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Grape seed proanthocyanidin extract or *spirulina platensis* alleviates blood biochemical and hepatic molecular derangements of experimentally-induced thyroid dysfunction in rats

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ABSTRACT

Hyper- or Hypothyroidism is an overactive or underactive thyroid gland that prevents the body from operating properly. The potential therapeutic effects of spirulina platensis and GSPE on experimental hypothyroidism and hyperthyroidism in rats were evaluated. A total 96 rats were divided into two main experimental groups: Experiment A: carbimazole-induced hypothyroidism (1.8 mg/kg b. wt.) and experiment B: induced hyperthyroidism (50, 100, 200 µg/kg b. wt.) for the first three weeks, respectively. The administered GSPE dose (150mg /kg b. wt./day) and Spirulina (300 mg/kg b. wt./day) for 3 weeks. The hyperthyroidism experiment (A) six sets of rats were used: Group 1 (control normal), Group 2 (hyperthyroidism), Group 3 (GSPE Protected): GSPE administered for the first 3 weeks and continued with thyroxine for another 3 weeks. Group 4 (GSPE treated): thyroxine administered for 3 weeks, followed by GPSE as in group 3. Group 5 (Spirulina Protected); spirulina and thyroxine administration as in group 3. Group 6 (Spirulina treated): spirulina and thyroxine administration in group IV. The hypothyroidism experiment (B) rats divided also into 6 groups like to the above design in hyperthyroidism experiment (A) but carbimazole dose was stable (1.8 mg/kg b. wt.) in the 3 weeks. In hyperthyroidism spirulina and GPSE significantly increased serum total cholesterol, triacylglycerols with down regulation of liver Caspase-8 and significant upregulation of Bcl2 gene. In hypothyroidism spirulina and GPSE exhibited down regulation in liver miRNA 224, PKCα with significant upregulation of miRNA 382 gene in hypothyroid rats. Spirulina and grape seed may treat and prevent hyperthyroidism or hypothyroidism in rats.

1. INTRODUCTION

The thyroid gland is the site for the synthesis of the thyroid hormones. The amino acid tyrosine is the source of two hormones that contain iodine. In the liver, thyroid hormones regulate cholesterol homeostasis, production of bile acids and the metabolism of fatty acids (Mullur et al., 2014). In contrast to hypothyroidism, which is characterized by elevated cholesterol levels, decreased lipolysis, and increased gluconeogenesis, hyperthyroidism generates a hyper-metabolic state that is marked by decreased cholesterol levels, increased lipolysis, and increased gluconeogenesis (Cicatiello et al., 2018). L-thyroxine and carbimazole, two medications used to treat thyroid conditions, are also utilized in animal experiments to experimentally cause hypothyroidism and hyperthyroidism (Treesh and Khair, 2014). Drugs that include l-thyroxine affect oxidative stress and have an impact on lipid profiles (TC, TG, HDL, LDL, VLDL). Hyperthyroidism is a hyper-metabolism resulting from increased freeT4 and/or free T3 serum levels (Işman et al., 2003). This hypermetabolic state is associated with an increase in the prooxidant to antioxidant ratio, which leads to oxidative stress (Kim 2012; Hashem 2016). People with hyperthyroidism might end up with common health problems such as cardiovascular diseases (heart failure and increased risk of heart attack), diabetes mellitus, oxidative damage to the liver and osteoporosis (Kim et al., 2012). On the other hand, deficiency, or absence of Thyroid hormone (TH) causes hypothyroidism. One of the most prevalent thyroid conditions in people, either congenital or acquired, is this one (Ayuob, 2016). All bodily processes slowdown in the hypothyroid state, which is a complex hormonal dysfunction (Hayat et al., 2010). In hypothyroidism, the basal metabolic rate is decreased, and the production of reactive oxygen species (ROS) is increased (Rabeh and El-Ghandour, 2016). Metabolic depression resulting from hypothyroidism has been associated with a decrease in oxidant production (Işman et al., 2003). Hypothyroidism was induced by the anti-thyroid drug carbimazole, which is used in the treatment of human hyperthyroidism. (Deshpande et al., 2002).

Spirulina (SP) is considered an excellent nutritional supplement with many health benefits. SP also contains phycocyanin, a powerful antioxidant which gives spirulina

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its rich green colour (Lissi et al., 2000). SP is well documented for its clinical importance in diabetes, hypertension, and cancer (Palaniswamy and Veluchamy, 2018), besides its antioxidant, immune-modulating, anti-microbial (Finamore et al., 2017). Many woody plants, including grape seeds and white pine, contain proanthocyanidins (PAs), oligomers and polymers of monomeric flavonoids. In comparison to vitamins C, E, and -Carotene, PAs contained in (GSPE) exhibit much higher protection against oxygen free radicals and possess a wide range of biological, pharmacological, and chemo-protective activities (Bagchi et al., 2002).

Several studies have shown that, by scavenging superoxide and hydroxyl radicals, Proanthocyanins have strong antioxidant and anticancer properties. They can also stop drug-induced liver and kidney damage (Engelbrecht et al., 2007). This study investigated the effect of experimental hypothyroidism and hyperthyroidism on molecular derangements and intracellular pathways alterations in the thyroid and liver. Moreover, the possible protective effects of spirulina and GSPE on thyroid dysfunction in rats were explored.

2. MATERIAL AND METHODS

2.1. Experimental animals

A total 96 male rats, aged 8–12 weeks old and weighing about 140–160 g, were employed in this investigation. Separate wire mesh cages with good ventilation, humidity control, and a 12-hour light/dark cycle was employed to house the rats. Clean drinking water was available at all times. Rats were separated for 15 days so they could acclimate before the trial started. The experimental protocols were also approved by the Animal Care and Use Committee at Benha University and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Approval no. BUFVTM 02-8-21).

2.2 Chemical and Antioxidant agents

2.2.1. Chemicals

2.2.1.1. Thyroxine (Eltroxin)® 5o µg tablets, was manufactured by GlaxoSmithKline GmbH (Germany). Rat model of the hyperthyroid received thyroxine at dose of (50, 100, 200 µg /kg b. wt.) orally for one, two and three weeks of study, respectively (Guerrero *et al.*, 1999)

2.2.1.2. Carbimazole:

Carbimazole (NeoMercazole® 5 mg tablets, was manufactured by Amdipharm (Dublin, Ireland). Rat model of the hypothyroid received carbimazole with a dose of 1.8 mg/kg b. wt./day administered orally over three weeks (Sakr *et al.*, 2012).

2.2.2. Antioxidant compounds

2.2.2.1. Spirulina platensis:

Pure *Spirulina platensis* powder was obtained from (National Research Center-- Dokki-Egypt). Dosage: rat 1.0 mL of a suspension of 300 mg/kg body weight of spray-dried powder of spirulina platensis dissolved in distilled water (Simsek et al., 2009).

2.2.2.2. Proanthocyanidin extract from grape seeds:

Grape Seed Proanthocyanidin (GSPE) was obtained from Al Debeiky Pharma Company for Pharmaceutical industries (Al Obour, Cairo, Egypt). DMSO 7 % was used to dissolve the GSPE, and sterile saline solution was used to dilute it to the right concentration. 150 mg/kg b.wt. of GSPE was used as the dosage (Yousef *et al.*, 2009).

2.3 Design of experiment:

Rats were split into two main experimental groups, housed in individual cages, and classed as follows:

- *Experiment A*: Eltroxin- induced hyperthyroidism (50, 100, 200 µg /kg b. wt.) for first three weeks, respectively. Hyperthyroidism experiment (A): six sets of rats were used: Group 1 (control normal), Group 2 (hyperthyroidism) 50 µg /kg b. wt., Group 3 (GSPE Protected): GSPE administered for the first 3 weeks and continued with Thyroxine for another 3 weeks. Group 4 (GSPE treated): Thyroxine administered for 3 weeks, followed by GPSE as in group 3. Group 5 (Spirulina Protected); Spirulina and Eltroxin administration as in group 3. Group 6 (Spirulina treated): Spirulina and Eltroxin administration as group IV.

- Experiment B: carbimazole-induced hypothyroidism (1.8 mg/kg b. wt.). The administered GSPE dose (150 /kg b. wt. /day) and Spirulina (300mg/kg b. wt./day) for 3 weeks. Hypothyroidism experiment (B) rats divided also into 6 groups similar the above design in Hyperthyroidism experiment (A) but carbimazole dose is stable (1.8 mg/kg b. wt.) in the 3 weeks.

2.4 Sampling

Blood samples, liver tissue specimens were collected from all Hyperthyroid and Hypothyroid animal groups 24 hours after the last dose of GSPE and spirulina administrations.

2.4.1 Blood samples

Serum was isolated from blood samples by centrifugation at 2500 rpm for 15 minutes after ocular vein punctures were used to collect the blood samples. When used for the later study of total cholesterol and triacylglycerols in the experiments (A) and (B) were separated by an automatic pipette, received in samples tubes, and preserved in a deep freezer at -20 °C.

2.4.2. Tissue specimen

After being collected, the liver tissues were placed in Eppendorf tubes, instantly frozen in liquid nitrogen, and maintained at – 80 °C until RNA extraction for Bcl-2 and caspase 8 determination in hyperthyroidism experiment (A) as well as PKCα, Micro- 224 and MicroRNA 382 gene expression in hypothyroidism experiment (B).

2.5 Analysis

2.5.1. Biochemical analysis (Serum):

Serum total cholesterol and triacylglycerols were determined by the methods of Ellefson and Caraway (1976) and Stein (1987), respectively.

2.5.2. Molecular investigation (Liver):

Using real-time quantitative polymerase chain reaction analysis (real-time qPCR), it was possible to identify the mRNA expression levels of the liver Caspase-8, Bcl-2, and PKC in the rat liver (Table 1). The load was managed with β -actin. Following the manufacturer's instructions, total RNA was extracted from the heart using the High Kit for extraction of pure RNA (Thermo Scientific, Fermentas, #K0731) RNA Extraction kit. Revert Aid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA) was used to reverse transcribe each cDNA sample. Then, real-time quantitative PCR amplification was performed on Faststart Universal SYBR Green Master (Roche, GER). The target gene was normalized with β-actin by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). miRNA224and miRNA382 were determined using real-time PCR with SYBR Green and U6 as an internal control.

Thermo Scientific, USA, # K0221), a miRNA-specific forward primer (Table 2), and a universal reverse primer supplied with the Quanti-Mir RT kit were used to amplify the extracted cDNA in accordance with the manufacturer's instructions.

Table 1 The forward and reverse primer sequences for qPCR primers.

Gene	Forward primer (/5 —>/3)	Reverse primer (/5 —>/3)
Caspase 8	CTGGGAAGGATCGACGATTA	CATGTCCTGCATTTTGATGG
Bcl-2	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC
PKCα	TTTGTTACTTTCTCTTGTCCGGGT	ACATTCATGTCGCAGGTGTCGCA
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

Table 2 The forward and reverse primer sequences are utilized in qPCR.

Gene	Primer sequence (/5 ------ /3)
miRNA224	CAAGTCACTAGTGGTTCCGTT
miRNA382	GAAGTTGTTCGTGGTGGATTCG
U6	TGACACGCAAATTCGTGAAGCGTTC
Universal reverse primer	CCAGTCTCAGGGTCCGAGGTATTC

2.6. Statistical evaluation

All of the data were provided as Mean ± SEM. Duncan's multiple range test was used to produce individual comparisons, and one-way analysis of variance (ANOVA) was used to assess statistical significance using SPSS 18.0 software in 2011. (DMRT). Values were deemed statistically significant when P≤ 0.05.

3. RESULTS

3.1. Effect of Hyperthyroidism:

A notable reduction in serum total cholesterol and triacylglycerols concentrations was observed in Eltroxin induced hyperthyroidism at all doses. Treatment and protection with GSPE (G3 & G4), spirulina (G5 & G6) to hyperthyroid rats showed a noticeable rise in serum total cholesterol and triacylglycerols levels as compared hyperthyroid non treated group (Table 3 and Figures 1, 2)

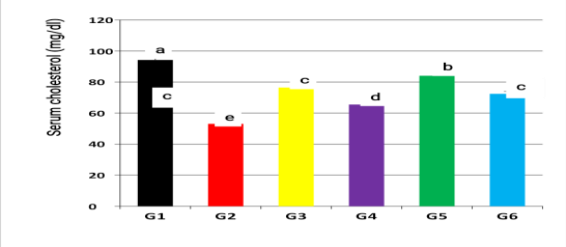

Fig. (1): Effect of Spirulina or GSPE treatment on serum total cholesterol concentration in experimental model of hyperthyroidism in rats.

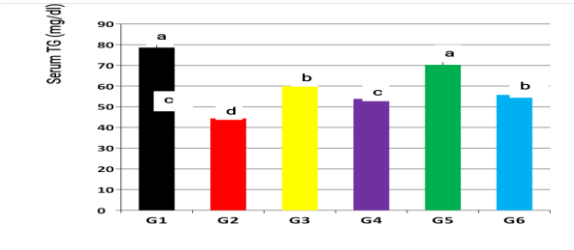

Fig. (2): Effect of Spirulina or GSPE treatment on serum triacylglycerols concentration in experimental model of hyperthyroidism in rats.

A significant upregulation in Caspase 8 and downregulation in Bcl-2 were observed in Eltroxin induced hyperthyroidism. Treatment and protection with GSPE (G3 & G4) and spirulina (G5 & G6) to Eltroxin induced hypothyroidism in rats exhibited a significant down-regulation in Caspase 8 with upregulation in Bcl-2 as compared with hyperthyroid non treated group (Table 4 and Figures 3, 4).

3.2 Effect of hypothyroidism:

A significant upregulation in miRNA 224 and PKCα and downregulation in miRNA 382 were observed in Carbimazole induced hypothyroidism. Treatment and protection with GSPE (G3 & G4) and spirulina (G5 & G6) to Carbimazole induced hypothyroid rats exhibited a significant downregulation in miRNA 224 and PKCα with upregulation in miRNA 382 as compared with hyperthyroid non treated rats (Table5 and Figures 5, 6 and 7).

4. DISCUSSION

A substantial drop in serum total cholesterol and triacylglycerols concentrations had been seen in Eltroxin-induced hyperthyroidism. Similarly, Lee et al. (2019) noted that TXN administration resulted in a considerable reduction in serum TC (total cholesterol) and TG (triacylglycerols) levels in the hyperthyroidism. Hyperthyroidism is in connection with reduced total and HDL cholesterol levels, as well as a lower total/HDL cholesterol ratio and apoA I levels. These effects are reversible if the underlying thyroid disorder is treated (O'Brien et al., 1997). Treatment and protection with GSPE or spirulina to hyperthyroid rats showed a considerable increase in serum total cholesterol and triacylglycerols levels as compared to hyperthyroid group. This outcome was consistent with Albrahim et al. (2020), who claimed that GSE treatment to Eltroxin-induced hyperthyroid mice for three weeks significantly normalize hyperthyroidism animals that reduced TC and TG levels while normalizing TSH levels that are already high. The major increase in serum TC and TG levels after treatment with Spirulina or GSPE, confirmed the anti-hyperthyroidism activity of GSPE and Spirulina.

Moreover, Bolkiny et al. (2019) reported that in hypo- and hyperthyroid mice treated with costus root extract, their results showed a substantial improvement in serum cholesterol and triglycerides in hyperthyroid mice suggest that GSPE has the potential resistance against the negative role of TXN Inhibiting the factors that produce total cholesterol and triacylglycerols. A significant upregulation in Caspase 8 and downregulation in Bcl-2 were observed in Eltroxin- induced hyperthyroidism.

Table 3 Effect of protection and treatment with Spirulina or GSPE on serum total cholesterol and triacylglycerol concentrations of Eltroxin induced hyperthyroidism in rats.

Animal groups	Total Cholesterol (mg/dl)	Triacylglycerols (mg/dl)
Control non treated (G1)	94.17 ± 2.02^{a}	78.56 ± 1.90^{a}
Hyperthyroidism (G2)	53.09 ± 1.11^{e}	44.39 ± 1.06^{d}
Hyperthyroidism + GSPE protection (G3)	76.45 ± 1.63^{c}	60.05 ± 1.29^{b}
Hyperthyroidism + GSPE treatment (G4)	65.47 ± 1.39^{d}	53.80 ± 1.39^{c}
Hyperthyroidism + spirulina protection (G5)	84.04 ± 1.82^{b}	70.24 ± 1.73^{a}
Hyperthyroidism+ spirulina treatment (G6)	72.36 ± 1.55^{c}	55.71 ± 1.35^{b}

Data are presented as (Mean ± S.E). SE = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table 4 Effect of protection and treatment with Spirulina or GSPE on liver Caspase 8 and Bcl-2 gene expression of Eltroxin induced hyperthyroidism in rats.

Animal groups	Caspase 8 Fold change ± SEM	Bcl-2 Fold change ± SEM
Control non treated (G1)	1.00 ± 0.08^{e}	1.00 ± 0.05^{a}
Hyperthyroidism (G2)	5.70 ± 0.22^{a}	0.12 ± 0.01^{e}
Hyperthyroidism + GSPE protection (G3)	3.39 ± 0.16^{c}	0.49 ± 0.02^{c}
Hyperthyroidism + GSPE treatment (G4)	4.50 ± 0.14^{b}	0.27 ± 0.01^{d}
Hyperthyroidism + spirulina protection (G5)	2.89 ± 0.10^{d}	0.59 ± 0.03^{b}
Hyperthyroidism+ spirulina treatment (G6)	3.41 ± 0.13^{c}	0.46 ± 0.02^{c}

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05). SEM, Standard Error of Mean.

Table 5 Effect of protection and treatment with Spirulina or GSPE on liver miRNA 224, miRNA 382 and PKCα gene expression of Carbimazole induced hypothyroidism in rats.

Animal groups	MirRNA 224 Fold change ±SEM	MirRNA 382 Fold change ±SEM	PKCα Fold change ±SEM
Control non treated (G1)	1.00 ± 0.08^{e}	1.00 ± 0.06^{a}	1.00 ± 0.08^{e}
Hyperthyroidism (G2)	9.51 ± 0.42^{a}	0.06 ± 0.01^{e}	4.35 ± 0.18^{a}
Hyperthyroidism + GSPE protection (G3)	6.11 ± 0.28^{c}	0.51 ± 0.03^{c}	2.50 ± 0.12^{c}
Hyperthyroidism + GSPE treatment (G4)	7.57 ± 0.33^{b}	0.23 ± 0.01^{d}	3.41 ± 0.14^{b}
Hyperthyroidism + spirulina protection (G5)	2.99 ± 0.14^{d}	0.73 ± 0.04^{b}	1.83 ± 0.10^{d}
Hyperthyroidism+ spirulina treatment (G6)	6.19 ± 0.29^{c}	0.42± 0.02^{c}	2.60 ± 0.13^{c}

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05). SEM, Standard Error of Mean.

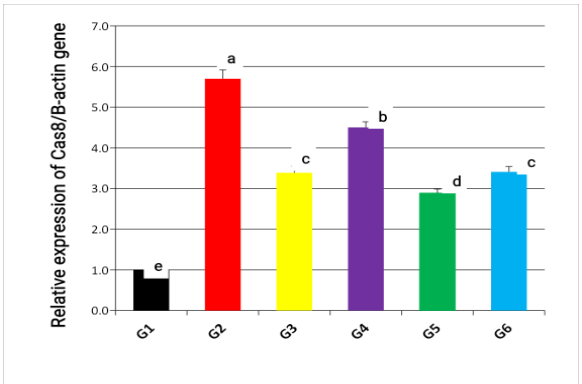

Fig.(3) Effect of Spirulina or GSPE treatment on liver caspase8 gene expression of experimental model of hyperthyroidism in rats.

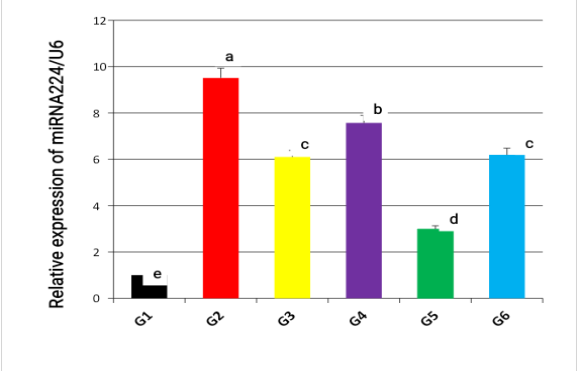

Fig.(5) Effect of Spirulina or GSPE treatment on miRNA 224 gene expression in experimental model of hypothyroidism in rats.

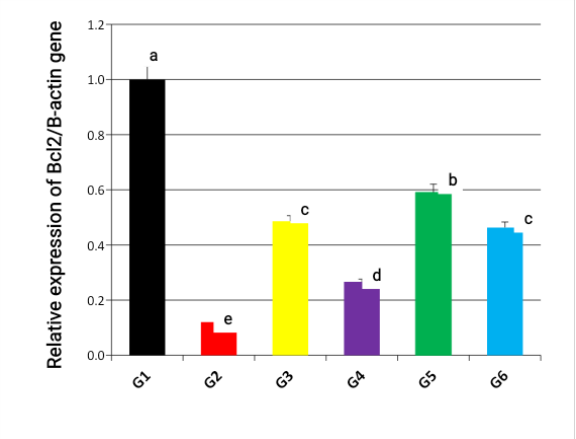

Fig.(4) Effect of Spirulina or GSPE treatment on liver Bcl-2 gene expression of experimental model of hyperthyroidism in rats.

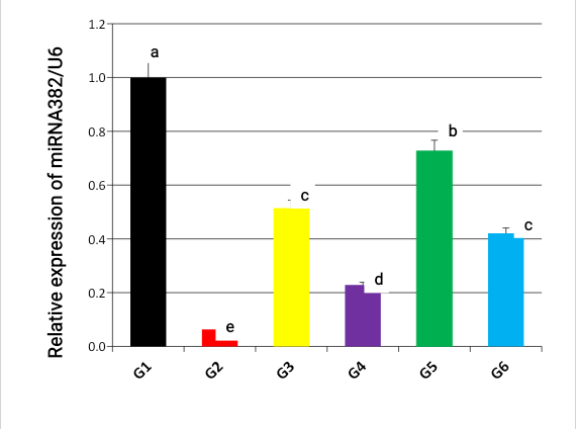

Fig.(6) Effect of Spirulina or GSPE treatment on miRNA 382 gene expression in experimental model of hypothyroidism in rats.

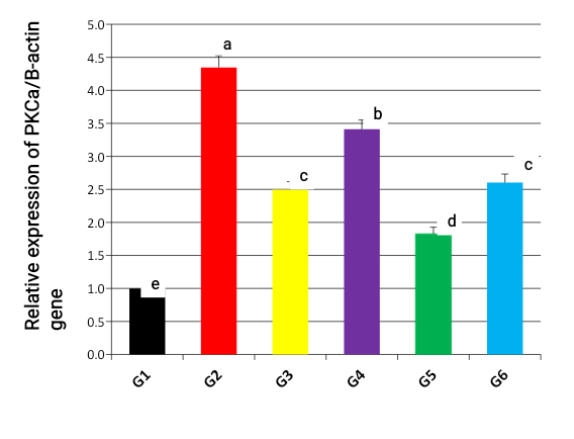

Fig.(7) Effect of Spirulina or GSPE treatment on PKCα gene expression in experimental model of hypothyroidism in rats.

The gene expressions of apoptotic mRNA, caspase-8 mRNA, and caspase-9 mRNA were substantially diminished and anti-apoptotic gene such as Bcl2 was significantly enhanced in the brain and liver of the fetuses after pregnant dams were orally administered with TXN. In some liver diseases, apoptosis results in the death of a considerable part of hepatocytes, impairing liver function. Hepatocyte damage may result in the production of apoptotic bodies and activation of Kupffer cells, which in turn may encourage inflammatory and fibrogenic responses (Hassa et al., 2018). Treatment and protection with GSPE or spirulina in Eltroxin-induced hyperthyroidism in rats exhibited a significant downregulation in Caspase 8 with upregulation in Bcl-2 as compared with hyperthyroid rats. This may be due to the fact that a mitochondria-mediated apoptotic pathway was found to link apoptosis to an increase in mitochondrial cytochrome c release, the Bax: Bcl-2 ratio, and caspase activation (Sharifi-Rad et al., 2021). Similarly, (Ebrahim, 2020) reported that the hippocampus of diabetic rats showed reduced oxidative stress and neuronal death in response to dietary spirulina or GSE's antioxidant and/or anti-inflammatory properties. A major downregulated in Caspase-8 and upregulated in Bcl-2 were detected during treatment of TXN-induced DCM in rats with GSE and Spirulina (Sharifi-Rad et al., 2021). They have demonstrated a function in either controlling the activity and the level of expression of the apoptotic initiator of caspase-8 and other genes, including caspase-3, -9, Bax, Cyt-c, TNF-α, and NF-B genes to the lowest and upregulated the level of expression of Bcl2 gene (Ebrahim, et al., 2020).

A significant upregulation in miRNA 224 and PKCα and downregulation in miRNA 382 were observed in Carbimazole induced hypothyroidism. Treatment and protection with GSPE and spirulina to Carbimazole induced hypothyroid rats exhibited a significant downregulation in miRNA 224 and PKCα with upregulation in miRNA 382 as compared with hyperthyroid non treated rats. Carbimazole is well known for causing epigenetic modifications that hinder regular metabolism (Pisera-Fuster et al., 2020). As a result, the current study sought to evaluate the various miRNA epigenetic patterns associated with CMZ-induced hypothyroidism. Similarly, Peixoto et al. (2021) established that the miRNA 224 action results explained the long-term effects of early nicotine exposure (6 mg/Kg), which acts as a hypothyroidic agent like effector like Carbimazol (Alhowail et al., 2021). Also, Capriglione et al., (2022) investigated the profile of miRNAs including miR382-5p found in exosomes secreted in serum of 58 papillary thyroid cancer patients (PTC). Furthermore, (Eunjin et al., 2014; Nie et al., 2021) demonstrated that miR-382-5p directly targeted the 3-UTR of human NR1H4 mRNA and that its level was shown to be elevated in HCC tissues and to be inversely associated to NR1H4 mRNA levels utilizing a luciferase reporter experiment. Overexpression of miR-382-5p facilitated the malignant proliferative cycle of HCC cells by inhibiting the expression of FXR. Additionally, Tian et al., (2022) confirmed that administration of 2, 2′-dipyridyl disulphide that lowered thyroid hormone levels has significantly increased the gene expression of protein kinase C (pkc), here as the hormones decreased, the level of PKCa expression is increasing. Hypothyroidism raises the expression of Protein Kinase C, a regulator of cardiac contractility important in the myocardial cells' ischemia-reperfusion process. These processes explain hypothyroidism's resistance to ischemia-reperfusion, together with the inhibition of apoptotic c-Jun N-terminal Kinases (JNKs) (Pantosetalm, 2008). In addition to McQuillin et al. (2007) showed that thyroid hormone specifically suppresses PKC and vice versa in the neonatal heart, as well as PKC in the adult heart. Changes in PKC caused by thyroid hormone are crucially permissive in the regulation of neurogenic responsiveness in ventricular cardiomyocytes. Lei et al. (2022) found that the wet and dry feed of Spirulina interestingly have a modulate on the PKCα. Collectively, the findings of this study clearly showed that the therapeutic properties of GSPE and Spirulina can modulate the upregulation of Caspase-8 in hyperthyroidism and the lowering of miRNA 224, PKCα in hypothyroidism. GSPE and Spirulina also enhancing the level of total cholesterol and triglycerides and Bcl-2 expression in hyperthyroidism as well as improving miRNA 382 in hypothyroidism, suggesting that they have multiple beneficial effects.

5. CONCLUSION

In conclusion, GSPE and Spirulina platensis had a potential therapeutic effect in thyroid dysfunction, through alleviating microRNA and PKCα that essential for thyroid hormone homeostasis and thyroid functions. Moreover, GSPE and Spirulina platensis mitigate liver damage and Thyroid disruptors via anti-apoptotic activities.

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