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Western Blotting and Bicinchonic Acid Assessment for Improvement of Sperms Mitochondrial Functions by Fenugreek, Extract and Nanoparticles

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Abstract

This study was aimed for assessment the effect of fenugreek extract and fenugreek NPs on the concentration of total protein in testicular tissues through application of two assays, Western blotting and Bicinchonic Acid. Totally, 60 Wister rats were selected, prepared and equally divided into three groups; negative control that remains without any treatment and given only distilled water daily; T1 that orally treated with a daily dose (100) mg/kg of extract fenugreek, and T2 that orally treated with a daily dose (100) mg/kg of fenugreek NPs. After an experimental period continued for 60 days, all study animals were euthanized with chloroform to collect of testis samples that subjected for homogenization and measurement of proteins concentration targeting of SDHA and CS proteins. The findings revealed that there is a significant increase in concentration of total protein in both fenugreek extract and fenugreek NP groups when compared with the value of control group. However, no significant variation was found between values of both fenugreek extract and fenugreek NP groups. Expression of mitochonderial expretion of SDHA protein was elevated in fenugreek extract group and fenugreek NP groups compared with control group. A comparison of values of treatment groups was revealed an elevation in values of fenugreek NP group more than fenugreek extract group. For CS protein, insignificant variation was reported between values of fenugreek extract and fenugreek NP groups when compared to value of control group. In conclusion, Western blotting demonstrated that is a powerful tool for detection and characterization of targeted proteins. Also, BCA revealed a high sensitivity and simple immunoassay for screening of increased total testicular protein. However, isolation of active components in fenugreek, and synthesizing of specific NPs based on one type of these components can provide new therapies with different characteristics. Also, the augmentation of fenugreek NPs

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and proper regulation of steroidogenesis and mitochondrial biogenesis related genes is notably need to be furthermore studied.

Keywords: Succinate dehydrogenase, Citrate synthase, Chitosan, Immunoassay, Iraq

Introduction

Sperms are composed of important component of mitochondrial parts. Anifandis *et al*. (2017) was found a link between sperm mitochondrial defects and decreased sperm motility. The molecular level has revealed that deletions and other changes to mitochondrial DNA can result in decreased sperm functionality and male infertility (Bahrehmand Namaghi and Vaziri, 2017). Comparing sperm from asthenozoospermic samples to control samples revealed that sperm from asthenozoospermic samples contain abnormally high levels of specific mitochondrial RNAs, as well as, transcripts encoding mitochondrial proteins that are encoded by the nucleus (Ferramosca *et al*., 2021). Another significant discovery has been the relationship between the activity of sperm mitochondrial enzymes including the mitochondrial electron transport chain complexes in addition to a variety of sperm parameters such as concentration, vitality and motility among other characteristics (Zhu *et al*., 2019). Also, many researchers have been discovered the citrate synthase (CS) and succinate dehydrogenase (SDHA) as both of which are nuclear-encoded enzymes involved in the Krebs cycle, exhibited the strongest relationships with one another out of all of the enzymes studied (Beyramzadeh *et al*., 2017; Fišar *et al*., 2019). In relation to citrate synthase (which is commonly used to indicate the presence or absence of mitochondria), the researchers have discovered that, in the future, mitochondrial volume, rather than distinct enzymatic activities in samples of varying quality may serve as a primary explanation for the correlations in question (Soren *et al*., 2018; Kang *et al*., 2020).

One of the medicinal plants that has been used since antiquity in the traditional medicine and for which significant therapeutic properties have been mentioned is fenugreek (*Trigonella foenum-graecum*), (Srinivasa and Naidu, 2021). Fenugreek contains a number of chemical constituents including steroidal sapogenins such as diosgenin component that found in the oily embryo of fenugreek. There are two furastanol glycosides, F-ring opened precursors of diosgenin that have been reported in fenugreek as also hederagin glycosides (Chaudhary *et al*., 2018). Alkaloid like trigocoumarin, nicotinic acid, trimethyl coumarin and trigonelline are present in stem. The mucilage is a standing out constituent of the seeds (Aher *et al*., 2016).

Nanoparticles (NPs) represent an active area of research and a techno-economic sector with full expansion in many application domains. NPs have gained prominence in technological

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advancements due to their tunable physicochemical characteristics such as melting point, wettability, electrical and thermal conductivity, catalytic activity, light absorption and scattering resulting in enhanced performance over their bulk counterparts (Hu, 2015). NPs have drawn increasing interest from every branch of medicine for their ability to deliver drugs in the optimum dosage range often resulting in increased therapeutic efficiency of the drugs, weakened side effects and improved patient compliance (Khan et al., 2018).The controlled release of pharmacologically active drugs to the precise action site at the therapeutically optimum degree and dose regimen has been a major goal in designing such devices (Simonazzi *et al*., 2018). Hence, this study was aimed for assessment the effect of fenugreek extract and fenugreek NPs on the concentration of total protein in testicular tissues through application of two assays, Western blotting and Bicinchonic Acid.

Materials and methods

Ethical approval

The current study was performed under the license of the Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, Al-Qasim Green University (Al-Qasim, Babil, Iraq).

Preparation of fenugreek extract and NPs

Initially, a total 300 g of fenugreek seeds were purchased from the local market of herbal medicine in AL-Qasim city, grinded using a blender max. The powder of fenugreek seeds was dissolved then into a totally 1000 ml of alcohol solution, hydroalcoholic acid (70%), with mechanical shaking by magnetic starrier at 55°C for 6 hours. The mixture was filtered, dried under the vacuum for 20 hours, incubated at 37ºC for 36 hours, and stored in deep freezes at -20ºC (Banso and Adeyemo, 2006). Chitosan was used to prepare of the NPs by the ionic gelation method with some modifications (Agarwal et al., 2018; Shafiei et al., 2019).

Study design and sample collection

Sixty Wister rats were randomly selected and subjected for preparatory period; during which, they kept at 23 ± 2ºC, fed on basal laboratory diet and tap water, and exposed to 12/12 light and dark conditions. After one week, the study animals were equally divided into three groups as following:

1. First group (Negative control group): Rats of this group still without any treatment and given only distilled water daily.
2. Second group (T1): Rats of this group treated with a daily dose (100) mg/kg of extract fenugreek that given orally by stomach tube.

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3. Third group (T2): Rats of this group treated with a daily dose (100) mg/kg of fenugreek NPs that given orally by stomach tube (Al-Zamely and Kshash, 2021). After an experimental period continued for 60 days, all study animals were euthanized with chloroform and subjected for sampling of testis.

Bicinchonic Acid Assay

The concentrations of total proteins in homogenized testicular tissues were determined following the manufacturer's instructions of the Bicinchoninic Acid Assay Kit (Elabscience, China). Briefly, all samples were diluted, pipetted into the wells of the plate, and the BCA reagents were added. The plate was covered, incubated at 37°C for 30 min, and read at an optical density (OD) of 562 nm using a Microplate reader (BioTek, USA). The linear standard curve of mean absorbance was used to measurement of the concentration of protein in all samples.

Western blotting assay (SDS-PAGE)

All buffers and reagent used in this assay were imported from the Elabscience Company (China). The samples were taken, and the tissues were washed thoroughly with pre-cooled PBS Buffer to remove the surface blood and internal debris. Then, the tissues were weighted and smashed with adding of an appropriate ratio of RIPA Lysis Buffer to homogenizely lyse the tissue. After homogenization, the sample was shacked and lysed on the ice for 30 min, and then, sonicated for 1 min to make cells fully lysis and reduce the viscosity of sample. The lysate was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was taken. The protein concentration was measure as mentioned in Bicinchonic Acid Assay method. According to molecular weight of targeted proteins, separation gel was prepared, and the test samples were added to each well with adding 5µl of Precision Plus Protein™ Dual Color Standards Marker to a reserved well in order to verify the target molecular weight and the extent of membrane transfer. Electrophoresis Buffer was added and electrophoresis was carried out at 80V for 2-3 hours till bromophenol blue reaches the bottom of the gel. The PVDF Membrane was soaked in methanol for 1 min to activate it, and then soaked in Transmembrane Buffer. The transmembrane conditions were adjusted, and the transmembrane process was carried out at low temperatures. After the transmembrane, the PVDF Membrane was taken out carefully and washed with TBST Buffer for 1 min. The PVDF Membrane was soaked with TBST Buffer and incubated at room temperature for 1.5 hour. According to dilution ratio, the TBST Buffer was used to dilute the primary antibody according to the antibody manual. The PVDF Membrane was soaked in the primary antibody working solution, incubated overnight at 4°C, and then, the PVDF Membrane was washed with TBST Buffer for 3 times. According to the recommended dilution ratio, secondary antibody was diluted with TBST Buffer,

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incubated at room temperature for 1 hour, and then, the PVDF Membrane was washed with TBST Buffer for 3 times. The A and B Buffers were mixed in the Excellent Chemiluminescent Substrate Detection Kit at the ratio of 1:1 as working solution, and the PVDF Membrane was taken out from the TBST Buffer. The PVDF Membrane was placed inside the X-ray cassette with adding of ECL working solution continuously on the PVDF Membrane. Protein bands intensity was measured using Image Studio Lite software version 5.2, and the unit used for bands measurement was Arbitrary units (AU).

Statistical analysis

All collected data were analyzed by ANOVA at $P<0.05$ in GraphPad Prism Software (version 6.0.1), (Al-Gharban and Al-Taee, 2016; Gharban et al., 2019).

Results and discussion

Bicinchonic Acid Assay

The results of total protein concentration seen that there was a significant increase ($p<0.05$) in values of both fenugreek extract (19.87 ± 1.245) and fenugreek NP (20.93 ± 2.128) groups when compared with the value of control (15.33 ± 0.895) group. However, no significant variation was found between values of both fenugreek extract and fenugreek NP groups (Table 1; Figure 1). Based on our data, BCA revealed a high sensitivity and simple immunoassay to screening of increased total testicular protein as a result of administration of fenugreek extract and fenugreek NPs which in agreement with that reported recently (Li *et al*., 2018; Zhou *et al*., 2020; Singh *et al*., 2022). Sedha *et al*. (2015) delineated that apoptosis of germ cells and generation of oxidative stress can further compromise capacity of leydig cells to be steroidogenic and ability to differentiate of germinal epithelium into normal spermatozoa. Hamza *et al*. (2016) advocated that the aqueous extract of fenugreek seeds appreciatively attenuate the testicular damage induced by cisplatin. Fenugreek extract have shown various curative qualities suchlike hypocholesterolaemic, antibacterial, antioxidant, antidiabetic agent, hepatoprotective effect and anticancer (Wani and Kumar, 2018). Neha *et al*. (2019) reported that fenugreek extract showed a protective effect against lipid peroxidation in testicular tissue. Kaur and Sadwal (2020) demonstrated that the administration of fenugreek was improved the deleterious effects on male reproductive system as indicated by improved sperm parameters.

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Table (1): Concentration of total protein among study groups

Standard concentrations of BSA[1] (mg/ml)	[2] Reading data Absorbance unite (nm)		Samples Codes	[2] Reading data Absorbance unite (nm)		Final Concentration of total protein (µg/µl)
3	2.694	2.613	W1	1.594	1.669	16.9
2.4	2.22	2.238	W2	1.464	1.523	15.3
1.8	1.749	1.758	W3	2.303	1.391	13.8
1.2	1.272	1.275	R1	1.914	1.993	20.8
0.6	0.756	0.822	R2	2.058	1.944	21.4
0.3	0.435	0.429	R3	1.65	1.685	17.4
0	0.177	0.171	B1	1.35	2.311	25.1
			B2	1.865	1.845	19.6
			B3	1.771	1.679	18.1

[1] Bovine serum albumin standard concentrations

[2] Absorbance reading using Microplate reader at 562 nm wavelength

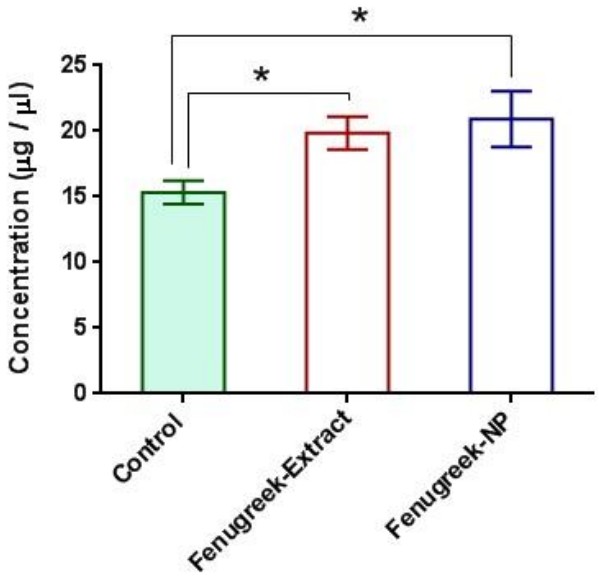

Figure (1): Values (M±SE) of total protein concentration among study groups

Further, sperm motility and sperm concentration were elevated in rat administered the extract demonstrating the antioxidant potential of fenugreek that helps to decrease the oxidative stress. Sadogh *et al*. (2022) mentioned that taking fenugreek seed oil drops improves the sperm count in

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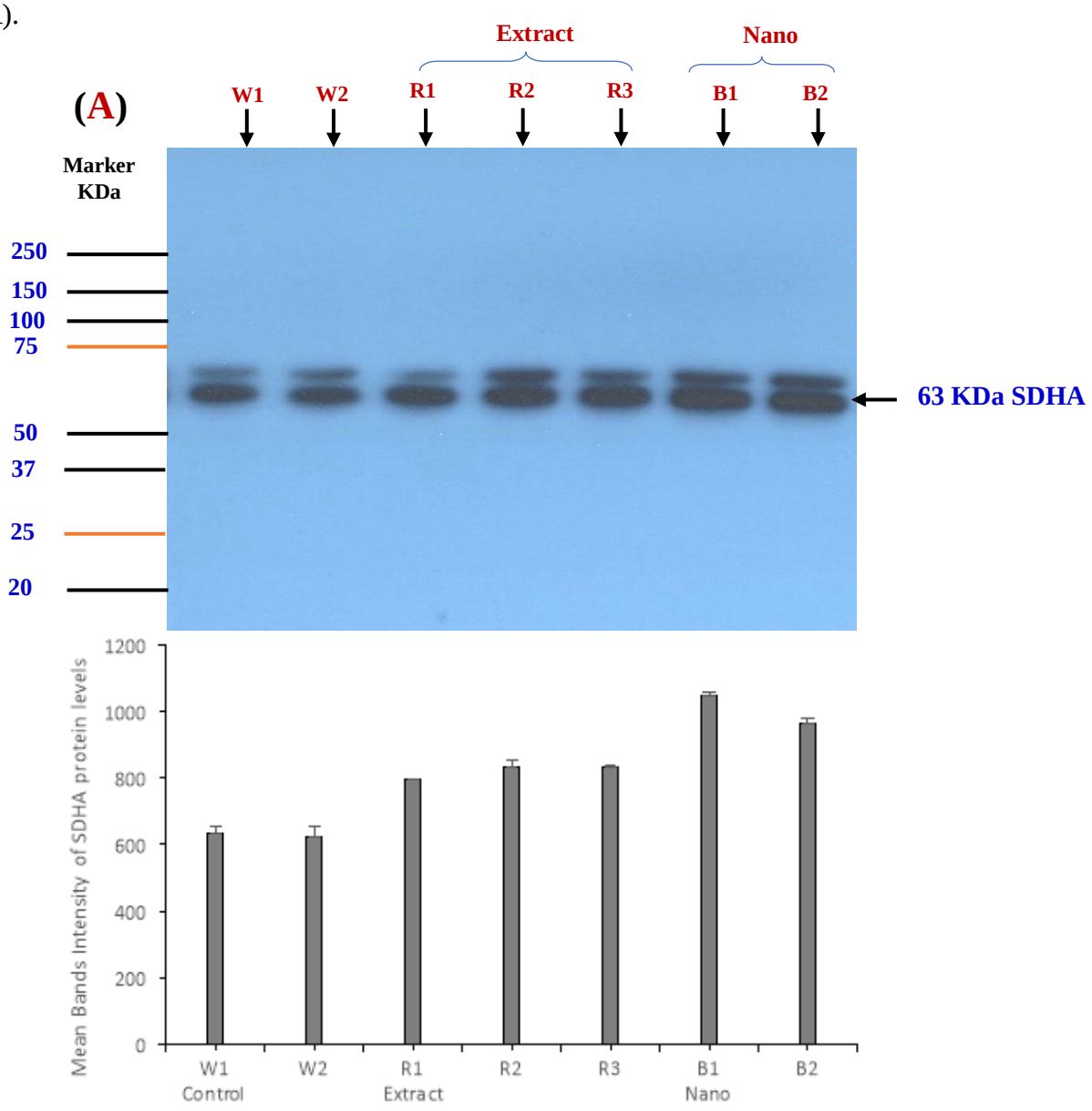
men with a low concentration of sperm.

Western Blotting Assay

The results of the current study showed that there was a significant increase (P< 0.002) in expression of mitochonderial expretion of SDHA protein in fenugreek extract group (819.67 ± 11.465) and fenugreek NP (1006 ± 13.537) groups compared with control (629.17 ± 26.308) group. A comparison between the result of the treatment groups revealed that there were significant elevation (P<0.0001) in values of fenugreek NP group more than fenugreek extract group (Figure 2A).



For CS protein, insignificant variation was reported between values of fenugreek extract (1023 ± 52.009) and fenugreek NP (984.83 ± 20.726) groups when compared to value of control

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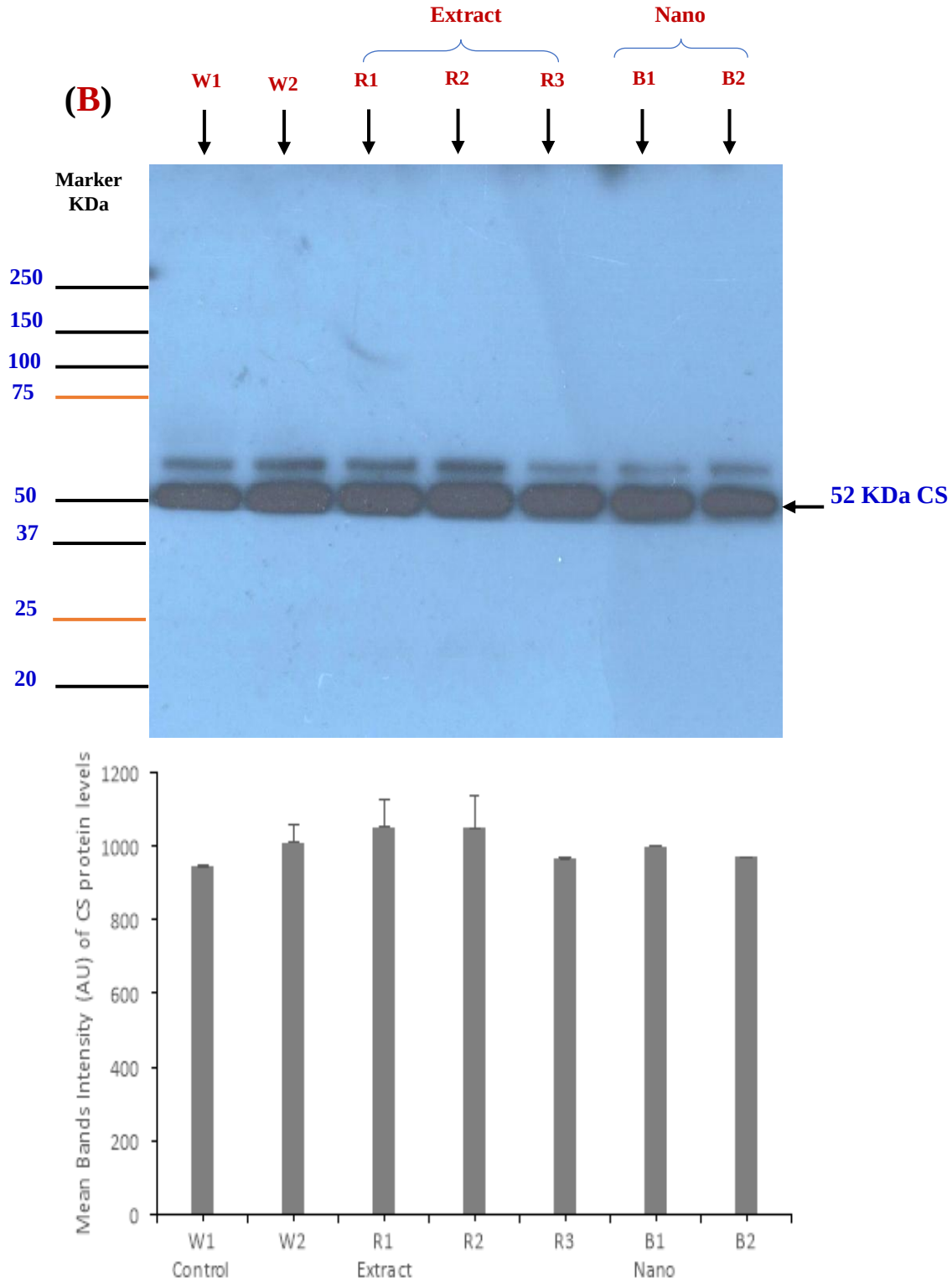
group (977.67 ± 27.818) at P<0.485 and P<0.809, respectively. In comparison between the treatment groups, no significant differences (P>0.05) were detected between values of fenugreek extract and fenugreek NP groups (Figure 2B).



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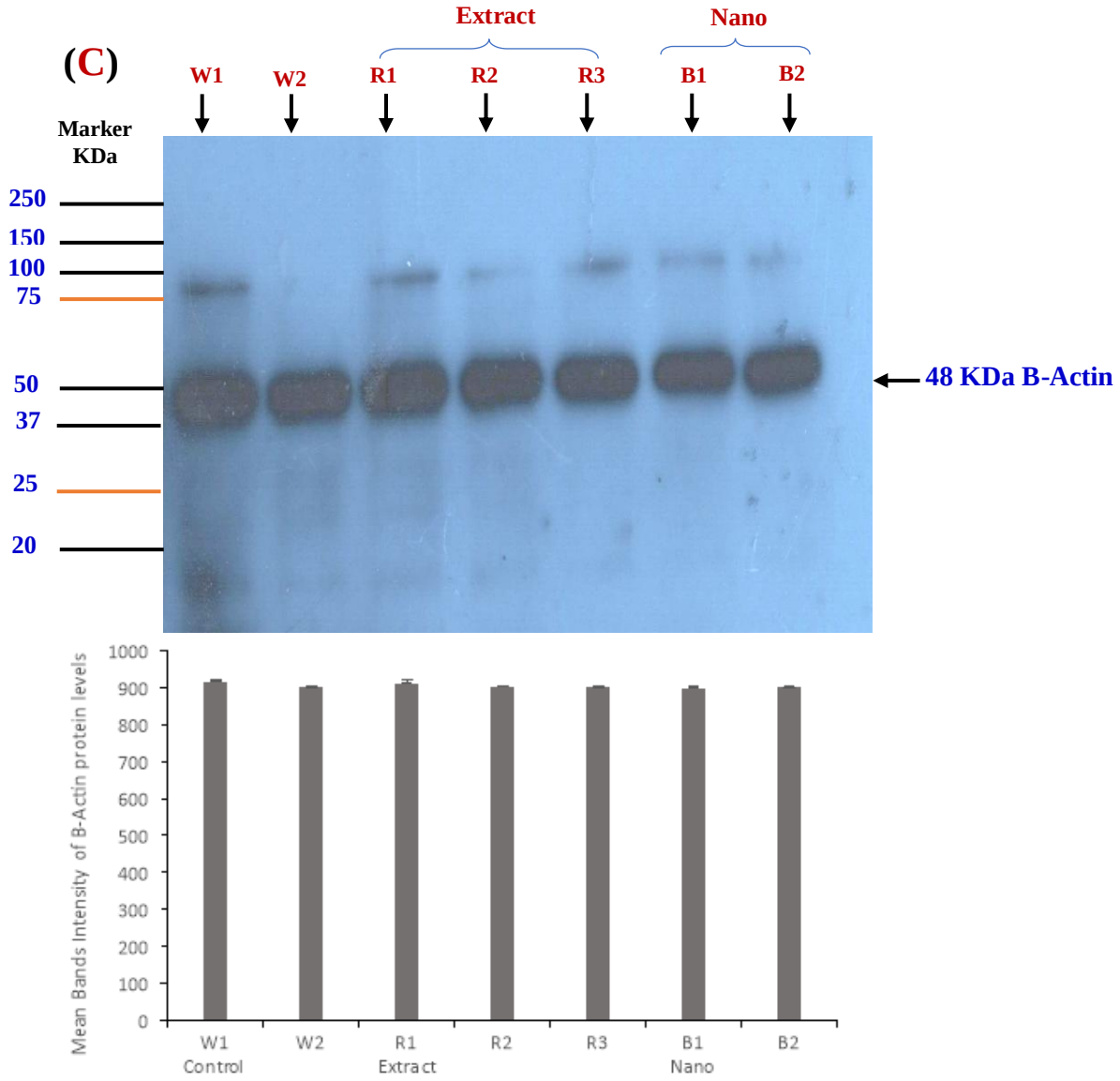

Figure (2): Mitochonderial expretion of SDHA and CS proteins in treated group with extract (R1, R2, R3) or NPs (B1, B2) compeared to control group (W1, W2) for epidedmus of mature wister male rat during spermatogenesis.

(A): The image showing level of SDHA protein in fenugreek extract and fenugreek NPs groups compared to non treated control group. SDHA protein increased significantly in both treated group in compare with the control group. Moreover, the fenugreek NPs goup showed a significant high increasing in level of SDHA compared with the fenugreek extract group

(B): The image showing the level of CS protein in the fenugreek extract and fenugreek NPs groups compared to non treated control group, in which, no significat changes were seen between

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fenugreek extract, fenugreek NPs and control groups.

(C): The image showing the level of B-Actin as a housekeeping gene that presents the equelizing of pipetting error and non effecting of cell homeostasis during treated process.

Western blotting assay is a powerful tool to detect and characterize a multitude of proteins, especially those proteins that are of low abundance. It offers various specific advantages such that the wet membranes are pliable and are easy to handle compared to gels, easy accessibility of the proteins immobilized on the membrane to different ligands, only small amount of reagents are required for transfer analysis, multiple replicas of a gel are possible, prolonged storage of transferred patterns, prior to use, becomes possible, and the same protein transfer can be used for multiple successive analyses (Kurien and Scofield, 2015; Neris *et al*., 2021).

The activity of spermatozoa mitochondria has been correlated with spermatozoa motility and thus with fertilization potential. Moreover, many studies demonstrated that the mitochondrial capsule localizes a variety of signaling proteins to the mid-piece and, thus, could play a critical role in spermatozoa function (Lu *et al*., 2017; Wang *et al*., 2020; Gallo *et al*., 2021). Further analysis of the identified proteins with reference to their biological function showed that the vast majority of the identified proteins were engaged in cell metabolism and energy production; while the other identified proteins were involved in sperm tail structure/motility, protein metabolism, mitochondrial maintenance, spermatogenesis, sperm maturation, intracellular trafficking, and immunity (Nowicka-Bauer *et al*., 2018). In this study, the findings of western blotting assay were supporting and confirmed results obtained by the BCA, in which, fenugreek extract and fenugreek NPs were participated significantly in increasing of SDHA protein but not CS protein that showed insignificant changes in their concentration among different study groups. Several experiments have been conducted in order to address these issues (Ibrahim et al., 2019; Kaur and Sadwal, 2020; Mansour *et al*., 2021). Jagtap *et al*. (2022) mentioned that fenugreek helps with reproductive problems and a variety of other disorders, and that the liquid extract of fenugreek increases sperm mortality and cation sperms channel proteins. Other study concluded that fenugreek seeds improved male sexual functioning, improved sexual life, and regulated orchid hormones and lactogen (Khanna *et al*., 2021). Rao (2020) mentioned that the pharmacological actions of fenugreek are attributed to a diverse array of phytonutrients, with the main groups being steroidal saponins that thought to be responsible for androgenic effects and have been used previously as precursors for the synthetic production of steroid hormones. Worldwide, there was a number of animal and human studies introduced fenugreek extract as a food supplement boosting testosterone (Heufelder *et al*., 2009; Aswar *et al*., 2010; Hamden *et al*., 2010; Nguyen *et al*., 2017), but the results of these studies

are not consistent.

Torra-Massana *et al*. (2021) concluded that the sperm samples from patients with fertilization failure present altered abundance of different proteins, including mainly mitochondrial proteins. By using the western blotting assay, Italiano *et al*. (2012) identified the loss of SDHA protein expression in tumor cases whereas expression was retained in the non-tumor cases referring that these results were expected because mutations that resulted by tumor which lead to a truncated SDHA protein. The *SDHA* gene encodes the major catalytic subunit of the succinate dehydrogenase complex II; and the germline mutations in *SDHA* are associated with neurodegenerative diseases such as an early-onset encephalopathy, optic atrophy, ataxia and myopathy (Di Donato, 2009; Ghaoui and Sue, 2018).

Conclusion

This study provided new insights into the mitochondrial expression of SDHA and CS proteins using the Western Blotting and Bicinchonic Acid assays. The administration of both fenugreek extract and fenugreek NPs were revealed a significant protein expression with in particular with using of fenugreek NPs that did not cause any side effects along the periods of the study. However, isolation of active components in fenugreek, and synthesizing of specific NPs based on one type of these components can provide new therapies with different characteristics. Alsom the augmentation of fenugreek NPs and proper regulation of steroidogenesis and mitochondrial biogenesis related genes is notably need to be furthermore studied.

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