard conditions of the animal house of Ophthalmology Research Institute, Giza, Egypt with a 12h light/dark cycle at temperature 21±0.5°C and relative humidity 55±5. During a 10 days period, they had nutrition diet contains (salt mixture 4%, corn seed oil 10%, vitamins mixture 1%, corn starch 70%, casein 10% and cellulose 5%). The experimental animals were randomly divided into five groups of ten animals. G1; Normal Control (NC) group, G2; Positive Control (PC) group was treated with single dose of MTX (20 mg/kg.b.w) i.p injection, G3; treated group was treated with single dose of MTX (20 mg/kg.b.w) i.p injection + An-AgNPs (10 mg/kg.b.w), G4; treated group was treated with single dose of MTX (20 mg/kg.b.w) i.p injection + An-AgNPs (15 mg/kg.b.w), G5; treated group was treated with single dose of MTX (20 mg/kg.b.w) i.p injection + An-AgNPs (20 mg/kg.b.w). The treated animals were kept for 10 days. Then, food intake and body weight were measured at the beginning and during the experimental period. After the treated period, a blood samples were taken from the orbital plexus using a mean of heparinized capillary glass tubes to obtain the blood serum, each sample was centrifuged (1500 xg at 4°C for 30 min).

2.4. Biochemical assays in serum

2.4.1. TC, TG, HDL, LDL assays

Triglycerides (TG) (Fossati and Prencipe, 1982), Total cholesterol (TC) (Allain et al., 1974), High density lipoprotein (HDL-C) (Lopez-Virella et al., 1977) and low-density lipoprotein (LDL-C) (Friedewald et al., 1972) were determined by kits obtained from bio diagnostic company, Dokki, Giza, Egypt.

2.4.2. sALT, sAST, ALP and LDH assay

Serum transaminases ALT and AST (Alanine transferase and Aspartate transferase) were measured calorimetrically according to the method described by Reitman and Frankel (1957). Serum alkaline phosphatase (ALP) and lactate dehydrogenase activities (LDH) were determined according to Bablok et al. (1988).

2.4.3. TBA assay

The method describes by Mihara and Uchiyama (1978) was used to determine the thiobarbituric acid (TBA). A mixture of 3 mL phosphoric acid (1%, pH 2.0) and 1 mL of TBA (0.6%) were added into 0.5 mL of the blood serum in airtight tubes and kept for 45 min in a boiling water bath. Then, cooled the samples in ice and add 5 mL butanol along with through the mixture mixing. Using 1000 xg centrifugation to separate the butanol phase and transferred into glass cuvettes. Spectrophotometer (Bauch and Lomb, Spectronic-20) was used to measure the color

of the TBA chromogen at 520 and 532 nm. The value of TBA was observed by measuring the difference between absorbance value at 520 and at 532 nm, which represents the concentration of malondialdehyde, and was taken as the measure of lipid peroxidation.

2.4.4. SOD and GSH assay

The measurement of serum Superoxide dismutase (SOD) enzymatic activity was followed as reported by Ohkuma et al. (1982). Glutathione (GSH) was tested according to the method described by Ellman (1959). GSH was interacted with 5,5'-dithiobis-2-nitrobenzoic acid. After that, the product absorbance spectra were measured at 410 nm (maximum absorbance). The $\mu\text{mol/g}$ tissue was used to express the final results.

2.5. Statistical analysis

The mean SEM was used to express the results and ANOVA (one-way analysis of variance) was used to measure the intergroup variation, which followed by Fischer's LSD test. Statistical significance was considered at ($P \leq 0.05$). Then, we used the Jandel Sigma Stat Statistical Software version 2.0 to analyze the statistical value.

3. Results and Discussion

Figure 1a and 1b show the FTIR spectroscopic analysis of silver nitrate only (1A), silver nano-particles/ anthocyanins nanocomposite (2A) and anthocyanines only (3A). This figure shows a difference between the three spectrums as follow: 1) The characteristic bands of silver nitrate which appear at 2715.7, 2409, 1772.6 and

1379 cm^{-1} (in 1A) are disappear in 2A and that appeared at 721 cm^{-1} shifted to 802.4 cm^{-1} in 2A mostly indicates a good interaction between anthocyanines molecules and the silver nano-particles. 2) Stretching vibration of OH groups in anthocyanines give broad band at 3392.7 cm^{-1} (in 3A) shifted to 3433 cm^{-1} (in 2A) with about 40.3 cm^{-1} . This indicates the breaking of some H-linkages of anthocyanines due to its interaction with the silver surface. 3) Three characteristic bands of anthocyanines appeared around 2931.8 cm^{-1} (in 3A) are shifted to lower absorption bands around 2924 cm^{-1} (in 2A) due to interaction with silver surface. 4) Stretching vibration of C=O and C=C in 3A appeared at 1716.6 and 1666.5 cm^{-1} respectively, disappeared and new peak appeared at 1743.6 cm^{-1} in 2A probably due to the interaction with the electron Plasmon resonance of silver nano-particles. 5) Four new peaks appeared from 1564-1421.5 cm^{-1} in 2A spectrum. 6) Characteristic bands of anthocyanines appeared from 1118.7 to 1000 cm^{-1} are disappeared in 2A spectrum. All the above mentioned observations confirm a good interaction between anthocyanines molecules and silver nano-particles in their nano-composite sample. This takes place probably due to the binding of natural molecules from C at the surface of the metallic nanoparticles (Fox Junior, 1968). The vibrational frequencies for glycosidic units appear between 1000-1250 cm^{-1} . These bands suggest that the anthocyanines are the organic molecules bounded as ligands at gold/silver nanoparticles (Crisan et al., 2013). Our results agree with Olenic and Chiorean (2015) they studied the interaction of organic molecules with gold/silver nanoparticles by FTIR spectroscopy and reported that for nanomaterials, the stretching vibrations of OH characteristic to associated OH-groups are shifted to higher values (3396 cm^{-1} for natural extracts and 3426/3435 cm^{-1} for gold/silver nanomaterials) with about 20 cm^{-1} . This indicates that more H linkages are broken. C=O and C=C stretching vibrations in extracts are at 1718 cm^{-1} and 1594 cm^{-1} . After the reduction of $\text{Au}^{3+}/\text{Ag}^{+}$ at Au^0/Ag^0 with anthocyanins, the peaks are shifted and intensively reduced in intensity (1719 cm^{-1} and 1629 cm^{-1}).

Morphology of An-AgNPs was studied by SEM tool as shown in Figure 2. The particles are in bundles ranging in size 70-130nm (0.07-0.13 μm). The size and dimensions of

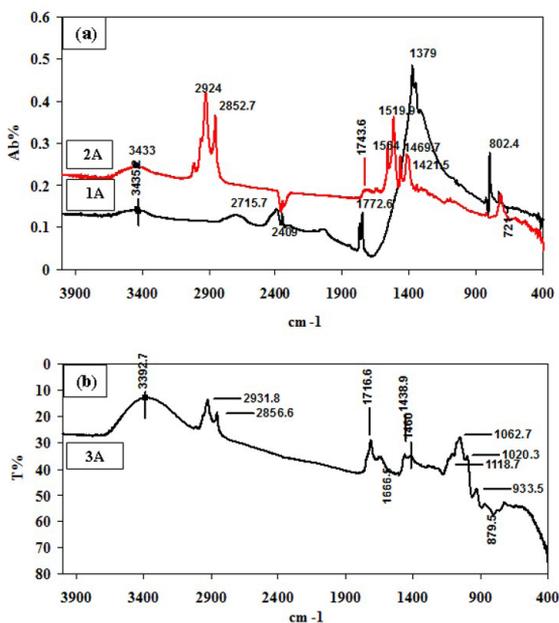


Figure 1. FTIR spectroscopic analysis of (a) silver nitrate (1A) and silver nano-particles/ anthocyanins nano-composite (2A), (b) anthocyanines only (3A).



Figure 2. SEM image of prepared An-AgNPs.

nanoparticles 9–82 nm for AgNPs-C, and they have an almost spherical or elongated shape (Olenic and Chiorean, 2015).

The results of TC, TG, HDL-c and LDL-c in blood serum are shown in Table 1. Levels of TC, TG and LDL-c blood serum were significantly ($P \leq 0.05$) increased in positive control (PC) than normal control (NC), while HDL-c blood serum level was significantly ($P \leq 0.05$) decreased (Hendawy et al., 2015). On other hand, administration with An-AgNPs (10, 15, 20 mg/kg b.w) +MTX significantly ($P \leq 0.05$) reversed these results to near NC values. Moreover, An-AgNPs (20 mg/kg b.w) + MTX administrated group gave a result close to NC values. MTX causes many toxicities such as cardiovascular diseases (Baggott et al., 1993). Sabah and Yasmin (2013) studied the effect of MTX on mice and noted that TG and TC serum levels were significantly increase, while HDL serum level was decreased significantly ($P < 0.05$). Anthocyanin have many bioactivities such as anti-cardiovascular, antioxidant properties (Hou et al., 2013) and anti-atherosclerotic activity (Xia et al., 2006). Also, Guo et al. (2007) reported that black rice anthocyanin in rich extract has preventing metabolic syndrome property, by improving the lipid profile.

Date presented in Table 2 shows ALT, AST, ALP and LDH activities. The activity of ALT, AST, ALP and LDH blood serum in PC group were significantly ($P \leq 0.05$) higher than NC. Although, administration with An-AgNPs at various levels + MTX were significantly ($P \leq 0.05$) decreased their activities than those in the PC. An-AgNPs (20 mg/kg b.w) + MTX administrated group gave the best results, which it close with NC values. The increases in the activity of ALT and

AST blood serum levels may be due to MTX cytotoxic effect on liver cells which probably lead to liver cell membrane permeability causing increase the movement of ALT and AST enzymes to the serum of blood (Bonnefoi et al., 1989). Our results agree with Sabah and Yasmin (2013), they showed significant increase in ALP, GOT, and GPT levels in blood serum in mice group administrated by MTX than control group. The levels of ALT activity in MTX treated group was higher significantly ($p = 0.001$) than group of control-saline, this increase demonstrates the hepatotoxicity (Erdogan et al., 2015).

Table 3 shows the results of TBA, SOD and GSH activities. The results showed a significantly ($P \leq 0.05$), decreased in the activity of SOD and GSH levels in blood serum and significantly ($p \leq 0.05$) increased in the activity of TBA levels in blood serum in treated rats with MTX (20 mg/kg bw) (PC) than NC. However, the groups treated with An-AgNPs (10, 15, 20 mg/kg bw) + MTX reverted TBA, SOD and GSH values to normal levels. GSH levels significant reduction may lead to reduction of effectiveness in the defense system of antioxidant enzyme, which increased, the cell sensitizing to ROS (Babiak et al., 1998). Also, MTX decreasing the antioxidant enzymes levels and causes oxidative liver tissue damage by increasing lipid peroxidation in the tissue of liver (Vardi et al., 2010). Anthocyanin black rice contains tyanidin-3,5-diglucoside; cyanidin-3-, glucoside; cyanidin-3- rutinoside; peonidin-3-glucoside and peonidin-3- rutinoside (Zhu et al., 2018), they have many bioactivates such as anti-carcinogenic and antioxidants properties (Hou et al., 2013). Vardi et al. (2010)

Table 1. Effect of An-AgNPs on serum TC, TG, LDL-c and HDL-c concentration in rats treated with methotrexate.

Treatments	TC mg/dl	TG mg/dl	HDL-C mg/dl	LDL-C mg/dl
G1 (NC)	127.177 ^a ±0.207	65.538 ^a ±0.180	35.612 ^b ±0.238	91.580 ^a ±0.193
G2 (PC) MTX (20mg/kgb.w)	304.875 ^a ±0.191	97.715 ^a ±0.140	12.473 ^a ±0.181	292.407 ^a ±0.310
G3 (An-AgNPs 10 mg/kg b.w+MTX)	185.102 ^b ±0.076	77.152 ^b ±0.057	30.967 ^a ±0.225	154.135 ^b ±0.259
G4 (An-AgNPs 15 mg/kg b.w+MTX)	134.980 ^a ±0.162	69.177 ^a ±0.145	33.512 ^a ±0.185	101.468 ^a ±0.172
G5 (An-AgNPs 20 mg/kg b.w+MTX)	125.778 ^a ±0.120	66.097 ^a ±0.060	37.505 ^a ±0.165	88.333 ^a ±0.244
LSD	0.462	0.368	0.585	0.702

TC = Total cholesterol. TG = Triglycerides. LDL-c = Low density lipoprotein. HDL-c = High density lipoprotein. NC = Normal control. PC = Positive control. MTX = Methotrexate. An-AgNPs = black rice anthocyanins nano-composite. Each value is mean ± SD for ten rats in each group. Significantly different from controls ($p < 0.05$) by ANOVA multiple range test.

Table 2. Effect of An-AgNPs on serum ALT, AST, ALP and LDH concentration in rats treated with methotrexate.

Treatments	ALT mg/dl	AST mg/dl	ALP U/L	LDH IU/L
G1 (NC)	72.653 ^d ±0.238	173.038 ^a ±0.052	75.507 ^b ±0.197	277.968 ^a ±0.051
G2 (PC) MTX (20mg/kgb.w)	157.272 ^a ±0.185	268.083 ^a ±0.164	195.997 ^a ±0.059	530.088 ^a ±0.069
G3 (An-AgNPs 10 mg/kg b.w+MTX)	115.028 ^b ±0.065	215.402 ^b ±0.251	126.037 ^a ±0.115	360.403 ^b ±0.088
G4 (An-AgNPs 15 mg/kg b.w+MTX)	74.248 ^a ±0.143	178.145 ^a ±0.149	85.998 ^a ±0.249	299.907 ^a ±0.164
G5 (An-AgNPs 20 mg/kg b.w+MTX)	72.980 ^a ±0.047	173.990 ^a ±0.097	76.750 ^a ±0.125	280.242 ^a ±0.125
LSD	0.447	0.458	0.475	0.313

ALT = Alanine transferase. AST = Aspartate transferase. ALP = Alkaline phosphatase. LDH = Lactate dehydrogenase. NC = Normal control. PC = Positive control. MTX = Methotrexate. An-AgNPs = black rice anthocyanins nano-composite. Each value is mean ± SD for ten rats in each group. Significantly different from controls ($p < 0.05$) by ANOVA multiple range test.

Table 3. Effect of An-AgNPs on serum TBA, SOD and GSH concentration in rats treated with methotrexate.

Treatments	TBA nmol/L	SOD U/mL	GSH Mmol/g
G1 (NC)	1.722 ^a ±0.016	110.088 ^b ±0.118	8.202 ^b ±0.025
G2 (PC) MTX (20mg/kg b.w)	8.237 ^a ±0.039	37.375 ^a ±0.206	3.770 ^a ±0.051
G3 (An-AgNPs 10 mg/kg b.w+MTX)	3.790 ^b ±0.026	71.418 ^a ±0.098	6.340 ^a ±0.050
G4 (An-AgNPs 15 mg/kg b.w+MTX)	2.098 ^a ±0.016	109.163 ^a ±0.085	7.870 ^a ±0.031
G5 (An-AgNPs 20 mg/kg b.w+MTX)	1.800 ^a ±0.016	115.022 ^a ±0.039	8.443 ^a ±0.040
LSD	0.071	0.356	0.118

TBA = thiobarbituric acid. SOD = Superoxide dismutase. GSH = Glutathione. NC = Normal control. PC = Positive control. MTX = Methotrexate. An-AgNPs = black rice anthocyanins nano-composite. Each value is mean ± SD for ten rats in each group. Significantly different from controls ($p < 0.05$) by ANOVA multiple range test.

found that the administration of MTX increases MDA level and decreases SOD activity in liver. Saka and Aouacheri (2017) noted a significant reduction in antioxidant enzyme defense system (AEDS) biomarkers of group that intake high-doses MTX, especially the levels of GSH which leads to decrease of AEDS effectiveness, thereby sensitizing the cells to Reactive Oxygen Species (ROS).

4. Conclusion

From our results, we can conclude that anthocyanin Nano-composite showed a highly significant protective activity against MTX-included hepatotoxicity and reversing the MTX effects on enzymatic and non-enzymatic biomarkers similar to NC, which indicate that anthocyanin Nano-composite is an important mechanism by preventing liver against the side effects of MTX as chemotherapy.

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