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Ameliorative Impacts of *Dactyloctenium aegyptium* and *Parapholis incurva* Ethanolic Extracts on Sodium Fluoride Induced Pathological Alterations in the Thyroid Gland in Male Rats

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Abstract

The present study aimed to demonstrate the ameliorative impacts of *Dactyloctenium aegyptium (D. aegyptium) and Parapholis incurva* (*P. incurva*) alcoholic extracts on hormonal, histological, and ultrastructural changes in rat thyroid gland induced hypothyroidism by daily oral administration of sodium fluoride (NaF) (5 mg/kg) for 28 days. Forty-Two adult male albino rats were randomly divided into 6 equal groups (7 rats each): the control group, NaF group (5 mg/kg/d), *D. aegyptium* group (100 mg/kg/d), *P. incurva* group (100 mg/kg/d), NaF + *D. aegyptium* group and NaF + *P. incurva* group. At the end of the experiment, the animals were sacrificed and blood samples were subjected to hormonal assay for triiodothyronine (T3) and thyroxine (T4) and thyroid-stimulating hormone (TSH) levels. Malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD), and calcium were assessed. Specimens of thyroid gland tissues were processed for histological, histochemical, immunohistochemical and scanning electron microscopy examinations. Further chemical profiling of the both extracts were determined using HPLC. Phytochemically, 14 phenolic compounds including 10 phenolic and 4 flavonoids were identified from *D. aegyptium* while 13 phenolics were identified from *P. incurva* comprising 8 phenolic and 5 flavonoids. NaF induced significant alterations in hormonal (decrease in serum T3 and T4 levels and increase in serum TSH levels) and antioxidant status, as well as, calcium levels when compared with control group. Histopathological (many empty follicles lined by flat cells, hemorrhage and disrupted basement membrane), histomorphometric, histochemical, immunohistochemical and ultrastructural (thyroid calcification) alterations were also recorded, which all were markedly improved in groups that received *D. aegyptium* or *P. incurva* extract combined with NaF. *D. aegyptium and P. incurva* extracts are useful in combating thyroid gland calcification and toxicity induced by NaF due to their high flavonoids and phenolic compound content.

Keywords: Calcitonin, Colloid, Scanning electron microscopy, T3, T4, Thyroid calcification, TSH.

1. Introduction

The thyroid gland is responsible for controlling the body's metabolic rate and is vital to human health. The thyroid gland is composed of spherical thyroid follicles whose walls made up of large number of cuboidal cells, also known as follicular cells that secrete thyroid hormones. Triiodothyronine (T3) and thyroxine (T4) are tyrosine-based thyroid hormones that regulate metabolism [1]. Thyroid cells absorb iodine to make T3 and T4. The normal thyroid gland produces about 80% T4 and about 20% T3, although T3 is strengthened [2].

T3 and T4 are required for the appropriate development, function, and differentiation of all human cells. In general when thyroid hormone binds to its intranuclear receptor, it activates the genes for increasing metabolic rate and thermogenesis. Thyroid problems can have a wide range of consequences that affect almost every physiological system [3]. Small C

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cells, also known as parafollicular cells, are found between thyroid follicles and secrete polypeptide hormone known as calcitonin. Calcitonin secretion is triggered by a rise in serum calcium levels, and calcitonin protects against hypercalcemia [4]. Calcium is necessary for various intracellular and extracellular activities as well as skeletal support in the body [5].

Many studies have demonstrated that exposure to certain endocrine disrupting chemicals alters thyroid structure and function, which is linked to an elevated risk of a variety of negative health outcomes, including mental disorders and cancers of various types. When exposed to endocrine disrupting substances, the colloidal content of thyroid follicular epithelial cells decreases, the endoplasmic reticulum is damaged, cytoplasmic vacuolation occurs, and the nuclei become irregular [6].

Sodium fluoride (NaF) has certain negative effects on the musculoskeletal system, tooth tissue, kidney, liver, brain, and endocrine glands, among other organs [7]. Drinking water and decay-preventive chemicals typically contain NaF [2]. Egypt is among the top 21 countries where fluorosis is endemic, with tap water from contaminated groundwater sources serving as the main source of fluoride exposure [8]. The thyroid gland is one of the most sensitive organs to excessive amounts of fluoride. Fluoride influences thyroid activity and development in a variety of ways [9].

People nowadays prefer complementary and alternative medicine, particularly herbal medications, because they have fewer side effects and are less expensive. Medicinal plants have drawn attention due to the phytochemicals' natural antioxidant activity, which reduces free-radical-induced oxidative damage. Consumption of edible plant parts high in total phenolic and antioxidant chemicals is beneficial in preventing oxidative damage [10].

Dactyloctenium aegyptium (D. aegyptium) is a widely used plant in traditional therapies around the world, with several pharmacological investigations demonstrating its anticancer, antipyretic, anti-inflammatory, and antioxidant potentialities, as well as gastrointestinal effects [11]. *Parapholis incurva* (*P. incurva*) is a widespread plant in northern Africa, Europe, and Asia [12]. To the best of our knowledge, there are no any chemical or biological studies on *P. incurva* until now, moreover, literature survey has shown that there are no scientific records in the biological effects of *D. aegyptium* and *P. incurva* in thyroid gland of experimental animals. The aim of this study was to estimate the chemical constituents of *D. aegyptium* and *P. incurva* and to study the possible protective effect of *D. aegyptium* and *P. incurva* on the NaF-induced histological, histochemical, ultrastructural and hormonal alterations in the thyroid gland.

2. Material and Methods

2.1. Chemicals:

NaF was obtained from LOBA Chemie Pvt. Ltd., Mumbai, India. NaF was supplied as a white powder form and dissolved in distilled water. NaF was given orally by gavages once a day at a dose of 5 mg/kg bw/day [13].

The used standard phenolic compounds for HPLC analysis were purchased from Sigma-Aldrich (Germany) including eight phenolic acids, chlorogenic, gallic, caffeic, coumaric, ellagic, syringic, ferulic, and cinnamic acids, three phenolic acid derivatives, propyl gallate, vanillin, and caffeine, and six flavonoids, naringenin, quercetin, catechin, pyrocatechol, rutin, and 4,7-dihydroxy isoflavone. Also the HPLC solvents involving acetonitrile and trifloroacetic acid were bought from Sigma-Aldrich (Germany). The di-distilled H_2O, used in HPLC, was acquired via the Hamilton water distillation system (Hamilton Laboratory Glass Ltd., Kent, England).

2.2. Plant Materials and extraction process:

The aerial parts of *D. aegyptium* and *P. incurva* (family: Poaceae) were collected from Kafr-Saad, Damitta, Egypt, during the flowering time. The collection and identification of the two plants were performed by Prof. Ahmed M. Abd-El-Gawad, professor of Taxonomy, Botany Department, Faculty of Science, Mansoura University, Egypt. Voucher sample specimens (DA-X317-015-M1921 and PI-X317-015-M1922) were placed in the herbarium of the Faculty of Science, Mansoura University, Egypt. The plant then was left to complete air dried.

Air-dried powder aerial parts of the two plants, *D. aegyptium* and *P. incurva* (500 g, each), were separately extracted with 70% ethanol, filtered, and dried under vacuum with temperature at 40 ^{o}C to produce dark black gum (24.5 and 21.0 g, respectively).

2.3. High Performance Liquid Chromatography (HPLC) Analysis

The HPLC analysis of the ethanolic extracts of the two plants performed according to the procedure previously reported by Abd-El Gawad et al. [14]. In brief, the performing of the HPLC analysis was happened by the Agilent 1260 series utilizing a C18 column chromatography (4.6 × 250 mm i.d., 5 μm) for separation. The separation was eluted via H_2O as eluent (A) and trifloroacetic acid (0.02%) in acetonitrile as eluent (B) with 1 mL min−1 flowing rate. The elution was performed

automatically with following step gradient: eluents (A): (B) (4:1, 0–5 min), eluents (A): (B) (3:2, 5–8 min), eluents (A): (B) (1:1, 8–12 min), eluents (A): (B) (2:3, 12–14 min), and eluents (A): (B) (1:4, 14–16 min). The detection of the multi-wavelength was carried out at 280 nm. A 10 µL from each plant extract solution was injected at 35 °C as column temperature.

2.4. Assessment of the acute toxicity of the two plant extracts

According to the OECD guideline 4235 [15], acute toxicity assessments of the two plant extracts were conducted using 56 Swiss albino mice divided into 7 groups of eight animals each. A single dose of 1 g/kg BW was given as a starting dose and then raised to 6 g/kg BW was supplied orally to groups 1 and 2 from the aqueous solution of ethanolic extract of *D. aegyptium*. Groups 3 and 4 were given a water solution of ethanolic extract of *P. incurva* orally using the same method. The rats in the control group (group 5) were given distilled water orally by the same gradient of the extracts. For 48 hours, overall, the mice were observed for mortality, clinical changes in all bodies, and their behavioral patterns.

2.5. Animals

Forty-Two adult male albino rats obtained from Laboratory Animal House, National Research Centre, Giza, Egypt, weighing 120 -130 g were utilized in this study. The animals were kept in stainless steel group cages at 22 ± 2ºC with a 12hr/light/dark cycle and were given a week to acclimate before being used in the experiment. During the investigation, all rats were given an unrestricted supply of food (rats nutritional pellets made by Cairo Company of Oil & Soap, Egypt) and water was allowed ad libitum. All rats were treated in accordance to the approved guide for the treatment and management of experimental animals of National Research Centre, Giza, Egypt.

2.6. Experimental Design

The rats were separated into six groups of seven rats each and given the following treatments for 28 days:

Group I: rats were given drinking water orally through a gastric tube for 28 days and served as control.

Group II: rats were given NaF orally by a gastric tube dissolved in distilled water at dose (5mg/kg) for 28 days [13].

Group III: rats were orally administered *D. aegyptium* extract alone at dose (100 mg/kg, 2% of LD_{50}) for 28 days.

Group IV: rats were orally administered *P. incurva* extract alone at dose (100 mg/kg, 1.7% of LD_{50}) for 28 days.

Group V: rats were co- administered with NaF at dose (5 mg/kg), and *D. aegyptium* extract at dose (100 mg/kg) for 28 days.

Group VI: rats were co- administered with NaF at dose (5 mg/kg), and *P. incurva* extract at dose (100 mg/kg) for 28 days.

2.7. Biochemical study

Retro-orbital blood samples were taken in capillary tubes and were centrifuged at 3000 rpm for 20 minutes. Clear sera were collected into tiny glass tubes and kept at 20°C until they were analyzed. The serum was tested for the following markers:

(1) Hormonal assay was performed to assess serum levels of T3, T4 using commercially available Chemiluminescence Immunoassay (LifeSpan BioSciences, Inc., Seattle, USA) and thyroid-stimulating hormone (TSH) using Rat ELISA Kit (Cusabio Technology LLC, Houston, Texas) [16].(2) Lipid peroxidation marker malondialdehyde (MDA) was determined colorimetrically (absorbance 534 nm) with a commonly produced kit (Biodiagnostic, Dokki, Giza, Egypt) [17].

(3) Glutathione (GSH) was determined colorimetrically (absorbance 405 nm) with an available commercial kit (Biodiagnostic, Dokki, Giza, Egypt) [18].

(4) Superoxide dismutase (SOD) was measured colorimetrically (absorbance 560 nm) with a commercially available kit (Biodiagnostic, Dokki, Giza, Egypt) [19].

(5) Calcium $(Ca)^{2+}$ was determined colorimetrically (absorbance 560 nm) with a commercially available kit (Biodiagnostic, Dokki, Giza, Egypt) [20].

2.8. Histological Study

At the end of experiment, all animals were sacrificed and their thyroid glands were removed. Right-sided thyroids lobes were fixed in 10% formal saline for 24 hours, then dehydrated in ascending grades of alcohol, cleared with xylene, and embedded into paraffin. Then 4–5 µm sections were cut, and hematoxylin and eosin (H & E) staining was performed [21].

2.9. Histochemical Studies

Paraffin sections 5 microns thickness stained with Periodic Acid Shiff's (PAS) reagent to demonstrate total carbohydrates [22], Mercury Bromophenol Blue to demonstrate total protein [23] and Feulgen to demonstrate DNA content in thyroid follicles [24].

2.10. Immunohistochemical Study

For anti-calcitonin immunostaining, the paraffin sections were processed using the streptavidin–biotin complex (Strep ABC) immunohistochemical procedure according to the manufacturer's

instructions. The sections of 4 µm paraffin sections were deparaffinized in xylene and rehydrated in a descending series of ethanol. The specimens were microwaved for 10 minutes for antigen retrieval in a citrate buffered solution (pH 6.0). Endogenous peroxidase was removed by incubating for 10 minutes in10% H_2O_2 in PBS (pH 7.4). After washing, the specimens were blocked for 20 minutes at room temperature in ready-to-use normal goat serum. The sections were incubated with calcitonin antibody-2 (Rabbit Polyclonal Antibody) (Sigma Aldrich Co., Cairo, Egypt). Antibodies diluents were used to dilute primary antisera (1:10000). An AEC (3-amino-9-ethyl carbazole) substrate kit (TA-004-HAC; Labvision) was used to assess peroxidase activity. The sections were rinsed in PBS. When the primary antibody was replaced with PBS, a negative control was obtained. Mayer's hematoxylin was used as a counterstain for the sections [25].

2.11. Histomorphometric Evaluaion and Image Analysis

To obtain the histomorphometric data in this study, five non-overlapping randomly-selected fields (100x) in each slide were examined via Leica Qwin 500 Image Analyzer Computer System (England)., All digital images were analyzed via semi-quantitative scoring system (Image J software, Java based application for analyzing images) at image analysis unit, Pathology Department, National Research Centre, Giza, Egypt, and the average was recorded to calculate each of the following:

1- Measurement of the follicular diameter in H&E stained sections.

2- The mean area percentage (%) of PAS stained sections.

3- The mean area percentage (%) of Mercury Bromophenol Blue stained sections.

4- The mean area percentage (%) of Feulgen stained sections.

5- The mean area percentage (%) of Calcitonin stained sections.

Such data were subjected to bio statistical analysis.

2.12. Scanning Electron Microscope Study

Left-sided thyroid lobes were fixed for 4 hours in a solution of 4% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.3), then post-fixed for 2 hours in osmium tetraoxide O_sO_4, and finally washed three times in the same buffer (sodium cacodylate buffer).The samples were dehydrated using a graduated ethanol series from 10 to 100%, 10 minutes in each one except the finely one 100% for 30 minutes for three changes (each one for 10 minutes). After that, it was dehydrated using a critical point dry instrument and liquid carbon dioxide CO_2. The specimens were mounted on cupper stub with double- sided sticky tape, coated with gold S150A sputter coater –Edwards- England. The specimen was studied with a scanning electron microscope (Quanta FEG 250, Europe union) at Electron Microscopic Unit, National Research Centre, Giza, Egypt [26].

2.13. Statistical analysis

Statistics were estimated using Graph Pad Prism 8 software. Data were represented as mean ± SEM. Differences between means were estimated by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons across the distinct studied groups. Significant differences are considered as $p < 0.05$. The normality test was performed using Shapiro-Wilk Test and the data was normally distributed.

3. Results

3.1. Acute Toxicity

The single dose of *D. aegyptium* EtOH extract (1–2 g/kg BW) in the first two days and for the next two weeks did not cause any mortality in the mice compared to the control negative group (Table 1). Between 3 and 6 g/kg/day, mortality was seen when the extract was administered orally. At a dosage of 5 g/kg/day, 50% of the mice were killed (LD_{50}). On the other hand, at concentrations of 4-6 g/kg/day of *P. incurva* EtOH extract, mice began to die. However, over the first two days, the LD_{50} dose that killed 50% of the mice was just 6 g/kg (Table 1) [27].

Table (1): Toxicological results of the ethanolic extracts of *D. aegyptium* and *P. incurva*.

	Mortality during 48 h	
Dose/Day	*D. aegyptium*	*P. incurva*
1 g/kg	0/8	0/8
2 g/kg	0/8	0/8
3 g/kg	1/8	0/8
4 g/kg	2/8	1/8
5 g/kg	4/8	2/8
6 g/kg	4/8	4/8
Dist. H_2O	0/8	0/8

3.2. High Performance Liquid Chromatography (HPLC) Analysis

The ethanolic extract of *D. aegyptium* was subjected to the HPLC analysis under the conditions listed in experimental section. Present results exhibited that the ethanolic extract of this plant is very rich with flavonoids, phenolic acids and phenolic derivatives **(Figure 1 A).** All identified compounds were summarized in **Table (2)** with the concentration of each compound by µg/g. Fourteen compounds were identified including 8 phenolic acids, 2 phenolic derivatives, and 4 flavonoids. The present study is the first chemical evaluation of *P. incurva* aerial parts. Present findings showed that the phenolic constituents including flavonoids, phenolic acids and phenolic derivatives are present in the ethanolic extract of this plant with significant concentrations **(Figure 1B and Table 2).** Thirteen phenolic components comprising 5 phenolic acids, 3 phenolic derivatives, and 5 flavonoids were characterized.

Table (2): Phenolic compounds of *D. aegyptium* and *P. incurva* extracts derived *via* HPLC.

No	Rt	Compound name	Conc. (µg/g)*	
			D. aegyptium	*P. incurve*
Phenolic acids				
1	3.114	Gallic acid	4042.41±211.4	6473.36±116.7
2	3.441	Chlorogenic acid	1218.67±109.2	--------
3	4.798	Caffeic acid	206.71±34.6	558.07±68.9
4	5.195	Syringic acid	339.94±41.5	--------
5	6.655	Ellagic acid	946.05±62.7	3282.44±104.6
6	7.453	Coumaric acid	379.34±52.1	134.42±28.7
7	9.329	Ferulic acid	275.60±21.2	--------
8	11.201	Cinnamic acid	280.32±13.3	322.79±39.8
Phenolic acid derivatives				
9	3.893	Caffeine	--------	507.55±81.8
10	8.050	Vanillin	358.54±18.4	224.21±58.1
11	10.442	Propyl gallate	872.49±67.8	895.59±83.2
Flavonoids				
12	3.678	Catechin	6583.44±196.1	7451.42±184.5
13	5.496	Rutin	--------	3508.09±123.7
14	10.221	Naringenin	16391.16±87.6	2210.25±151.4
15	10.628	4`,7-Dihydroxy isoflavone	454.05±48.2	825.37±95.1
16	11.118	Querectin	8553.45±163.1	3304.08±139.3

*average concentration of two replications ± standard deviation

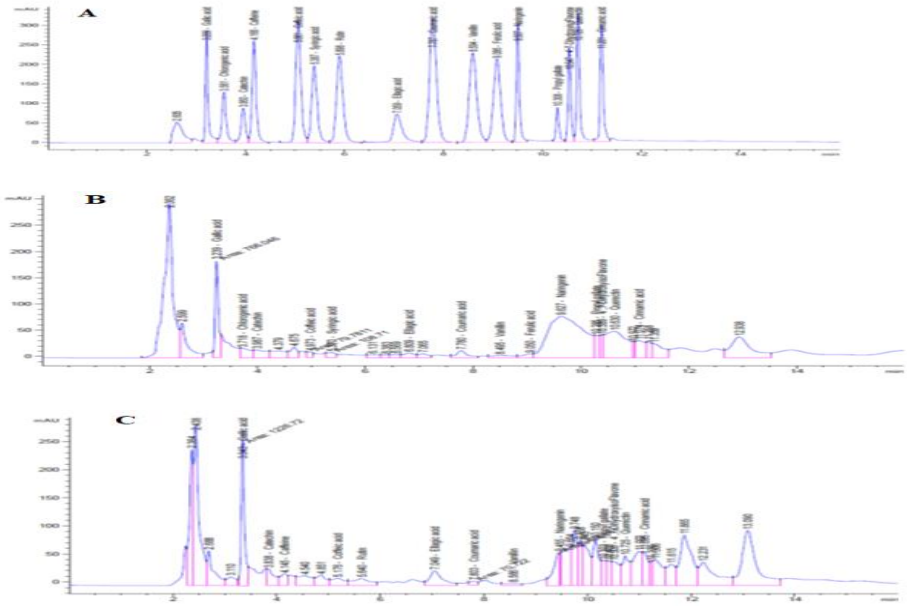

Figure (1): Shows HPLC chromatograms of A) EtOH extract of *Dactyloctenium aegyptium*, B) mixture of the 17 standard compounds and C) EtOH extract of *Parapholis incurva*

3.3. Biochemical results

3.3.1. Serum T3, T4 and TSH

The Na–F-treated group showed a significant decrease in serum T3 (-52%) at *P< 0.0001* compared with the control group. *D. aegyptium* extract treated group showed a significant decrease (-19.7%) in serum T3 at *P< 0.05* when compared with control group. *P. incurva* extract treated group showed no significant difference (-10.9%) in serum T3 level as compared to control. NaF-treated rats that received *D. aegyptium* extract showed a significant decrease in serum T3 at *P< 0.0001* when compared with control group and significant increase (50%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received *P. incurva* extract showed a significant decrease in Serum T3 at *P< 0.0001* when compared to control group and significant increase (49%) at *P< 0.0001* when compared with NaF group. There are no significant differences among NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 2a).**

Regarding T4 hormone, Na–F-treated group showed a significant decrease in serum T4 (-45.7%) at *P< 0.0001* compared with the control group**.** *D. aegyptium* extract treated group showed a significant decrease (-16%) in serum T3 at *P< 0.05* when compared with control group. *P. incurva* extract treated group showed significant decrease (-9.5%) in serum T3 at *P< 0.05* when compared with control group. NaF-treated rats that received *D. aegyptium* extract showed a significant decrease at *P< 0.001* in serum T4 when compared with control group and a significant increase (62.2%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* extract showed a significant decrease at *P< 0.0001* in serum T4 when compared with control and a significant increase (60%) at *P< 0.0001* when compared with NaF group. There are no significant differences among NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 2b).**

The Na–F-treated group showed a significant increase in serum TSH level (160.78%) at *P< 0.0001* compared with the control group**.** *D. aegyptium* or *P. incurva* extract treated group showed a significant increase (80.4% and 90.2%) in serum TSH at *P< 0.05* when compared with control group. NaF-treated rats that received *D. aegyptium* extract showed a significant increase in serum TSH at *P< 0.0001* compared with control group and significant decrease (-48%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* extract showed a significant increase in serum TSH at *P< 0.0001* compared with control group and significant decrease (-47.2%) at *P< 0.0001* compared to NaF group. There were no significance differences between NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 2c).**

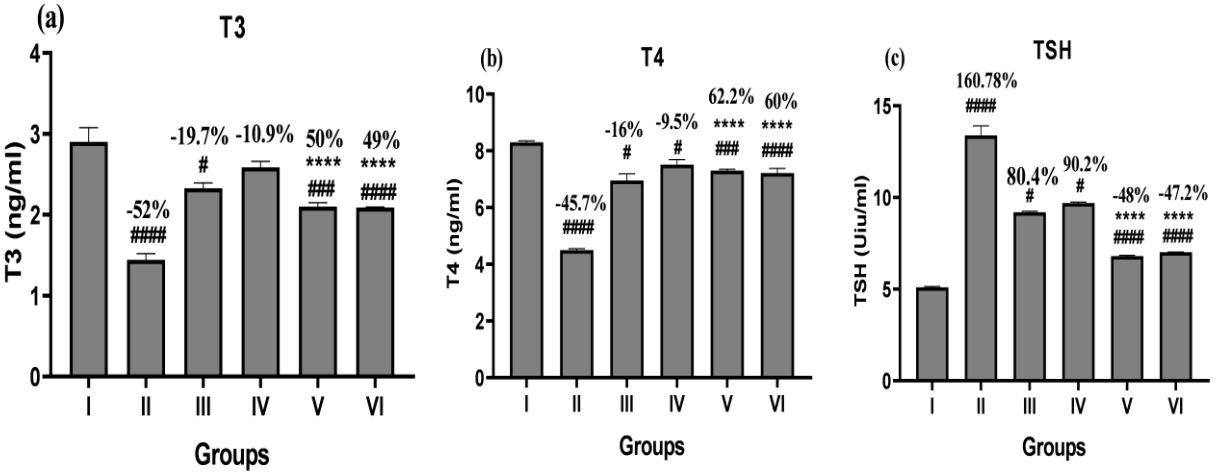

Figure (2): Shows changes in T3, T4 and TSH hormone concentrations following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#}$ *P< 0.05*; $^{\#\#\#}$ *P< 0.001*; $^{\#\#\#\#}$ *P< 0.*0001 compared to control group. **** *P< 0.0001* compared to NaF treated group.

I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group; **V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

3.3.2. Serum MDA

The Na–F-treated group showed a significant increase in serum MDA level (283%) at *P< 0.0001* when compared with the control group. *D. aegyptium* or *P. incurva* extract treated group showed a significant increase (95% and 110.9%) in serum MDA level at *P< 0.05* when compared with

control group. NaF-treated rats that received ***D.*** *aegyptium* extract showed significant increase in serum MDA level at *P< 0.0001* level as compared to control group and significant decrease (-48%) at *P< 0.0001* when compared with NaF group, while NaF-treated rats that received ***P.*** *incurva* extract showed no significant differences when compared with control group with significant decrease in serum MDA level (-27%) at *P< 0.0001* in serum MDA level when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* showed significant increase in MDA level (41%) at *P< 0.0001* compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 3a).**

3.3.3. Serum GSH and SOD

The NaF-treated group showed a significant decrease in serum GSH level (-60.6%) at *P< 0.0001* when compared with the control group. *D. aegyptium* or *P. incurva* extract treated group showed a significant decrease (-10.8%) and (-30.2%) in serum GSH at *P< 0.05* when compared with control group. NaF-treated rats that received ***D.*** *aegyptium* extract showed significant decrease in serum GSH level at *P< 0.0001* compared to control group and significant increase (64%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* extract showed no significant difference was recorded as compared to control group with significant increase in serum GSH level (156%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* showed a significant increase in serum GSH level (55.6%) at *P< 0.0001* as compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 3b).**

Regarding serum SOD, NaF-treated group showed a significant decrease in serum SOD level (-55%) at *P< 0.0001* when compared with the control group. *D. aegyptium* extract treated group showed no significant difference (-27.8%) in serum SOD when compared with control group. *P. incurva* extract treated group showed a significant decrease (-33%) in serum SOD level at *P< 0.05* when compared with control group. NaF-treated rats that received ***D.*** *aegyptium* extract showed significant decrease at in serum SOD level *P< 0.0001* as compared to control group with no significant difference as compared to NaF group. NaF-treated rats that received ***P.*** *incurva* extract showed significant decrease in serum SOD level at *P< 0.05* when compared with the control group and significant increase (58%) at *P< 0.05* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* showed increase (50%) in serum SOD level as compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 3c).**

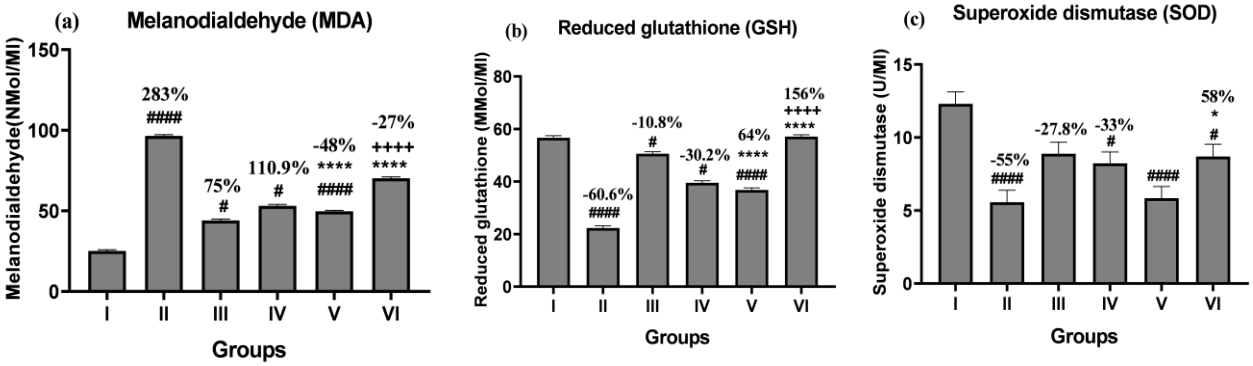

Figure (3): Shows changes in serum MDA, GSH and SOD concentrations following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#}P< 0.05$; $^{\#\#\#\#}P< 0.0001$ compared to control group. $^{*}P< 0.05$; $^{****}P< 0.0001$ compared to NaF treated group. $^{++++}P< 0.0001$ compared to NaF + ***D.*** *aegyptium* treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group; **V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

3.3.4. Serum calcium

The Na–F-treated group showed a significant increase in serum calcium level (198.9%) at *P< 0.0001* compared with the control group. *D. aegyptium* or *P. incurva* extract treated group showed no significant difference (-13.7% and -27.2%) in serum calcium level when compared with control group. NaF-treated rats that received ***D.*** *aegyptium* showed no significant difference as compared to control with significant decrease in serum calcium (-79%) at *P< 0.0001* when compared with NaF group. NaF-treated rats that received ***P.*** *incurva* showed no significant difference as compared to control with significant decrease in serum calcium level (-76%) at *P< 0.0001* when compared with NaF group. NaF-treated rats There were no significance differences between NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 4).**

3.4. Histological and histomorphometric results:

Light microscopic examination of sections of thyroid glands of control rats showed normal histological structure of thyroid gland. The thyroid gland formed of multiple follicles filled with acidophilic homogenous colloid. The lining follicular cells were flattened to cuboidal in shape with oval to rounded pale nuclei. Few numbers of parafollicular cells and blood capillaries were observed in between the follicles **(Figure 5A).** Examination of sections of thyroid glands obtained from NaF-treated rats showed many empty follicles lined by flat cells with apoptotic epithelial lining and intra-follicular desquamated cells, and excess stroma with large areas of hemorrhage **(Figure 5B)**. Disrupted basement membrane with fusion of the lumina and mildly congested blood vessels were also observed **(Figure 5C)**. Follicle diameter was significantly decreased (-15%) at $p<0.05$ as compared to control group **(Figure 6)**. Microscopic examination of sections of thyroid glands obtained from rats treated with *D. aegyptium* extract, or with *P. incurva* extract revealed the normal histoarchitecture of thyroid tissues and no histopathological alterations were recorded **(Figures 5D and 5E).** *D. aegyptium* extract, or *P. incurva* extract groups also showed no significant difference in follicle diameter as compared to control group. No significant difference in follicle size was recorded between *D. aegyptium* and *P. incurva* groups **(Figure 6)**.

Examination of sections of thyroid glands of NaF-treated rats that received ***D. aegyptium*** extract showed restoration of the normal histological structure. Average follicles lined by cuboidal epithelial cells and filled completely with bright eosinophilic colloid were observed **(Figures 5F).** NaF-treated rats that received ***D. aegyptium*** group showed no significant difference in follicle diameter as compared to control group **(Figure 6)**.

Histopathological examination of sections of thyroid glands of NaF treated rats that received ***P. incurva*** extract showed marked improvement of thyroid histological structure. Average follicles were lined by cuboidal cells and filled completely with bright eosinophilic colloid as compared to NaF treated group **(Figure 5G)**. Moreover, NaF and ***P. incurva*** cotreated group showed significant increase (26%) at $p< 0.05$ in follicle diameter as compared to NaF treated group with no significant difference as compared to control group. NaF-treated rats that received ***P. incurva*** showed increase (14%) in follicle diameter compared to NaF-treated rats that received ***D. aegyptium*** **(Figure 6).** No significant difference in follicle diameter was observed between NaF-treated rats that received ***D. aegyptium*** and NaF-treated rats that received ***P. incurva*** **(Figure 6).**

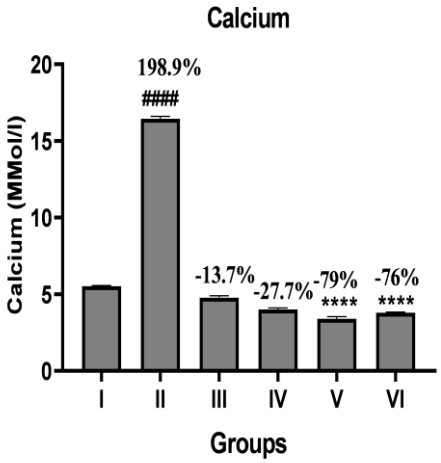

Figure (4): Shows changes in serum Calcium concentrations following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#\#\#\#}P< 0.0001$ compared to control group. $^{****}P< 0.0001$ compared to NaF treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group; **V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

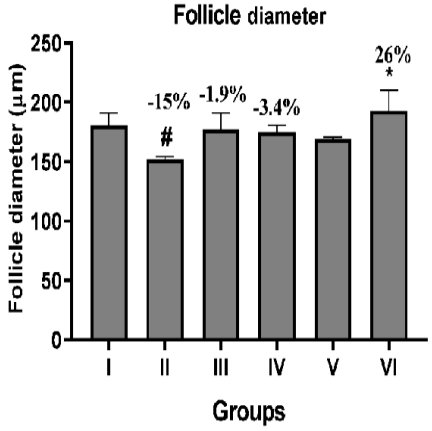

Figure (6): Shows changes in thyroid follicle diameter following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#}P< 0.01$ compared to control group. $^{*}P< 0.05$ compared to NaF treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group**; V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

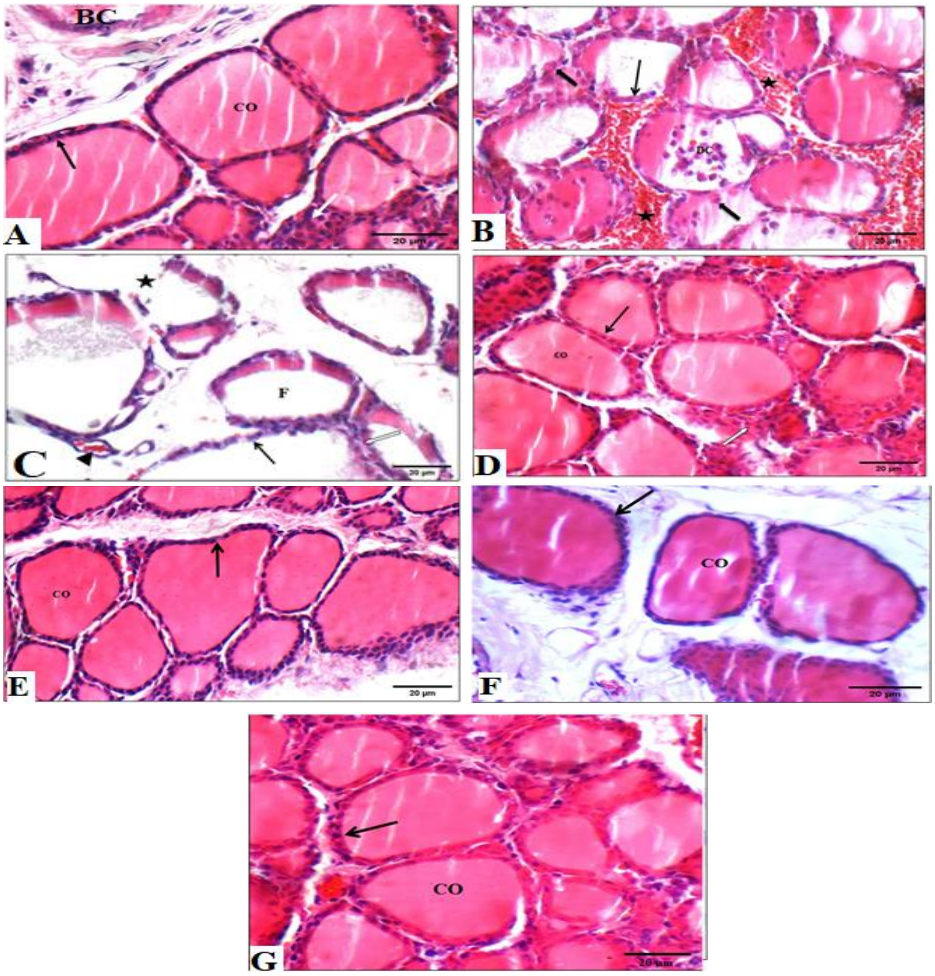

Figure (5): Light microscope photomicrographs showing histological alterations in thyroid gland of different experimental groups (H&E stain, scale bar: 20 µm). **(A)** Section from a control rat showing normal follicles with cuboidal epithelial lining (black arrow) filled completely with bright eosinophilic colloid (co), parafollicular cells (whit arrow) and blood capillaries (BC). **(B)** Section from a NaF treated rat showing many empty follicles lined by flat cells (black arrow) with apoptotic epithelial lining (thick arrow), intra-follicular desquamated cells (red arrow), and hemorrhage (stars). **(C)** Section from another NaF treated rat showing completely empty follicles (F) with apoptotic epithelial lining (white arrow), disrupted basement membrane with fusion of the lumina (star) and excess stroma with mildly congested blood vessels (arrow head). **(D)** Section from ***D.*** *aegyptium* treated rat showing the normal thyroid tissues with normal follicles (black arrow) filled completely with colloid (CO), and normal para-follicular cells (white arrow). **(E)** Section from ***P.*** *incurva* treated rat showing average-sized follicles (arrow) and filled completely by colloid (CO). **(F)** Section from of NaF treated rat that received ***D.*** *aegyptium* showing average follicles (arrow) and filled completely with colloid (CO). **(G)** Section from of NaF treated rat that received ***P.*** *incurva* showing average-sized follicles (arrow) filled completely with colloid (CO).

3.5. Histochemical results

3.5.1. PAS reaction

Microscopic examination of sections of thyroid glands of control rats showed follicles filled completely by dense magenta-stained positive colloid for PAS, and strong PAS reaction in thyroid follicular wall, and within parafollicular cells **(Figure 7A)**. Examination of sections of thyroid glands of NaF-treated rats showed a weak PAS reaction in colloidal material and inbetween thyroid follicles. Analyses of digital images revealed a significant decrease (-40.3%) at $P< 0.0001$ in PAS-positive materials (%) area by comparing with control group **(Figures 7B and 8)**. Microscopic examination of sections of thyroid glands of rats treated with ***D.*** *aegyptium* extract showed a strong PAS reaction in colloid contents, but data obtained from digital images analyses recorded a significant decrease at $p< 0.01$ in PAS-positive materials (%) area as compared to control group **(Figures 7C and 8)**. Histochemical

examination of sections of thyroid glands of rats treated with ***P.*** *incurva* extract, or with *P. incurva* extract showed normal follicles filled completely by dense magenta-stained positive colloidal materials, with insignificant statistically difference as compared to control group **(Figures 7D and 8)**. **Moreover,** insignificant difference was recorded when comparing and analyzing digital images obtained from *D. aegyptium* and *P. incurva* groups **(Figure 8)**. Examination of sections of thyroid glands of NaF-treated rats that received ***D.*** *aegyptium* extract showed follicles filled completely by moderate magenta-stained positive colloid for PAS with significant increase (40%) at *p< 0.0001* in PAS-positive materials (%) area as compared to NaF treated group **(Figures 7E and 8).** Thickness of follicle walls, colloid and thickness between follicles were observed **(Figures 7E)**.

. Microscopic examination of sections of thyroid glands of NaF treated rats that received ***P.*** *incurva* extract showed strong PAS reaction in colloid with significant increase (51.4%) at *p< 0.0001* in PAS-positive materials (%) area as compared to NaF treated group **(Figures 7F and 8)**. Thickness of follicle walls, colloid and thickness between follicles were decreased **(Figure 7F)**. NaF-treated rats that received ***P.*** *incurva* showed increase (9.3%) in PAS-positive materials (%) area compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 8).**

No significant difference was recorded between NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 8)**.

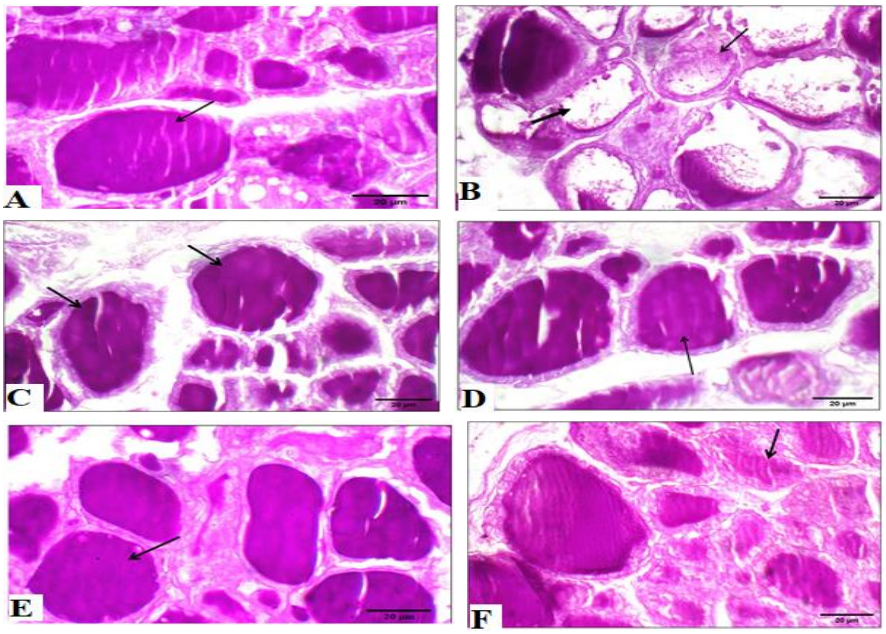

Figure (7): Light microscope photomicrographs showing histochemical alterations in thyroid gland tissues of different experimental groups (PAS stain, scale bar: 20 µm). **(A)** Section from control group showing follicles filled completely by dense magenta-stained positive colloid for PAS (arrows). **(B)** Section from NaF treated group showing many empty follicles (arrow) and others filled incompletely by faint magenta-stained positive colloid for PAS. **(C)** Section from ***D.*** *aegyptium* treated group showing follicles filled completely by dense magenta-stained positive colloid for PAS. **(D)** Section from ***P.*** *incurva* treated group showing follicles filled completely by dense magenta-stained positive colloid with scattered peripheral vacuoles (arrow). **(E)** Section from of NaF treated rat that received ***D.*** *aegyptium* showing follicles filled completely by dense magenta-stained positive colloid for PAS with (arrow). Thickness of follicle walls, colloid and thickness between follicles are increased. **(F)** Section from of NaF treated rat that received ***P.*** *incurve* showing follicles filled completely by dense magenta-stained positive colloid for PAS. Thickness of follicle walls, colloid and thickness between follicles are decreased.

3.5.2. Total protein

Microscopic examination of sections of thyroid glands of control rats showed normal distribution of total protein in form of deeply stained fine blue granules in most of follicular and para-follicular cells **(Figure 9A).** Examination of sections of thyroid glands of NaF-treated rats showed faint blue granules in most of follicular and para-follicular cells with significant decrease at *P< 0.0001*(-23.28%) in total protein (%) stained area compared with the control group **(Figures 9B and 10)**. Sections of thyroid glands of rats treated with ***D.*** *aegyptium* or with ***P.*** *incurva* showed deeply stained blue granules in most of follicular and para-follicular cells with insignificant difference as compared to control group **(Figures 9C, D and 10)**. There was no No significant

difference recorded between *D. aegyptium* and *P. incurva* groups **(Figure 10)**.

Examination of sections of thyroid glands of NaF-treated rats that received ***D.*** *aegyptium* extract showed deeply stained blue granules in few follicular and para-follicular cells with significant increase (16%) at *P< 0.001* in total protein (%) area compared with NaF treated group and significant decrease at *P< 0.001* in total protein (%) area compared with the control group **(Figures 9E and 10)**. Histochemical examination of sections of thyroid glands of NaF treated rats that received ***P.*** *incurva* extract showed strong reaction as indicated by dense blue stain and significant increase (24.3%) at *P< 0.0001*(%) area in total protein compared with NaF treated group and No significant difference as compared to control group **(Figures 9F and 10)**. NaF-treated rats that received ***P.*** *incurva* showed a significant increase (7%) at *P< 0.05* area (%) in total protein as compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 10)**.

3.5.3.DNA content

Microscopic examination of sections of thyroid glands of control rats showed normal distribution of DNA content in nuclei of follicular cells and parafollicular cells **(Figure 11A)**, while sections of thyroid glands of NaF-treated rats showed mild positivity for Feulgen in nuclei of follicular cells with significant decrease (-33%) at *P< 0.0001* in DNA stained area (%) compared to control group **(Figures 11B and 12)**. Microscopic examination of sections of thyroid glands of rats treated with ***D.*** *aegyptium* or ***P.*** *incurva* extracts showed strong positivity for Feulgen in nuclei of follicular cells and parafollicular cells **(Figures 11C and D)**. There was no significant difference recorded between *D. aegyptium* and *P. incurva* groups **(Figure 12).**

Examination of sections of thyroid glands of NaF-treated rat that received ***D.*** *aegyptium* extract showed marked positivity for Feulgen in nuclei of follicular cells and parafollicular cells with significant increase (22.4%) at *p< 0.0001* in DNA stained area (%) as compared to NaF treated group and significant decrease at *p< 0.0001* as compared to control group **(Figures 11E and 12)**. Microscopic examination of sections of thyroid glands of NaF treated rats that received ***P.*** *incurva* extract showed showing strong reaction for Feulgen in nuclei of follicular cells and parafollicular cells with significant increase (35.9%) at *p< 0.0001* in DNA stained area (%) as compared to NaF treated group and significant decrease at *p< 0.05* as compared to control group **(Figures 11F and 12)**. NaF-treated rats that received ***P.*** *incurva* showed a significant increase (10%) at *P< 0.05* in DNA content (%) area as compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 12).**

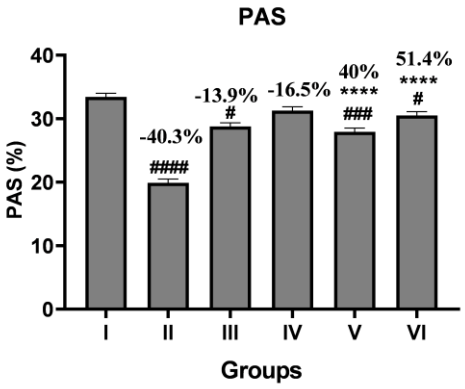

Figure (8): Shows changes in the in PAS-positive materials (%) following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group.. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#}P< 0.01$; $^{\#\#\#}P< 0.001$; $^{\#\#\#\#}P< 0.0001$ compared to control group. $^{****}P< 0.0001$ compared to NaF treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group**; V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

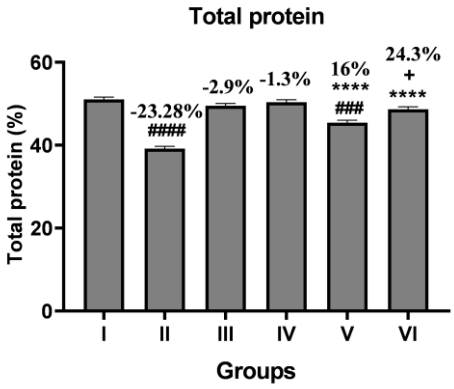

Figure (10): Shows changes in total protein stained area (%) following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. $^{###}P< 0.001$; $^{####}P< 0.0001$ compared to control group. $^{****}P< 0.0001$ compared to NaF treated group. $^{+}P< 0.05$ compared to NaF + ***D.*** *aegyptium* treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group**; V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

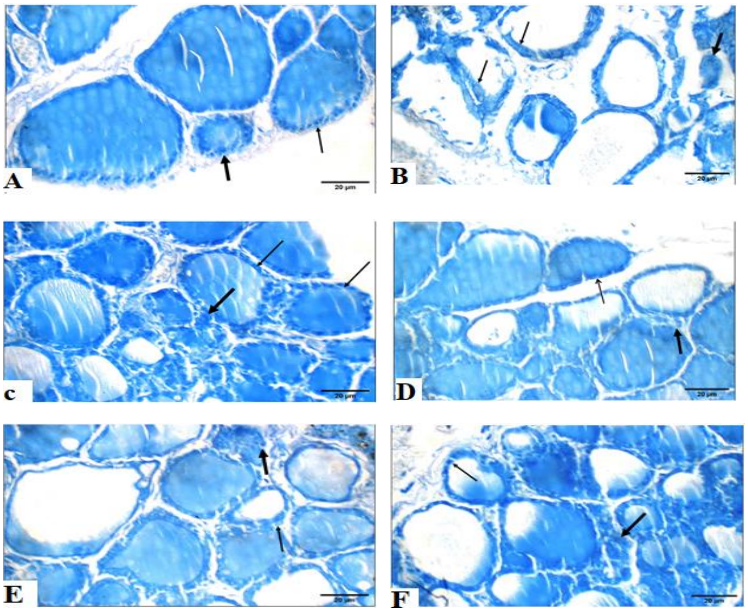

Figure (9): Light microscope photomicrographs showing histochemical alterations in thyroid gland tissues of different experimental groups **(Bromophenol blue stain, scale bar: 20 µm). (A)** Section from control group showing deeply stained fine blue granules in most of follicular (thin arrow) and para-follicular cells (thick arrow). **(B)** Section from NaF treated group showing faint blue granules in most of follicular (thin arrows) and para-follicular cells (thick arrow). **(C)** Section from ***D.*** *aegyptium* treated group showing deeply stained fine blue granules in most of follicular (thin arrow) and para-follicular cells (thick arrow). **(D)** Section from ***P.*** *incurva* treated group showing strong reaction in most of follicular (thin arrow) and para-follicular cells (thick arrow) (arrow). **(E)** Section from of NaF treated rat that received ***D.*** *aegyptium* showing increase in protein content in most follicular (thin arrow) and para-follicular cells (thick arrow) as indicated by dense blue stain. **(F)** Section from of NaF treated rat that received ***P.*** *incurva* showing marked increase in protein content in most follicular (thin arrow) and para-follicular cells (thick arrow).

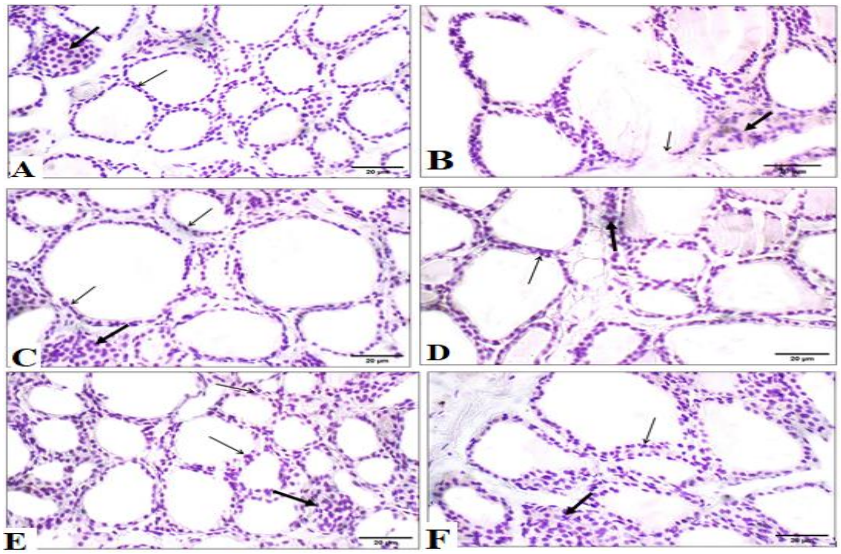

Figure (11): Light microscope photomicrographs showing histochemical alterations in thyroid gland tissues of different experimental groups **(Feulgen stain, scale bar: 20 µm). (A)** Section from control group showing marked positivity for Feulgen with average DNA distribution in nuclei of follicular cells (thin arrow) and parafollicular cells (thick arrow). **(B)** Section from NaF treated group showing mild positivity for Feulgen in nuclei of follicular cells (thin arrow) and parafollicular cells (thick arrow). **(C)** Section from ***D.*** *aegyptium* treated group showing marked positivity for Feulgen in nuclei of follicular cells (thin arrows) and parafollicular cells (thick arrow). **(D)** Section from ***P.*** *incurva* treated group showing strong reaction for feulgen in nuclei of follicular cells (thin arrow) and parafollicular cells (thick arrow). **(E)** Section from of NaF treated rat that received ***D.*** *aegyptium* showing increase in DNA content in Feulgen in nuclei of follicular cells (thin arrow) and parafollicular cells (thick arrow). **(F)** Section from of NaF treated rat that received ***P.*** *incurva* showing marked increase in DNA content in nuclei of follicular cells (thin arrow) and parafollicular cells (thick arrow).

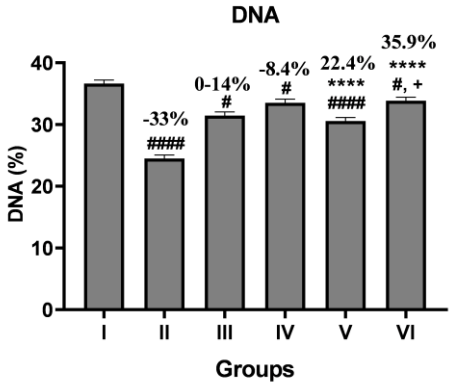

Figure (12): Shows changes in DNA stained area (%) following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group$^{\#\#\#\#}P< 0.0001$ compared to control group. $^{****}P< 0.0001$ compared to NaF treated group. $^{+}P< 0.05$ compared to NaF + ***D.*** *aegyptium* treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group**; V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

3.6. Immunohistochemical demonstration of Calcitonin

Microscopic examination of sections of thyroid glands of control rats showed weak cytoplasmic reactivity for calcitonin in para-follicular cells **(Figure 13A)**. Examination of sections of thyroid glands of NaF-treated rats showed strong cytoplasmic reactivity for calcitonin in para-follicular cells with significant increase (38.7%) *at P< 0.0001* in calcitonin (%) area compared to control group **(Figures 13B and 14)**. Microscopic examination of sections of thyroid glands of rats treated with ***D.*** *aegyptium* or with ***P.*** *incurva* showed weak cytoplasmic reactivity for calcitonin in para-follicular cells with no significant difference as compared to control group **(Figures 13C, D and 14)**. No significant difference was recorded *D. aegyptium* and *P. incurva* groups **(Figure 14).** Examination of sections of thyroid glands of NaF-treated rat that received ***D.*** *aegyptium* extract showed moderate cytoplasmic reactivity for calcitonin in para-follicular cells with significant decrease (-42.9%) in calcitonin stained area (%) at *p< 0.0001* compared to NaF treated group **(Figures 13E and 14)**. Microscopic examination of sections of thyroid glands of NaF treated rats that received ***P.*** *incurva* extract showed weak cytoplasmic reactivity for calcitonin in para-follicular cells with significant decrease(-38.8%) at *p< 0.0001* in calcitonin stained area (%) as compared to NaF treated group **(Figures 13F and 14)**. NaF-treated rats that received ***P.*** *incurva* showed increase (7.1%) in calcitonin stained area (%) compared to NaF-treated rats that received ***D.*** *aegyptium* **(Figure 13).** No significant difference was observed between

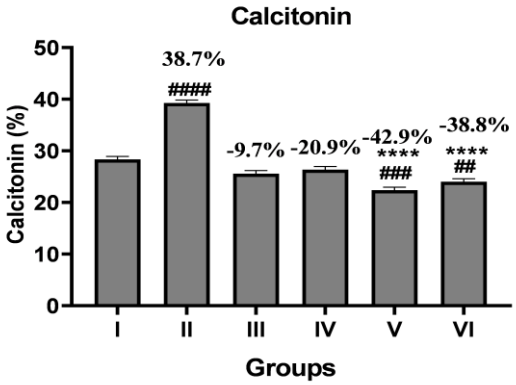

Figure (14): Shows changes in calcitonin stained area (%) following NaF (5mg /Kg), *D. aegyptium* (100 mg / kg) and *P. incurva* (100 mg / kg) treatment for 28 days. The results are expressed as mean ± SEM, n = 7 rats/group. $^{\#\#}P< 0.01$; $^{\#\#\#}P< 0.001$; $^{\#\#\#\#}P< 0.0001$ compared to control group. $^{****}P< 0.0001$ compared to NaF treated group.
I: The control group; **II:** NaF group**; III:** *D. aegyptium* group; **IV:** *P. incurva* group**; V:** NaF + *D. aegyptium* group and **VI:** NaF + *P. incurva* group.

NaF-treated rats that received ***D.*** *aegyptium* and NaF-treated rats that received ***P.*** *incurva* **(Figure 14)**.

3.7. Scanning Electron microscopic observations

Scanning electron microscopic examination of sections of thyroid glands of control rats showed normal ultrastructure of thyroid gland covered by a thick fibrous connective tissue capsule. Parenchyma of thyroid gland was composed of spherical follicles which were separated from each other by interfollicular or interstitial connective tissue made up of collagen and reticular fibers. Fibroblast and parafollicular or "C" cells were present. The lumens of thyroid follicles were filled with gel-like round substances called colloid substances. The colloid particles were uniform and homogenous and size of particles. The follicular epithelium cells of the thyroid gland were squamous to low cuboidal in shape **(Figures 15A and B).** In the present study, there were some irregular follicles observed that can be due to the plane of the section of the follicles or tissue shrinkage. Examination of sections of thyroid glands obtained from rats treated with *D. aegyptium*, or with *P. incurva* revealed normal thyroid ultrastructure, without any histopathological alterations **(Figures 15C and D).** On the other hand, examination of sections of thyroid glands of NaF-treated rats showed marked calcification of colloid of thyroid follicles. Hemorrhage, and increase in number of macrophages as indicator of inflammation were clearly observed. NaF also induced decrease in thyroid follicle size and increased connective tissues between thyroid follicles **(Figures 15E and F).** Microscopic examination of sections of thyroid

glands obtained from NaF groups co-treated with *D. aegyptium* extract, or with *P. incurva* extract revealed the preservation of ultrastructure of thyroid tissues as normal with marked decrease in calcification as compared with NaF treated group. It was shown an increase in colloid in follicular cells again and increase in follicle size as normal **(Figures 15G and H).** However, few red blood cells and macrophages were still observed in *D. aegyptium* treated group by comparing with *P. incurve* treated group **(Figures 15 I and J).**

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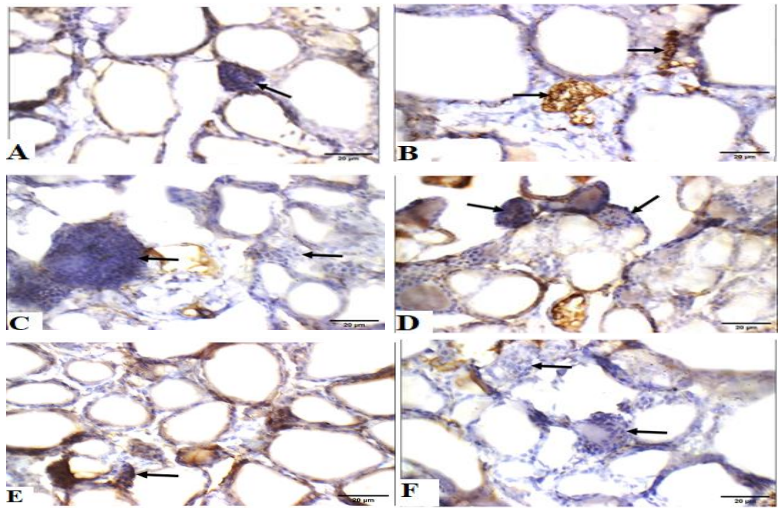

Figure (13): Light microscope photomicrographs showing immun**o**histochemical alterations in thyroid gland tissues of different experimental groups **(Anticalcitonin immunostaining, scale bar: 20 µm). (A)** Section from control group showing showing weak cytoplasmic reactivity for calcitonin in para-follicular cells (arrows). **(B)** Section from NaF treated group showing strong cytoplasmic reactivity for calcitonin in para-follicular cells (arrows). **(C)** Section from ***D. aegyptium*** treated group showing weak cytoplasmic reactivity for calcitonin in para-follicular cells (arrow). **(D)** Section from ***P. incurva*** treated group showing showing weak cytoplasmic reactivity for calcitonin in para-follicular cells (arrows). **(E)** Section from of NaF treated rat that received ***D. aegyptium*** showing decrease in cytoplasmic reactivity for calcitonin in para-follicular cells (arrows). **(F)** Section from of NaF treated rat that received ***P. incurva*** showing decrease in cytoplasmic reactivity for calcitonin in para-follicular cells (arrows).

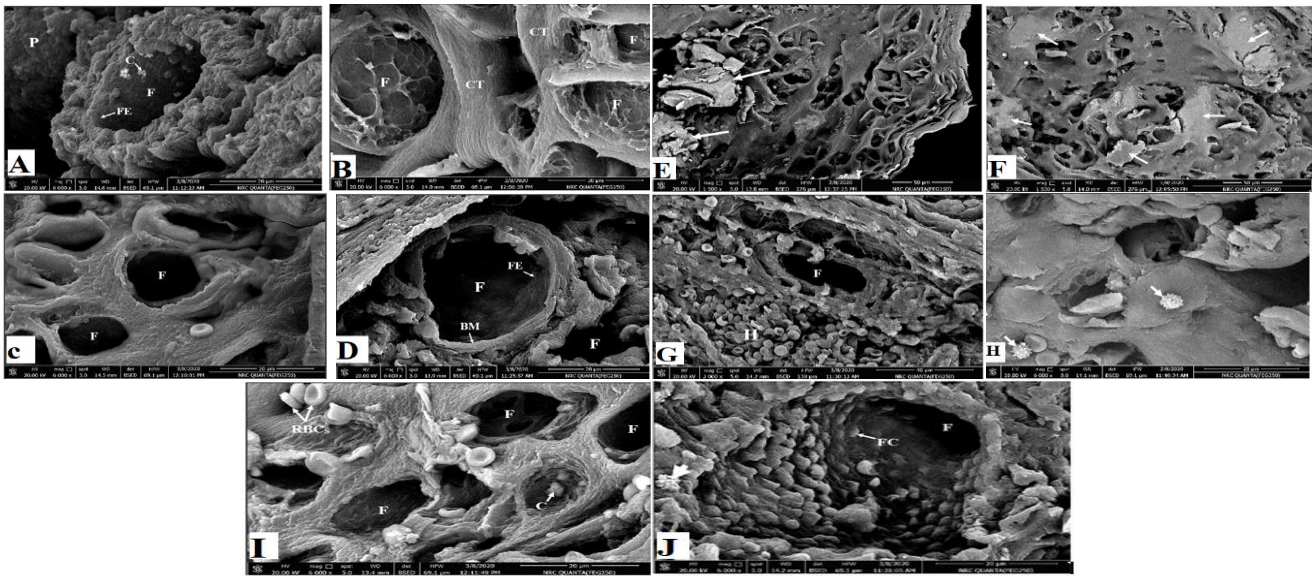

Figure (15): Scanning electron micrographs showing ultra-structural alterations in thyroid gland tissues of different experimental groups**. (A)** Micrograph of control group showing the internal surface of thyroid follicles (F) filled with the colloid particles (C) and lined by follicular epitheilium (FE). Parafollicular cells (P) are present. **(B)** Micrograph of control group showing the external surface of thyroid follicles (F) surrounded by connective tissue fibers (CT). **(C)** Micrograph of ***D. aegyptium*** treated group showing the normal ultrastructure of thyroid follicles (F) as control group. **(D)** Micrograph of ***P. incurva*** group treated showing thyroid follicles (F), follicular epithelium (FE) and basement membrane (BM). **(E&F)** Micrographs of NaF treated rat showing thyroid calcification (arrows). **(G&H)** Micrographs of NaF treated rat showing decrease in follicle size (F), large areas of hemorrhage and macrophages (Arrows). **(I)** Micrograph of NaF treated rat that received ***D. aegyptium*** showing thyroid follicles (F) filled with colloid particles (C).Few red blood cells (RBC_s) are present. **(I)** Micrograph of NaF treated rat that received *P. incurva* showing increase in size of thyroid follicles (F) again as control group. Follicular cells (FC) on the internal surface of thyroid follicles.

4. Discussion

The primary goal of this study was to examine the histological, histochemical, ultrastructural and hormonal alterations in the thyroid gland that might happen following NaF treatment. Furthermore, this study aimed to determine the chemical constituents and the potential protective effects of *D. aegyptium* and *P. incurva* extracts when given in combination with NaF.

In the recent years, Herbal plants have gained popularity as a result of their potent therapeutic properties and low risk of adverse effects. *D. aegyptium* belongs to the Gramineae (Poaceae) family and is widely naturalized throughout the tropics and subtropics. This plant is a highly valuable herb with a variety of medical properties. Furthermore, pharmacological studies revealed that the *D. aegyptium* plant had antioxidant, anti-inflammatory, anticancer, and antipyretic characteristics, as well as gastrointestinal benefits [28].

D. aegyptium (Koreeb) was recorded to have several classes of constituents including flavonoids, tannins, and others [11-28]. Present HPLC results exhibited that the ethanolic extract of this plant is very rich with flavonoids, phenolic acids and phenolic derivatives. The present results showed that querectin, catechin, gallic acid, naringenin and chlorogenic acid are the major compounds, while caffeic acid is the minor compound. These data are in agreement with those published data [11-29].

Chemical and physicochemical assessment of *D. aegyptium* gave the following values (on dry basis): crude protein: 7.25%; fibre: 33.74%; N-free extract: 45.32%; ether extract: 1.23%; total ash: 12.46%; ash solubility in HCl: 8.65%; CaO: 0.91%; P2O7: 0.49%; MgO: 0.70%; Na: 0.074%; and K2O: 3.75% [30]. The plant contained carbohydrates, proteins, amino acids, terpenoids, alkaloids, saponins, tannins, flavonoids, steroids, fixed oils and phenols, according to the phytochemical study [31]. Carbohydrates, proteins, amino acids, saponins, flavonoids, and tannins were found in an aqueous extract. Carbohydrates, proteins, amino acids, saponins, flavonoids, tannins, terpenoids, and alkaloids were found in the hydroalcoholic extract. Several solvents of *D. aegyptium* extracts revealed the significant difference of the output elution [32]. Quantitative investigation indicated that D. aegyptium leaf extract included alkaloids 0.540 ± 0.083, phenols 0.246 ± 0.041, Saponins 1.120 ± 0.047, and tannins 0.430 ± 0.032 mg/g dry weight. Cynogenic glycosides, oxalic acid oxalates, glutamic and aspartic acids, cystine and tyrosine were also found in *D. aegyptium* [33].

The results of previous study by Nagarjuna et al. [32] showed that animals treated with *D. aegyptium* showed significant decreases in blood glucose, HbA1c, and MDA levels, as well as significant increases in insulin, Hb, SOD, catalase, GSH, and body weight. *D. aegyptium* could be used to treat diabetes, according to the findings. Rekha and Shivanna [34] reported that the radical scavenging activity of the crude extract of *D. aegyptium* was assessed by DPPH method. The radical scavenging activity of *D. aegyptium* crude extract was significant (66.59%), but less when compare to standard, ascorbic acid (78.40%).

The present HPLC findings of *P. incurva* ethanolic extract estimated the phenolic constituents including flavonoids, phenolic acids and phenolic derivatives. Thirteen phenolic components comprising 5 phenolic acids, 3 phenolic derivatives, and 5 flavonoids were characterized. Catechin, gallic acid, querectin, ellagic acid and naringenin were assigned as the main compounds while coumaric acid is the minor compound. Our findings were the first chemical profiling of *P. incurva*.

As shown results, rats treated with NaF revealed a significant decrease in the serum levels of T3 (-52%) and T4 (-45.7%) as well as significant increase (160.78%) in TSH levels as compared with the control group. Similar findings were reported by previous studies [13, 35] who reported that NaF intake may cause hypothyroidism. In the current study, NaF caused a significant increase (283%) in MDA and a significant decrease in the levels of serum antioxidant enzymes GSH (-66.6%) and SOD (-55%) in comparison to the control group. These results are in harmony with other reported results [36]. In hypothyroid subjects with oxidative stress, MDA levels were shown to be higher [3]. Excess TSH is known to cause oxidative damage; these results also go with the results of Abdel-Wahhab et al. [37] who reported that MDA levels of the liver and kidney increased significantly in hypothyroid modeled rats.

Administration of *D. aegyptium* extract showed a significant decrease in serum T3 and T4 levels and significant increase in serum TSH level compared to control. Administration of *P. incurva* extract alone showed showed a significant decrease in serum T4 levels and significant increase in serum TSH level compared to control. Treatment with *P. incurva* extract alone had no effect on serum T3 levels.

Treatment with *D. aegyptium* or *P. incurva* extracts following NaF revealed an increase in thyroid hormones while the TSH level decreased (-48%). The results of the current study indicate that *D. aegyptium* or *P. incurve* extracts treatment enhances thyroid function by lowering thyroid hormones and reducing oxidative stress. The two plant extracts are high in antioxidants, group of phenolic compounds

that can improve thyroid hormones; this result is in line with that of Yu et al. [38].

In the current investigation, treatment of NaF groups with *D. aegyptium* or *P. incurva* extracts showed a significant decrease (-48%) and (-27%) in serum levels of MDA and significant increase (64%) and (156%) in serum levels of GSH. *D. aegyptium* or *P. incurva* extract treated group showed a significant increase in serum MDA level at $P< 0.05$ when compared with control group. *D. aegyptium* or *P. incurva* extract treated group showed a significant decrease in serum GSH at $P< 0.05$ when compared with control group. *D. aegyptium* extract treated group showed no significant difference in serum SOD when compared with control group. *P. incurva* extract treated group showed a significant decrease in serum SOD level at $P< 0.05$ when compared with control group.

Treatment of NaF groups with *P. incurva* showed significant increase (58%) in SOD serum levels. Such protective role of *D. aegyptium* and *P. incurva* extracts could be explained by its antioxidant effect. Catechin and Naringenin of *D. aegyptium* or *P. incurva* extracts are responsible for such antioxidant activity. Similar findings were observed by Zaki et al. [39] who reported that flavonoids of green tea, particularly catechin have antioxidant activity.

The present investigation showed that rats treated with NaF revealed a significant increase in the calcium serum levels (198.9%) compared to control group. These data are parallel to that achieved by Begic-Karup et al. [40] who indicated that calcium metabolism is thought to be affected by thyroid hormones. Yan et al. [41] reported that hypothyroidism affects calcium homeostasis and thus causing hypercalcemia, resulting in an elevated serum calcium level. *D. aegyptium* or *P. incurva* extract treated group showed no significant difference (-13.7% and -27.2%) in serum calcium level when compared with control group.

In the present study, histological alterations in thyroid gland after NaF exposure were confirmed by thyroid function alterations. The gland has a high capability for fluoride absorption and accumulation [13]. Histopathological results showed that the gland tissue parenchyma was severely damaged and destroyed, with large areas of haemorrhage. This could be related to severe inflammation in the thyroid gland, which causes vasoconstriction and blood vessel congestion. Such findings coincide with that of other studies [42-43]. Furthermore, the current investigation revealed small sized empty follicles with damaged basement membranes and lumina fusion. Similar findings have been reported by Badr El Dine et al. [3]. Some follicular lumina of NaF-treated group showed desquamated follicular cells. In accordance, some researchers reported that sloughing of degenerated follicular epithelial cells is possible [43]. The histological alterations of the rat thyroid gland following NaF treatment can be due to the increased TSH level. TSH is responsible for follicular cell proliferation and the production of growth factors that regulate folliculogenisis [8]. Fluoride is mutagenic due to the unrestrained multiplication of cells that it causes [44].

Jwad [42] found that NaF in drinking water alters the morphological structure of thyroid tissue in rats following 150 days of exposure to 50, 100, and 200 mg/L NaF. In the necrotic acinia with granulomatous lesion, fibrous connective tissue infiltrated with polymorphic nuclear cells "PNCs" was seen after 60 days. These occur as a result of the severe excessive effects of NaF, which disrupt thyroid hormone (T3, T4, and TSH) and cause an increase in oxygen radical generation and lipid peroxidation in the pathogenesis of fluoridated water, causing destruction of the cellular membrane by simple diffusion, cell damage, and aggregation of PNCs, particularly macrophages, in tissue. Also Wang et al. [45] reported that rats given high doses of NaF in their drinking water developed flattened follicular epithelial cells and hyperplastic nodules made up of thyroid para follicular cells. The alterations were further confirmed by morphometric and statistical investigations that demonstrated a significant ($p<0.05$) decrease in follicle size as compared to control group. Thyroid follicles may have decreased in size and partially collapsed due to the lack of colloid. This finding is consistent with that of Badr El Dine et al. [3], who found a decrease in colloid content as well as enhanced follicular number and vascularity in mice treated with NaF. The degenerative changes observed in the follicular epithelial cells studied can be explained by Barbier et al. [46] who suggested that fluoride interacts with a wide range of cellular processes including gene expression, cell cycle, proliferation and migration, respiration, metabolism, ion transport, secretion, apoptosis and oxidative stress.

Abdel-Wahhab et al. [37] showed that the cytoplasm of the follicular cells showed overall disorganization; the mitochondria were lost with scattered stacks of rough endoplasmic reticulum. Excessive fluorine damaged thyroid gland structures such as mitochondria and endoplasmic reticulum. Zhan et al. [47] studied the effects of fluoride on pancreatic acinar cells and found enlarged mitochondria with loss of cristae and dilated endoplasmic reticulum. Badr El Dine et al. [3] observed that lipid peroxidation generated by NaF disrupts a range of intra and extra-mitochondrial membrane systems, which may lead to apoptosis, which could explain the loss of mitochondria.

Antioxidant enzymes such as SOD, glutathione peroxidase, and catalase are thought to be inhibited by fluoride. Furthermore, fluoride can change glutathione levels, leading to an excess of ROS formation at the mitochondrial level, resulting in cellular component damage due to phospholipid, protein, and nucleotide peroxidation. As a result of the disruption of membrane stability and integrity, osmolality alterations and hydropic cell degeneration occur [8]. Scanlan et al. [48] reported that the destruction of follicular cells in the thyroid appears to be a cause of low thyroid hormones, and oxidative stress could also be a factor. This result described by that thyroid hormone discharge initiates with endocytosis of small quantities of colloid into vesicles transferred inside the follicular cells. Lysosomes then combine with these vesicles and release T4/T3.

In the present work, Administration of *D. aegyptium* or *P. incurva* extracts produced a significant improvement in the thyroid tissue histological structures, the follicular lumina contained colloid, and the follicular cells looked to be healthy in the control group. This result may be attributed to antioxidant, anti-inflammatory and anti-apoptotic activities of *D. aegyptium* and *P. incurva* extracts. This is in agreement with Abdelaleem et al. [8] who reported that the polyphenolic substances of curcumin provide significant protection against thyroid dysfunction. Our results are also in coincidence with Abdel-Wahhab et al. [37] who found that phenolic compounds of *Withania somnifera*, particularly caffeine and cinnamic acid, restored the structural alterations of the thyroid gland to nearly normal.

Concerning the present histochemical results, NaF treatment caused reduction in glycogen (-40.3%) and total protein (-23.28%) in follicular cells and the colloid, with a significant decrease in DNA (-33%) content in follicular cells. The present results are supported by Lu et al. [48] who found that NaF treatment causes an excessive production of reactive oxygen species (ROS) that destroy DNA, proteins, and lipids, resulting in a variety of biological effects such as changes in signal transduction, gene expression, mutagenesis, and apoptosis. It was hypothesised that the decrease in glycogen is thought to be related to its faster breakdown (glycogenolysis), which releases glucose into the circulatory system to fulfill the increased energy demands under stress [50]. Badr El Dine et al. [3] revealed degraded endoplasmic reticulum and nuclei in follicular cells, which could contribute to a decrease in total protein and DNA. Endonuclease enzymes are activated by lipid peroxidation, resulting in the destruction of nuclear DNA and nuclear degeneration [3-51]. Al-Amoudi [49] also found that Lambda-cyhalothrin treatment caused a significant decrease in thyroid hormone levels, which in turn caused a significantly lower level of glycogen, total protein, and DNA content.

In the current work, administration of *D. aegyptium* or *P. incurva* extracts with NaF showed significant increase in glycogen (40%) and (51.4%) and total protein (16%) and (24.3%) in follicular cells and significant increase in DNA content (22.4%) and (35.9%) in nuclei follicular cells. This result might be due to the antioxidant properties of the phenolic compounds of *D. aegyptium* and *P. incurve* extracts, notably gallic acid, that restore glycogen content in follicular cells and colloid to near-normal levels. These results run with the study of Blas-Valdivia et al. [52] who reported that gallic acid reduces oxidative stress, protein oxidation and DNA damage in hypothyroid rats. Reconstruction of the structure of endoplasmic reticulum and nuclei regained total protein and DNA content in follicular cells as normal. Al-Amoudi [50] also found that taking ginger extract caused thyroid hormone levels to rise to normal levels, which led to an increase in glycogen, total protein, and DNA content.

With regard to sections stained with anti-calcitonin antibody in NaF treated group, the present study revealed a significant increase (38.7%) in mean area% of calcitonin immunoreactive C cells compared with the control group. This could be owing to high TSH levels caused by NaF treatment, which causes immunopositive C cells to hyperplasia and hypertrophy. These findings coincided with those of Elkalawy et al. [53] who found larger C cells in the hyperactive thyroid, which were scattered in small groups or singly. These findings could indicate to a possible link between thyroid gland function and C cell activity. Thyroid hormones are believed to affect calcium metabolism [40]. Calcium serum levels were raised as a result of thyroid hormone problems. Enhanced blood calcium concentration stimulates calcitonin secretion [4], which appears to be the cause of increased calcitonin cytoplasmic reactivity in parafollicular cells.

In present study, co-administration with *D. aegyptium* or *P. incurva* extracts showed significant decrease (-42.9%) and (-38.8%) in immuoreactvity of calcitonin in parafollicular cells. This result could be due to free radical scavenging property of flavonoids and phenolic compounds of two plants extracts. Such result is in agreement with the study of Abd El-Twab and Abdul-Hamid [54] who reported that the polyphenolic components of curcumin improve immunohistochemistry of thyroid tissues.

In the present study, Scanning electron microscope study of NaF treated group showed haemorrhage, increase in number of macrophages and inflammation. It also showed decrease in thyroid follicle size, increase in connective tissues between thyroid follicles and thyroid calcification. These

results confirmed the present configuration of NaF-induced histologial alterations in thyroid follicles that revealed congestion of blood vessels, hemorrhage, inflammation and decrease in thyroid follicle size. El-Kerdasy et al. [55] found thyroid follicle destruction with macrophages infiltration and a significant increase in serum TSH levels, as well as that chlorpyrifos toxic effects on thyroid gland tissue which may be due to lipid peroxidation associated with stellate cell stimulation for collagen synthesis in between thyroid follicles. Abdelaleem et al. [8] reported that NaF causes major pathological changes in follicular cells and cellular components, resulting in decreased colloid secretion, as well as that the early reaction of TSH triggered thyroid follicular cells is ingestion of colloid material, which explains decreased luminal colloid.

Thyroid calcifications, which contain calcium oxalate, are frequently found in thyroid tissue. Calcium oxalate is most commonly found in mitochondria and the endoplasmic reticulum [56]. Abdel-Wahhab et al. [37] reported that NaF causes damage to cellular organelles such as mitochondria and the endoplasmic reticulum. This could result in a large amount of calcium oxalate being released into the extracellular colloid, causing colloid calcification.

On the microscopic level, three types of thyroid calcifications are discussed. Psammoma bodies emerge as non-polarized spherical lamellar calcifications. The second is a ring of unspecific eggshell calcifications of different sizes encircles the capsule in capsular calcification while the latter calcifications of the colloid of the follicle [56].

Thyroid calcification, hypothyroidism, hypercalcemia, and calcitonin levels were all found to be linked in this study. This finding is consistent with Yan et al. [41] who found hypothyroidism to be a potential cause of hypercalcemia via raising serum calcium levels. The secretion of calcitonin by parafollicular cells is stimulated by an increase in serum calcium levels [5]. These effects are linked to immunohistochmical findings of enhanced calcitonin expression in parafollicular cells. An elevated calcitonin serum level is also indicative of hypercalcemia, thyroid calcification and tumor progression according to Giannetta [57].

Ultra-structurally, rough endoplasmic reticulum cisternae were disturbed in follicular cells, which served degenerated mitochondria. Mitochondria with damaged cristae were also found, as well as a large number of lysosomes and vesicles. Cuboidal to high columnar cells were observed in some follicular cells [8]. In addition, Abdul Rahman and Fetouh [58] found that fluoride has been shown to alter the ultrastructure of the tissue of the liver, adrenal glands, and thyroid glands of fetuses, causing cell nuclei to be destroyed and their membranes to widen. According to Jacinto-Aleman et al. [59], high fluoride intake has been found as a risk factor for fluorosis and oxidative stress, which can lead to DNA breakage and apoptosis.

Co administration of *D. aegyptium or P. incurva* extracts with NaF showed improvement in thyroid ultrastructure represented by increase in follicle size and colloid content again and decrease in connective tissue between follicles. These results are in line with Yousef et al. [10] found that ginger extract functions as powerful antioxidant. Ginger contains gingerols, which are phenolic substances that decrease lipid peroxidation and nitrosative stress. Furthermore, it reduces the ultra-structural changes in thyroid gland of albino rats produced by cypermethrin. The follicular cell restored its original height, and the cytoplasm retained some of its organelles, including mitochondria, rough endoplasmic reticulum, and small dense apical vesicles. The basal lamina appeared undamaged and regular supporting the follicular cell. The nucleus looked regular in shape and euchromatic with well-formed nuclear membrane and conspicuous nucleoli. The restoration of mitochondrial structure leads to the repair of the antioxidant defense system as well as an increase in antioxidant enzymes that scavenge free radicals.

The present data showed no thyroid calcification when *D. aegyptium* or *P. incurva extracts* were supplemented with NaF. This finding is consistent Abdel Wahhab et al. [37] who reported that antioxidant activity of phenolic compounds ameliorates hypothyroidism by increasing serum T3 and T4 levels. This may lead to a decrease in serum calcium levels nearly similar to normal and thus protect against thyroid calcification.

In present study, co-administration with *D. aegyptium* or *P. incurva* extracts showed significant decrease in immuoreactvity of calcitonin in parafollicular cells. This result could be due to free radical scavenging property of flavonoids and phenolic compounds of extracts of two plants. Such result is in agreement with the study of Abd El-Twab and Abdul-Hamid [54] who reported that the polyphenolic compounds of curcumin improve immunohistochemistry of thyroid tissues.

The present data clearly indicated that administration of *D. aegyptium* or *P. incurva* extract alone had no adverse effects on histology, histochemistry and ultrastructure of thyroid gland. These data are parallel to that achieved by Nagarjuna et al. [32] who found no toxicity in rats and mice were given ethanolic extracts of *D. aegyptium* at levels up to 2000 mg/kg. In the current work, co administration of *D. aegyptium* or *P. incurva* extracts with NaF resulted in a significant improvement in thyroid gland tissues as compared to NaF treated

group. These histological effects associated as well with the morphometric and biochemical findings, which revealed a significant improvement in these characteristics.

In our study, *P. incurva* extract improved pathological alterations more than *D. aegyptium* extract. According to the findings of the current investigation, *D. aegyptium* extract administration improved thyroid function by lowering thyroid hormones and reducing oxidative stress. These impacts could be due the significant functions of the phenolic contents as antioxidant agents. Thirteen phenolic components comprising 5 phenolic acids, 3 phenolic derivatives, and 5 flavonoids were characterized. Gallic acid (6473.36 µg/g), caffeic acid (558.07 µg/g), cinnamic acid (322.79 µg/g) and naringenin (2210.25 µg/g), catechin (7451.42 µg/g), Rutin (3508.09 µg/g) were assigned as the main compounds. The main identified such as gallic acid, caffeic acid, cinnamic acid, naringenin, catechin, rutin played main roles as free radical scavengers and inflammation inhibitors [60]. These results are in line with Abdel-Wahhab et al. [37] who reported that animals treated with Ashwaganda extract resulted in a significant improvement in thyroid gland tissue as compared to group of hypothyroidism. Abdel-Wahhab et al. [37] stated that the Ashwagandha extract exhibited more improvement in pathological changes and thyroid function by ameliorating thyroid hormones via preventing the oxidative stress. The significance of Ashwagandha extract was attributed to the higher contents of antioxidant component.

5. Conclusion

For both the thyroid gland's function and ultrastructure, sodium fluoride had a deleterious impact, but the presence of *D. aegyptium* and *P. incurva* protected against these harmful effects. extracts. It might be suggested as a useful method to protect against hazards occurring at the thyroid structure caused by NaF exposure. A re-examination of the safety profile of the sodium fluoride ratio (1.5mg/l) used in fluoridation of water was advised by the study. There should be further research done to determine the efficacy and safety of *D. aegyptium* or *P. incurva*, the structural analogues of *D. aegyptium* and *P. incurva*, as well as the combination of *D. aegyptium* and *P. incurva* with existing medicines that may hold tremendous promise in the future.

6. Ethical approval: All animals received human care in compliance with the standard institutional criteria for the care and use of experimental animals according to the National Research Centre ethical committee.

7. Conflicts of interest

There are no conflicts to declare.

8. Abbreviations

D. aegyptium: *Dactyloctenium aegyptium*; E: Eosin; H: Haematoxylin; HPLC: High Performance Liquid Chromatography Analysis; MDA: Malondialdehyde; *P. incurva*: *Parapholis incurve*; PAS: Periodic Acid Shiff's; GSH: Reduced glutathione; NaF: Sodium fluoride; SOD: Superoxide dismutase; TSH: Thyroid-stimulating hormone; T4: Thyroxine; T3: Triiodothyronine.

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