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The Anti-hypothyroidism Regulating Role of *Ficus carica* L. Leaves Extract: *In-vitro, In-vivo,* and *In-silico* Studies

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Abstract

The study targets anti-hypothyroditic effect and phytochemistry of the *Ficus carica* L. leaves pet. ether extract. GC/MS data of the unsaponifiable fraction led to identification of 23 compounds (85.14%) while identified saponifiable fraction amounted 78.56%. TLC chromatography led to isolation of five triterpenes (α-amyrin, taraxerol, lupeol, 3, 22 dihydroxy-13 (18) oleanene and β- sitosterol). The extract exhibited promising *in vitro* anti-inflammatory response *via* fluorometric assay. The extract was orally given at a dose (500 mg/kg) where different biomarkers were measured *in vivo:* serum TSH, T3, T4, IL-6 and oxidative stress levels. *Ficus carica* pet. ether extract recorded amelioration in all parameters suggesting its significance against thyroid hypothyroidism induced in male rats as prophylaxis and treatment. Histological examinations of all the groups supported the results. Additionally, molecular docking studies reinforced the results, demonstrating that the major compounds exhibited high binding interactions with (COX-1), (5-LOX), (IL-6), and thyroid stimulating hormone receptor.

Key words: Anti-hypothyroidism, *Ficus carica*, GC/MS, triterpenes, TSH, IL-6, molecular docking.

1. Introduction

Family Moraceae, widely known as fig family or mulberry family, mainly consists of trees and shrubs, comprised of nearly 40 genera and 1000 species [1]. *Ficus* L. genus is considered one of the principal genera of the Moraceae including about 800 species. It almost spreads in the subtropical and topical areas. Our plant of interest, *Ficus carica* L. (common Fig) is one of the genus members that are widely distributed in Mediterranean regions [2,3]. The plant leaves and edible fruits are known to be rich in both primary and secondary metabolites, hence they obtained their high nutritional value. A diversity of bioactive compounds was reported from *F. carica* L. e.g. (flavonoids, phenolics volatile constituents, and sterols) [4]. The fruit has wide use in traditional medicine in cardiovascular diseases and as spasmolytic [5]. *F. carica* L. leaves are reported to exhibit many bioactivities such as hepato-protective, anti-inflammatory, anti-diabetic and anti-ischemic effects [6].

Regarding previous hormonal activity studies performed on *F. carica* L. leaves, recent studies affirmed that *F. carica* leaves methanol extract possessed curative effect on T3 (3, 5, 3'-tri-iodothyronine) and T4 (thyroxine) production in hypothyroditic rats at dose (500mg/kg). The activity was accredited to the enriched phytochemicals composition [7,8]. Moreover, a former study indicated that the ethanolic extract of *F. carica* ameliorated the sperm count and had other favorable results on enhancement of testis function [9]. Additionally, *F.*

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carica L. fruit extract at 50 mg/kg significantly improve both male and female fertility in rats [10].

To decrease the vulnerability to thyroid diseases, it is important to comprehend the connection between environmental stressors and the effectiveness of the hypothalamus-pituitary-thyroid (HPT) axis. Thyroid disorders are a significant type of hormonal imbalance that can have negative impacts on overall bodily health. The indications of hypothyroidism include tiredness, sensitivity to cold, joint pain, muscle spasms, dry skin, and so on. Despite being a frequently occurring hormonal disorder, treating hypothyroidism poses multiple challenges [11]. There are four typical instances of thyroid disorders that can occur: Hypothyroidism, which is a decrease in thyroid hormone levels in the blood; hyperthyroidism, which is an increase in thyroid hormone levels in the blood; thyroid nodule, which is a swelling in a specific part of the gland that can be either benign or malignant; and thyroid cancer [12]. These situations require a considerable amount of time to manifest and exhibit noticeable fluctuations in hormone levels within the blood.

Hypothyroidism is a prevalent thyroid disease that affects a significant number of individuals. In cases of hypothyroidism, there is an observed increase in thyroid-stimulating hormone (TSH) levels. This results in excessive stimulation of the follicular cells, leading to metabolic changes caused by the inadequate production of T3 and T4 hormones [13]. The thyroid gland is commonly referred to as the chief controller of metabolic processes in both humans and animals. The thyroid hormones regulate important functions for normal growth and development. They also play a role in monitoring body weight and energy usage [14,15].

In individuals who are in good health, the HPT axis operates effectively and in harmony to regulate the typical levels of thyroid hormones in the bloodstream. On a hypothalamic level, the release of thyroid releasing hormone (TRH) prompts the pituitary gland to secrete thyroid-stimulating hormone (TSH). TSH then stimulates the thyroid gland to release the precursor hormones (T3) and (T4) into the bloodstream. Furthermore, within the outer tissues, (T4) is transformed into (T3) and reverse T3 (rT3), which is believed to have no metabolic activity. The levels of TRH and TSH hormones secreted by the hypothalamus and pituitary are controlled by a negative feedback loop involving T4 and T3 hormones [16].

The main objective of this study is to scrutinize the lipoidal profile of *F. carica* L. leaves pet. ether extract and investigate its effect against a very widespread hormonal disorder; hypothyrodism with supportive molecular docking evaluation. This was accomplished through determining serum TSH, (T3), (T4), IL-6, in addition to oxidative stress in both treated and prophylactic groups in contrast to positive control one. Histological study was executed to compare the thyroid changes among the groups.

2. Material and methods

2.1. Phytochemical study

2.1.1. Plant materials

Fresh leaves of *F. carica L.* were assembled from Orman garden, Giza, Egypt in May 2021. Identification of plant material was established by Ms. Therese Labib, Botanical consultant Orman Botanical Garden, a voucher specimen (JSAP-2021) was saved at Pharmacognosy Dept., National Research Centre, Cairo, Egypt. The collected fresh leaves (2.5 Kg) were air dried, powdered and kept in firmly sealed bags.

2.1.2. Preparation of the pet. ether extract

F. carica dried powdered leaves (960g) were extensively extracted on cold with 4L of petroleum ether (40-60℃), concentrated utilizing rotary evaporator at temperature 45◦C, then reserved in well closed containers (6◦C).

2.1.3. Chemicals, kits and authentic terpenes and sterols references

All used material were of analytical grade, products of Sigma (USA), Merck (Germany), and Fluka (Switzerland). Drug inducing thyroid dysfunction was acquired from various international companies including Sigma in the United States, Merck in Germany, BDH in England, and Fluka in Switzerland. The kits utilized for measuring the levels of thyroid hormones were bought from Abia Diagnostic Company, Gmbh located in Berlin. IL-6 kit was obtained from Wuhan Fine Biotech. China's company, Co Ltd.

2.1.4. Investigation of lipoidal contents

2.1.4.1. Extract saponification

The extract (5 gm) was subjected to saponification procedures as described by Tsuda et al. [17] resulting in unsaponifaiable and saponifiable fractions. Further methylation for saponifiable fraction was done according to Finar [18]. Both fractions were subjected to GC/MS analysis.

2.1.4.2. GC/MS analysis of unsaponifiable and saponifiable fractions

GC/MS analysis was performed for characterization of unsaponifiable and saponifiable fractions by means of gas chromatography-mass spectrometry apparatus located at the Department of Medicinal and Aromatic Plants Research, NRC, under the upcoming criteria; TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA) Instrument joined with detector: (ISQ Single Quadrupole MS Spectrometer), TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 μm

film thickness). Consequently, helium served as carrier gas with 1.0 mL/min flow rate by the following temperature programming: 60ºC for 1 min; rising at 4ºC/min to 300 ºC and kept with time interval of 15 min. The temperature of both injector and detector were 280ºC. MS spectral data were performed through (EI) at 70 eV. The identification was underdone via mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library) analytical method.

2.1.4.3. Isolation and identification of the major compounds

The extract was loaded on preparative TLC using toluene– ethyl acetate (80:20 v/v). The plates were examined under the UV light at 254 nm and 365 nm resp., before spraying with 10% H_2SO_4. The colored bands were marked and gathered. The Rf values of the isolated compounds were determined in contrast to the available authentic terpenes and / or sterols for confirmation. The structure elucidation of some the obtained compounds was confirmed by different spectral analyses (H^1-NMR, IR and MS) [19].

2.2. Biological study

2.2.1. *In vitro* anti-inflammatory

The in-*vitro* inhibition influence of *F. carica* L. pet. ether leaves extract against the COX-1 (cyclooxygenase-1) and 5-LOX (lipoxygenase) isoenzymes was performed using fluorometric assay according to [20,21].

2.2.2. *In vivo* study

Animals

Forty male adult Wistar albino rats, each weighing approximately 100g, were acquired from the animal housing facility at the National Research Centre in Giza, Egypt. During the experiment, the animals were kept in a controlled environment that followed standard laboratory conditions. This included 12 hours of light and 12 hours of darkness each day, and the room temperature was maintained at 24°C. The rats were given unlimited access to tap water and were given standard commercial rat food for feeding. All of the research was carried out following the guidelines of the Animal Ethical Committee at the National Research Centre in Dokki, Cairo, Egypt. Approval was given under the ethical number (7448092023).

2.2.3. Acute toxicity studies

The median lethal dose (LD_{50}) value was determined by administering various doses of the extract orally, with the highest dose being 3000 mg/kg b. wt. The rat mortality and behavior were assessed for a period of two weeks. Acute toxicity experiments were conducted following the procedure outlined by Hu et al. [22].

2.2.4. Serum thyroid hormones assay

The quantification of thyroid hormones in serum was conducted using the ELISA technique with the assistance of the automated ELISA reader, Expert Plus UV, manufactured by biochrom. The estimation of TSH, T3and T4 was conducted using the methods outlined by Fisher, Nelson and Wilcox, and Ekins [23, 24, 25].

2.2.5. Serum IL-6 assay.

The level of Interleukin 6 (IL-6) was measured in serum using the Stat-Fax -2100, an automated ELISA reader manufactured by Awareness Technology.

2.2.6. Estimation of antioxidants and oxidative stress markers

The colorimetric assay method was used to measure the levels of nitric oxide in a thyroid tissue homogenate [26].

2.2.7. Lipid peroxide assay

It was evaluated in thyroid tissue homogenate using method of [27].

2.2.8. Total antioxidant was performed in serum according to [28].

2.2.9. Grouping and biochemical studies

- Following a one-week period of adjustment, the animals were separated into five groups, with each group consisting of eight rats.
-The initial group did not receive any additional substances and was used as a control group for comparison (G1).
-The second group was given a dosage of 500 mg/kg of body weight of extract. They were considered a normal group and were treated with the extract to observe its impact on normal conditions (G2).
-The third group was given propylthiouracil orally at a dosage of 50 mg / kg of body weight / day for 30 days and was used as a reference group (G3).
- The fourth group was given thyroid dysfunction caused by propylthiouracil for a duration of one month before being treated with *F. carica* pet. ether extract administered at a dosage of 500 mg/ kg of body weight / day for a duration of two weeks(G4).
-The propylthiouracil dosage given to the 5th group (G5) was 50 mg/kg. Medication (bd.wt/ day) was received for a duration of four weeks along with *F. carica* pet. ether extract taken orally at the dose of 500 mg / kg body weight /day, for two weeks. The rats were dissected and the blood was taken from the retro-orbital plexus of the eyes and centrifuged at 4000 r.p.m for 10 min. Serum was separated, stored at -20°C till it used in biochemical investigations. The thyroid gland was taken out and purified for biochemical examination and other parts were ' soaked in a solution containing 10% formalin and made ready for histological analysis. It is noteworthy to mention that this study needed no reference standard treatment drug group as the objective was determining the

improvement of prophylaxis and treatment regarding to the positive control group [29, 30, 31].

2.2.10. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The data were evaluated statistically by SPSS (windows 7, version 8, Chicago, IL) software computer program (one-way analysis of variance (ANOVA, post-hoc, Least Significant Difference (LSD) followed by co- state computer program for comparison of therapeutic group's means. Letters which are different considered significant at $P \leq 0.05$.

2.3. Molecular docking simulation

All protein targets were acquired from the Protein Data Bank (Table S1). We removed all ligands that were present, ions, and water molecules using the PyMOL. The receptor molecule was then modified by adding hydrogen atoms using Autodock Vina [32]. We acquired the structure of chemicals from the PubChem database. Using an Open Babel, each chemical was changed into a mol2. [33]. Furthermore, the internal degrees of freedom and torsions were optimized to be at their minimal values, and the polar hydrogen charges were allocated using the Gasteiger technique. Using Autodock tools, molecules were transformed to the pdbqt format. Polar-H atoms were introduced to the target prior to docking, and then Autodock tools were used to calculate the Gasteiger charges. To be utilized for docking, the macromolecule file was saved in pdbqt format. The AutoGrid produced ligand-centered maps with grid size of 90A^ x 90A˚ x 90A^. The Discovery Studio 4.5 software was utilized to analyze the target-ligand bond interactions. Additionally, the BIOVIA Discovery Studio was used to determine the compounds' physicochemical properties [34].

3. Results

3.1. Phytochemical study

3.1.1. Investigation of lipoidal content

The petroleum ether extract yielded 120g. Twenty g. were subjected for saponification process where unsaponifiable (7.5 g) and saponifiable (6.8g) fractions were further analyzed using GC/MS technique.

3.1.2. GC/MS analysis of unsaponifiable and saponifiable fractions

GC/MS quantitative technique was underdone using peak area integration. The identified compounds were listed in Tables 1 and 2, Figures Fig S1 and Fig. S2. Twenty-three compounds were recognized from the unsaponifiable fraction (USF), amounting (85.14%) of the total content. Nevertheless, phytol was the predominant compound (49.66%) followed by xanthotoxin (7.27%). On the other hand, the saponifiable fraction (SF, 6 fatty acid derivatives) represented (78.56%) of the total content**.** Methyl linoleate was found to be the major unsaturated fatty acid (28.07%) while methyl palmitate represented the main saturated fatty acid yielding (14.01%). Moreover, a previous study proved that phytol, β-sitosterol, β-amyrin, lupeyl acetate and lupeol beside palmitic, linoleic acid, linolenic and stearic acids were formerly recognized from *F. carica* L. leaves [35].

3.1.3. Isolation and structure elucidation of the major compounds

The extract was loaded repeatedly to TLC chromatography then, the collected bands were examined under UV at λ max 254 and 365 nm. The bands were further purified several times resulting in isolation of five compounds **(1-5)** that showed positive Salkowski's results and attained different colors with 10% sulphuric acid as spraying reagent (Fig.1).

β-Amyrin (3β-hydroxy-urs-12-en-3-ol; Compound 1)

It was obtained as white crystalline powder with melting point 187 °C. It is soluble in n-hexane and insoluble in methanol. EI-MS showed 426 (M^+) assigned to molecular formula $C_{30}H_{50}O$. The characteristic fragment (*m/z*) 189, 218(100%) and 203. FT-IR spectrum (KBr/cm^{-1}) showed absorption at 3321 for OH groups, 1646 for C=C conjugation, 1054 (C-O bond). H^1 -NMR (400 MHz, $CDCl_3$, δ, ppm) gave some distinctive signals; at 3.20 (H-3, dd), 5.11 ppm for (H-12, t, 1H), 1.89 (H-19, dd) 1.81 (H-22, m). It is worth mentioning that compound 1 was co-chromatographed with authentic β-amyrin and was compatible with the spectral data that stated in the literature [36,37, 38].

Taraxerol (13α-methyl-27-norolean-14-en-3β-ol; Compound 2)

It was attained in the form of white amorphous powder, m.p. 277 ^{0}C. Mass spectrum showed (m/z) 426 (M^+) assigned to molecular formula $C_{30}H_{50}O$. Other distinguishing fragments (*m/z*) were: of 411(14%), 393, 355, 341, 327, 302, 287, 269, 245, 231, 218, 204, 189, 175, 147, 135, and 107. Suffice it to say that the major peak at 204 characteristics for taraxerol originates from retro-Diels-Alder reorganization [39]. FT-IR (KBr, cm^{-1}): 3365for OH group, 2928 and 2851 for CH stretching, 1643 for C=C conjugation, 1038 (C-O bond). ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm): 1.60 (H-6, m, 1H), 1.66 (H-2, H-7, m 2H), 1.47 (H-11a, m,1H), 1.34 (H-12, m,1H) 5.48 (H-15, dd, 1H), 1.94 (H- 19, m,1H), 1.19 (H-21, m,2H), 1.00 (H-27, s, 3H), 0.91 (H-29, s,3H), 3.21 (H-31, m, 1H) [40]. Tarxerol has been isolated from other *Ficus* spp before [41, 42].

Table 1: Identified compounds by GC/MS analysis of the unsaponifiable fraction of *F. carica* leaves

No.	Compound	M. Wt.	Molecular formula	Base Peak	R_t	Relative Area%
	Non oxygenated hydrocarbons					
1	2,6-dimethyl- 1,5-Heptadiene	124	C_9H_{16}	69	1.47	1.30
2	Methyl undecane	170	$C_{12}H_{26}$	57	5.71	1.07
3	heptylcyclohexan	182	$C_{13}H_{26}$	83	6.11	1.11
4	Phenyldecane	218	$C_{16}H_{26}$	91	18.74	0.89
5	4,4,7,7-Tetramethyldeca-1,9-diene	194	$C_{14}H_{26}$	55	28.13	0.95
6	Pentane, 3,3-dimethyl-	100	C_7H_{16}	71	29.23	1.14
7	Octadecane	254	$C_{18}H_{38}$	43	29.31	0.75
8	1-Hexadecyne	222	$C_{16}H_{30}$	43	29.82	0.64
9	1-Octadecyne	250	$C_{18}H_{34}$	81	29.93	0.35
	Oxygenated hydrocarbons					
10	Oleyl Alcohol	286	$C_{18}H_{36}O$	82	5.77	4.16
11	Di-tert-butylphenol	206	$C_{14}H_{22}O$	191	22.29	1.84
12	2-Decenol	156	$C_{10}H_{20}O$	57	29.32	1.58
13	2-Nonenol	142	$C_9H_{18}O$	82	29.56	1.67
14	Phytol	296	$C_{20}H_{40}O$	71	29.58	49.66
15	Phytol acetate	338	$C_{22}H_{42}O_2$	82	29.93	1.30
16	Psoralene	186	$C_{11}H_6O_3$	186	31.47	4.60
17	Xanthotoxin	216	$C_{12}H_8O_4$	216	36.87	7.27
	Triterpene and sterol					
18	Brassicasterol	412	$C_{29}H_{48}O$	255	47.74	0.84
19	β-sitosterol	414	$C_{29}H_{48}O$	414	53.50	0.96
20	Taraxasterol	426	$C_{30}H_{50}O$	207	55.88	0.23
21	α-Tocopherol	430	$C_{29}H_{50}O_2$	165	56.24	0.14
22	Lupeyl acetate	43	$C_{32}H_{52}O_2$	468	56.42	0.31
23	Lanosta-8,24-dien-103-ol, acetate	69	$C_{32}H_{52}O_2$	468	59.89	0.27
Total identified compounds						**85.14%**

Lupeol; lup-20(29)-en-3β-ol (Compound 3)

It was isolated as yellowish powder with melting point 218-220 ^{0}C. EI-MS showed (*m/z*) 426 (M^+) ssigned to molecular formula$C_{30}H_{50}O$, other fragments (m/z) were at m/z: 189 and m/z 218 suggesting a pentacyclic triterpene structure. The other fragments at m/z161, 175, 234, 257 and 315 are often associated with lupeol. FT-IR (KBr, cm^{-1}): OH bond vibration of hydroxyl group (3521), C=O (1716). ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm): depicted the following: 3.18 (H-3, m, 1H), 4.70, 4.55 (H-29a, H-29b, s, 2H, exomethylene protons), 0.67, 0.73, 0.81, 0.89, 0.94, 1.01, and 1.35 (3H, s, seven CH_3 groups). ^{13}C-NMR (125 MHz, $CDCl_3$, δ, ppm): 37.71(C-1), 29.54 (C-2), 78.32(C-3), 39.12(C-4), 53.90(C-5), 18.61(C-6), 32.88(C-7), 41.11(C-8), 51.32(C-9), 35.81 (C10), 20.98(C-11), 26.11(C-12), 43.32(C-14), 35.31(C-16),49.21(C-17), 49.69(C18), 150.90 (C-20), 30.20(C-21), 40.65 (C-22), 28.45(C-23), 17.70(C-25), 18.45(C-26), 17.90(C-28), 108.93(C-29), 21.21(C30). By comparison with the previous literature [43], compound 3 was suggested to be lupeol

Table 2: Identified FAME by GC/MS analysis of the saponifiable fraction of *F. carica* leaves

No.	Identified Compound	Base Peak	Molecular Formula	M. Wt.	Rt	Relative area %
	Unsaturated					
1	Ethyl linolenate	79	$C_{20}H_{34}O_2$	306	9.39	11.36
2	Methyl oleate	55	$C_{19}H_{36}O_2$	296	13.89	15.48
3	Methyl linoleate	67	$C_{19}H_{34}O_2$	294	16.95	28.07
	Saturated					
4	Methyl palmitate	74	$C_{17}H_{34}O_2$	270	23.73	14.01
5	Ethyl palmitate	88	$C_{18}H_{36}O_2$,	284	27.16	6.63
6	Methyl stearate	74	$C_{19}H_{38}O_2$	298	28.88	3.01
Total identified compounds 78.56%						

3, 22-Dihydroxy-13(18) oleanene (Compound 4)

It was obtained as amorphous white powder. EI-MS (*m/z*) 442 (M^+) assigned to molecular formula $C_{30}H_{50}O_2$. Some major fragments (m/z) were: 427, 424 (M^+ - H_2O), 393 (M^+-CH_2OH and 2 H_2O) and 207 (OH bond cleavage of the 2 rings). FT-IR (KBr, cm^{-1}): 3512 (broad peak assigned for hydroxyl groups), 2928-2892 for CH stretching, 1612 for oleifinic C=C conjugation, 1041attributed to (C-O bond). ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm) gave the following signals δ: (H-3, dd, 1H), 3.61 (H-22, m,1H)0.81, 1.03 (H-23, s, 6H), 0.87 (H-24, s, 3H), 1.18 (CH-25, s, 3H,), 1.00 (H-26, s, 3H), 1.26 (H-27, s, 3H), 0.92 (H-28, s, 3H), 0.98 (H-29, s, 3H), (H-30, s, 3H). By comparing the above spectral data with the available literature, it could be noticed that this compound belongs to oleanane skeleton being a pentacyclic triterpene and has been previously isolated from other *Ficus* spp. [44]**.**

β- Sitosterol (Compound 5)

It was in the form of white needles. Its melting point was 149 °C. FT-IR (KBr, cm^{-1}) revealed absorption bands at 3452 (hydroxyl groups), 2932- 2863 for methylene, 1621 for oleifinicC=C,1459 assignable for methyl groups and 1052cm^{-1} for C-O bond. EI-MS showed M^+ at m/z 414 for molecular formula $C_{29}H_{50}O$. The other fragments showed at m/ z 396, 314, 271, 189,145, 137. Associating the obtained spectral data of compound 5 with that of the published data and the available standard authentic, it was identified as β-sitosterol which has been detected before in some *Ficus* spp and in *F. carica* leaves [45].

It is noteworthy that compounds **(1-4)** are isolated for the first time from leaves, although being formerly detected from *F. carica* different parts except for β-sitosterol that has been previously isolated from the leaves. In addition, compounds 1, 2, 4, 5 were co-chromatographed with authentic standards.

3.2. Results of biochemical investigation

3.2.1. *In vitro* anti-inflammatory activity

In vitro study of anti-inflammatory potential *F. carica* pet. ether extract against inflammatory markers COX-1 and 5-LOX showed the potent anti-inflammatory effect. The extract gave half-maximal inhibitory concentration (IC_{50}) = 0.347±0.39 ug/ml against COX-1 while, recorded IC_{50}= 0.447±0.57 ug/ml against 5-LOX as compared to anti-inflammatory standard drugs indomethacin (COX-1) and Zileuton (5-LOX) which exhibited IC_{50}= 0.227±0.01 ug/ml and 0.579±0.03 ug/ml, respectively (**Table 3, Fig.2**).

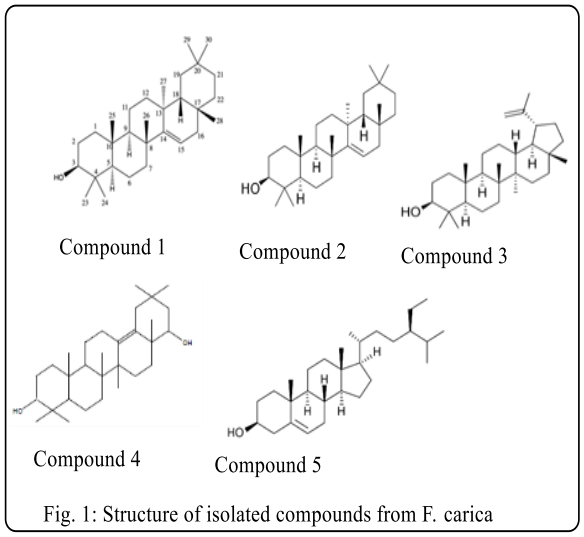

Fig. 1: Structure of isolated compounds from F. carica

isolated from the leaves. In addition, compounds 1, 2, 4, 5 were co-chromatographed with authentic standards.

Table 3: Anti-inflammatory potential of *F. carica* pet. ether extract against inflammatory markers COX-1 and 5-LOX

Sample	IC_{50} (ug/ml)	
	COX-1	**5-LOX**
***F. carica* extract**	0.347±0.39	0.447±0.57
Indomethacin	0.227±0.01	---
Zileuton	---	0.579±0.03

Statistical analysis is carried out using (ANOVA, LSD), combined with Co-state computer program

3.2.2. *In vivo* study

3.2.1. Acute toxicity results

Orally controlled of various dosages of *F. carica* extract gave no harmfulness indications recognized when surrendered to male rodents at dose 3000 mg/kg b. wt. No mortality was recorded after 24 hrs. also, during about fourteen days after extract infusion. Appropriately, the extract is viewed as totally safe when administered to animals.

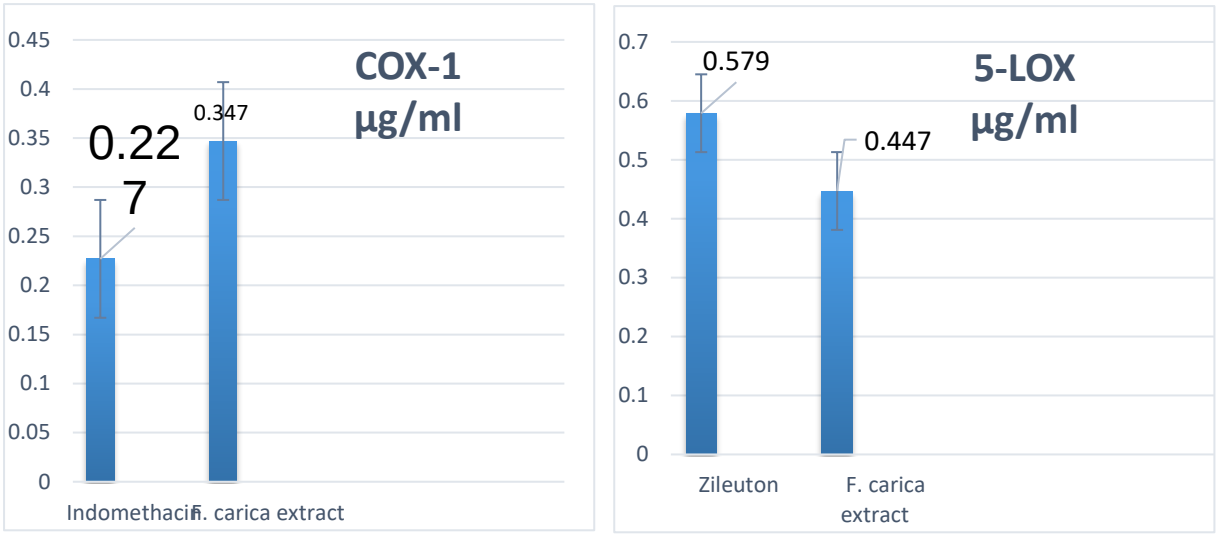

Fig.2: Anti-inflammatory potential of *F. carica* pet. ether extract against inflammatory markers COX-1 and 5-LOX.

3.2.2. Therapeutic and protective effects of *F. carica* pet. ether extract on oxidative stress markers

The present results recorded significant increase in nitric oxide and lipid peroxide levels in hypothyrodic rats (positive control) with increasing percentages of 108.51% and 184.83%, respectively

On the other hand, noticeable enhancement was detected post treatment with *F. carica* pet. ether leaves extract (59.57 and 91.48%, respectively for nitric oxide and lipid peroxide) whereas, the protective group recorded (47.47 and 126.66%, respectively) comparing to positive control. Furthermore, the present results showed significant decrease in total antioxidant capacity in hypothyrodic rats with percentage 55%, while noticeable improvement was detected post treatment group (35.23%) and the protective group by 51.14% (**Table 4**).

3.2.3. Therapeutic and protective effects of *F. carica* pet. ether extract on TSH, T3, T4 and IL-6

Additionally, present results recorded significant decrease in thyroid stimulating hormones levels (TSH, T3 and T4) in hypothyrodic rats with percentages 99.81%, 62.2 and 76.3%, respectively, while, noticeable enhancement was detected post treatment with *F. carica* extract (4.69, 54.87and 53.3%), respectively for TSH, T3 and T4). The protective group recorded (3.84, 50.87 and 39.3%, respectively, Table 5). While significant increase in IL-6 in hypothyrodic rats with percentage 132.32%. In contrast noticeable enhancement was detected post treatment with *F. carica* extract 71.72% compared with protective group 85.86%.

3.3. Histopathological Examination

Histopathological Examination of thyroid gland showed the following: G1 and G2: represent negative control rats and control rats receiving *F. carica* pet. ether leaves extract, respectively, showed normal histological structure of thyroid follicle with normal intraluminal colloid. G3: represent hypothyroidic rats, showed atrophy of follicles and flattening of some follicles with lining epithelium
and follicular configuration distortion. Also showed edema and dispersion of inter-follicular connective tissue. G4: represent hypothyrodic rats treated with the *F. carica* extract, showed mild edema of inter-follicular connective tissue with atrophy of some follicles. G5: represent the protective group of *F. carica* treated rats, showed few inactive follicles, most follicles with normal lining epithelium and normal colloidal material (Fig.3).

Table 4: Therapeutic and protective effects of *F. carica* pet. ether on oxidative stress markers

Parameters/ Groups	Control	Negative control	Positive control	Treated group	Protective group
Total antioxidant capacity (mM/gm tissue) % change % improvement	0.88±0.11[a]	0.88±0.11[a] 2.27	0.33±0.11[d] 55	0.64±0.14[c] 27.27 35.23	0.78±0.08[b] 11.36 51.14
Nitric oxide (µ mol/ gm tissue) % change % improvement	47±0.12[d]	50±0.08[d] 6.40	98±0.12[a] 108.51	70±0.09[b] 48.94 59.57	63±0.09[c] 34.04 47.47
Lipid peroxide (nmol/gm tissue) % change % improvement	290.2±0.09[d]	295.9±0.11[d] 1.72	826.5±0.08[a] 184.83	561±0.21[b] 93.45 91.48	459.2±0.16[c] 58.34 126.68

Statistical analysis is carried out using ANOVA (Ppost –hoc, LSD) combined with co-state computer program, where different letters are significant at *P ≤0.05.*

Table 5: Therapeutic and protective effects of *F. carica* pet. ether extract on TSH, T3, T4 and IL-6

Parameters/ Groups	Control	Plant control	Positive control	Treated group	Protective group
TSH (ulU/ ml) % change % improvement	3.20±0.44[a]	3.60±0.24[a] 12.5	0.005±0.001[d] 99.81	0.155±0.002[g] 95.16 4.69	0.128±0.001[g] 96 3.84
T3(ng/dl) % change % improvement	150.00±5.20[b]	144.00±4.76[b] 4.00	56.70±3.94[e] 62.2	139.00±6.22[b] 7.33 54.87	133.00±5.14[b] 11.33 50.87
T4(ng/dl) % change % improvement	10.00±0.69[c]	10.60±0.76[c] 6.00	2.37±0.14[f] 76.3	5.90±0.97[h] 41.00 35.30	6.30±0.10[h] 37.00 39.30
IL-6(pg/ml) % change % improvement	99.00±2.90[a]	90.80±3.22[a] 8.28	230.00±7.60[b] 132.23	159.00±8.00[c] 60.61 71.72	145.00±7.90[c] 46.46 85.86

Statistical analysis is carried out using ANOVA (post –hoc, LSD) combined with co-state computer program, where different letters are significant at $P \leq 0.05$. TSH: thyroid stimulating hormone, T3: 3, 5, 3'-tri-iodothyronine, T4: thyroxine and IL-6: interleukin 6.

Table 6: Score level of histopathological changes in thyroid gland of all therapeutic groups

Groups	**G1 &G2**	**G3**	**G4**	**G5**
In active follicles	0	3	2	1
Vacuolation of follicular lining epithelium	0	3	2	1
Necrosis and desquamation of follicular lining epithelium	0	2	1	0
Flattening of follicular lining epithelium	0	3	2	1
Edema and dispersion of inter- follicular connective tissue	0	3	1	0

The score system was designed as: score 0 = absence of the lesion in all rats of the group (n= 5), score 1 = (<30%), score 2= (<30% – 50%), score 3= (>50%).

3.4. Computational examination

Cyclooxygenase-1 (COX-1) is an enzyme that plays crucial roles in various physiological processes, including inflammation, pain modulation, and regulation of blood flow in different tissues. Through docking analysis, it has been determined that COX-1 exhibits a strong affinity for the isolated compounds, namely β-amyrin, taraxerol, β-sitosterol, lupeol, besides methyl linoleate, being the main identified fatty acid from GC/MS. These compounds displayed binding energies of -6.90, -6.80, -6.90, -7.10, and -7.30 kcal/mol, respectively, compared to Indomethacin's binding energy of -7.00 kcal/mol. The binding of these compounds involved the formation of hydrogen bonds with Asn382, Thr212, Arg120, and Tyr355. Additionally, hydrophobic interactions, including (Pi-alkyl) with His386, His207, Ile444, Leu408, Met391, Tyr404, His446, His388, Ile444, Leu294, Phe210, Phe409, Val291, Val447, Leu295, Ala527, Phe381, Val349, and Leu531, as well as (Pi-sigma) with

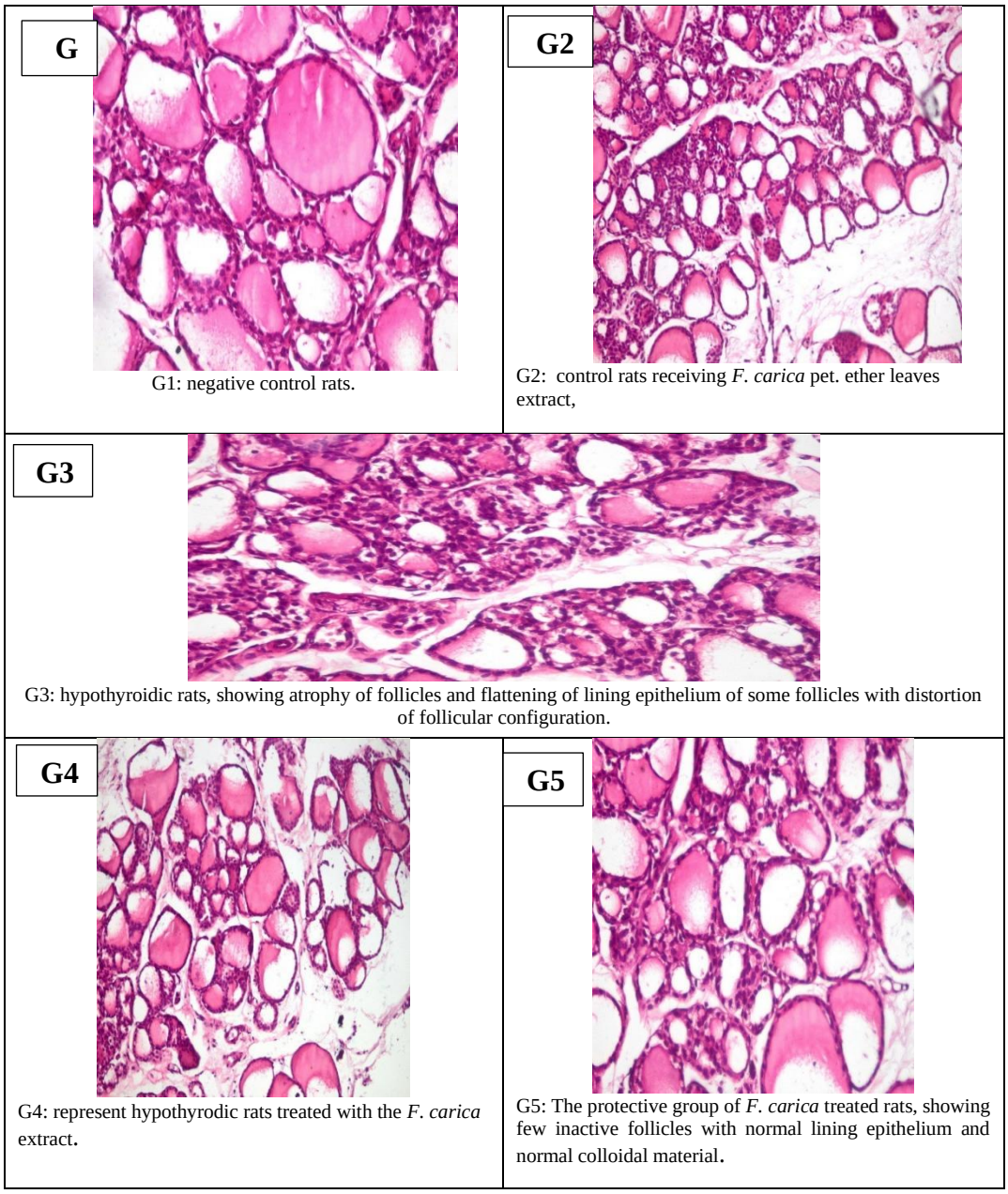

Fig.3: Histopathological examination of different studied groups.

Phe209, were observed. Notably, the residues Thr212, Asn382, Arg120, and His388 have positive effect on the binding energy. Also, these results strongly suggest that lupeol and methyl linoleate are the most promising compounds for further investigation as potential inhibitors of cyclooxygenase-1 (COX-1) (Fig. 4 and Table S2).

5-Lipoxygenase (5-LOX) is an enzyme that catalyzes the oxygenation of fatty acids. One of the primary roles of 5-LOX is as a mediator in inflammatory and immune responses. Docking analysis reveals that 5-LOX exhibits a strong affinity for the isolated compounds **(1-5)** and phytol, the major identified oxygenated hydrocarbon. These compounds demonstrated binding energies of -8.80, -8.60, -8.40, -8.50, -8.60, and -6.40 kcal/mol, respectively, compared to zileuton (-6.10 kcal/mol). No hydrophilic interactions were obtained with these compounds. However, non-hydrophilic interactions, such as (alkyl bonds) with Leu607, Ala606, Ala410, Leu414, Trp599, Phe169, Ile406, Lys409, Ile406, Leu368, and His432, as well as (Pi-sigma) interactions with Phe169, were observed. Notably, the residues Leu414, Ala410, Trp599, and Lys409 were found to enhance the binding affinity. Overall, these findings suggest that those compounds hold promise as potential inhibitors of 5-Lipoxygenase (Fig. 5 and Table S3).

Interleukin-6 Receptor alpha chain (IL-6) is a protein that plays a crucial role as a pro-inflammatory cytokine. IL-6 is involved in various physiological processes, including immune responses, inflammation, hematopoiesis. Based on the docking analysis, IL-6 has a strong affinity to the isolated compounds **(1-5)** and methyl linoleate with binding energies of -7.10, -7.40, -6.00, -7.00, -7.10 and -5.50 kcal/mol, respectively. These compounds formed hydrophilic interactions with Prp7, Cys174, Lys126, Gln9, Gln143, and Tyr140. Also, non-hydrophilic bonds were also observed including (alkyl bond) with Val128, Pro145, Pro7, Lys126, Cys174, Arg4, Pro3, Leu130, and Phe155, (carbon-Bond) with Pro7. The residues Val128, Pro145, and Pro7 were found to enhance the interaction energy. Overall, the results suggest that the aforementioned compounds are potential inhibitors of IL-6 protein (Fig. 6 and Table S4).

Thyroid Stimulating Hormone Receptor (TSHR) plays a crucial role in the regulation of thyroid gland function. Therefore, according to docking results, TSHR has affinity to the isolated compounds **(1-5)** and methyl linoleate with binding energies of -6.90, -6.90, -5.90, -6.10, -7.00 and -5.00kcal/mol, respectively. Only Methyl linoleate formed one hydrogen bond with His105. Also, non-hydrophilic bonds were also observed including (alkyl bond) with Phe130, Lys58, Ile60, Tyr82, His105, Arg109, Ile155, Ile85, (Pi-Sigma) with Phe130 and Tyr82. The residues Phe130, Tyr82, His105, and Arg109 were found to enhance the binding affinity. Overall, the results suggest that the previously stated compounds are promising as potential inhibitors of TSHR protein (Fig.7 and Table S5).

References 3.4.1. Pharmacokinetics and *in-silico* ADME prediction of compounds

The most promising compounds with the highest affinity for protein receptors have been associated with ADME and toxicity were studied. Table S6 and Figs. 8 and 9 display the physiochemical characteristics and ADMET prediction of the compounds. The chosen compounds were tested using the Lipinski rule, which included assessing parameters such molecular weight as: (MW), ALogP, HBA, HBD, RB, and PSA. Moreover, having MW less than 500, all the tested compounds were found to be Lipinski-compatible, demonstrating their tiny size, simple transferability, and effective absorption. Furthermore, every compound had enough rotatable bonds (RBs 1–10), which is essential for great structural flexibility. This is significant because compounds that have less than ten RBs have a higher probability of becoming bioavailable. An effective interaction with specific binding sites is increasingly dependent on the quantity of RBs present. The hydrogen bond acceptors (HBA) and donors (HBD) were also calculated for all identified compounds, and it was found that all tested compounds had less than 10 HBA and less than 5 HBD, indicating a favorable balance of HBA and HBD and a higher likelihood of oral bioavailability. Furthermore, each compound has been evaluated for its lipophilicity and water solubility. According to our research, β-amyrin, taraxerol, lupeol, and β-sitosterol poorly have solubility in water, while 3,22-dihydroxy-13(18) oleanane, phytol, and methyl linoleate compounds have moderate solubility. For these compounds, the range of Log S values is -8.64 to -4.97, which suggests a moderate water solubility. Furthermore, all compounds' lipophilicity parameters, or XLOGP3, seemed to fall between 6.24 and 9.87, which is the permitted range. Also, we have also evaluated pharmacokinetics of selected compounds. According to our findings, the tested compounds have high theoretical bioavailability and the potential to be a medication or drug-like agent. Nevertheless, none of the substances can pass across the blood-brain barrier. Moreover, 3,22-dihydroxy-13(18) oleanene and methyl linoleate have a high intestine absorption rate because they inhibit the CYP2D6 and CYP3A4 enzymes. Nonetheless, it seems that the study employed several techniques, such as the Lipinski, Ghose, Veber, Muegge, and Egan criteria, to assess the drug-likeness of the compounds. The fact that phytol and methyl linoleate compounds satisfied every condition of every drug-likeness technique is promising. It could be noted that the calculated bioavailability of all molecules being 0.55 is also a positive sign, suggesting that these compounds have the potential to be absorbed into the bloodstream and reach their intended target. It is suggested that they were non-irritant, non-tumorigenic, non-mutagenic, or harmful to reproduction. Additionally, the (TPSA) was used as a metric to analyze the features of drug transport. It was discovered that the compounds' TPSA values were low. TPSA is just one of several variables that might affect drug's absorption and bioavailability, thus it's vital to keep in mind that other variables like molecular weight, lipophilicity, and solubility should also be considered, Table (S7).

4. Discussion

As a matter of fact, the lipoidal content of *F. carica* leaves has not been evaluated as anti-hypothyroditic but, it was recorded for other hormonal disorders such as improvement of both male and female fertility in rats [9]. Nevertheless, *F. carica* leaves methanol extract proved its regulative role on T3 and T4 levels in hypothyroditic rats at dose (500mg/kg).

The studies attributed this activity to phytochemicals' presence of different phenolics [7, 8].

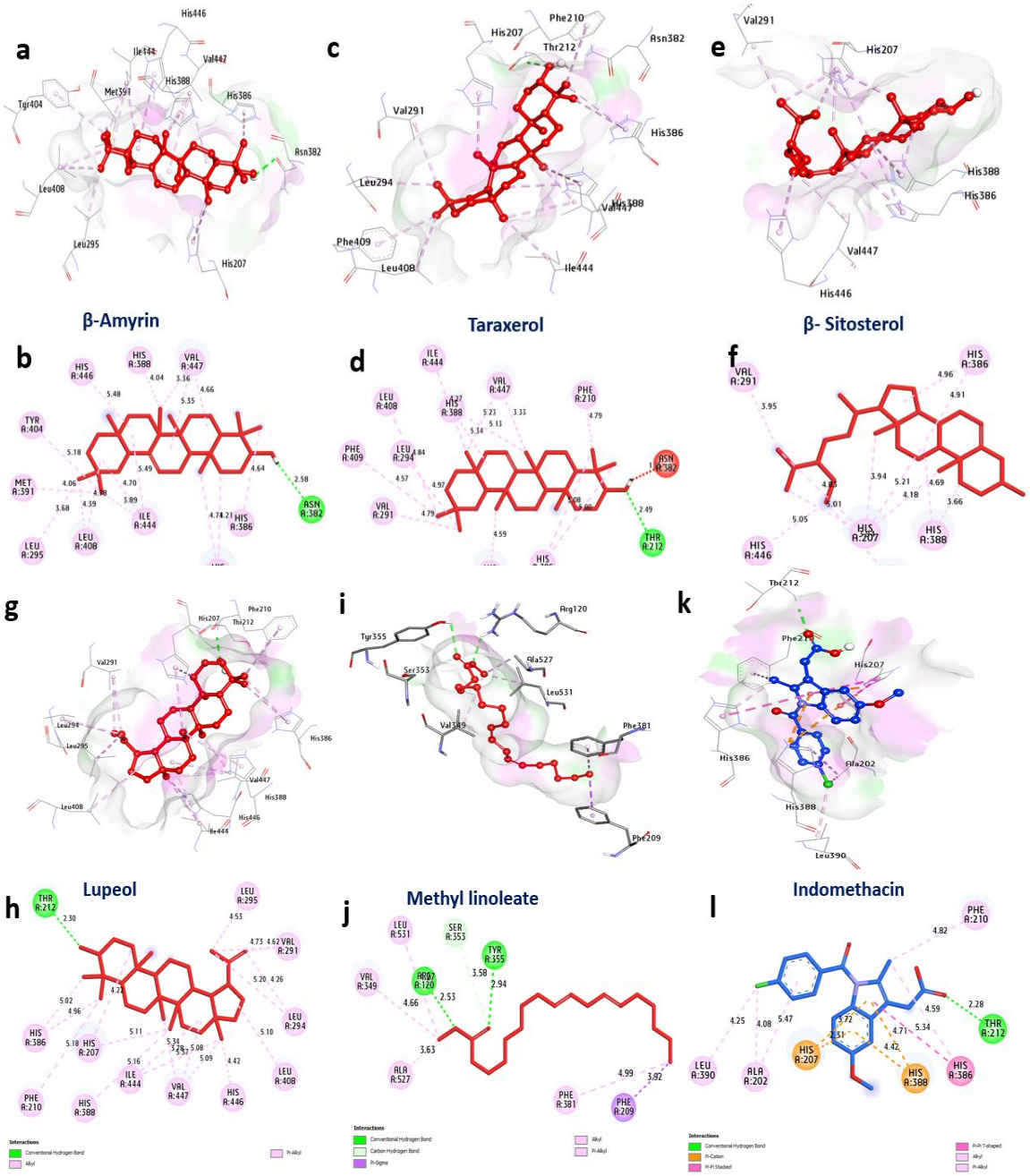

Fig. 4: 3D Interaction of compounds at the activity site of COX-1 (PDB: 6Y3C). (a and b) β-amyrin, (c and d) taraxerol, (e and f) β- sitosterol, (g and h) lupeol, (I and j) methyl linoleate and (k and l) indomethacin.

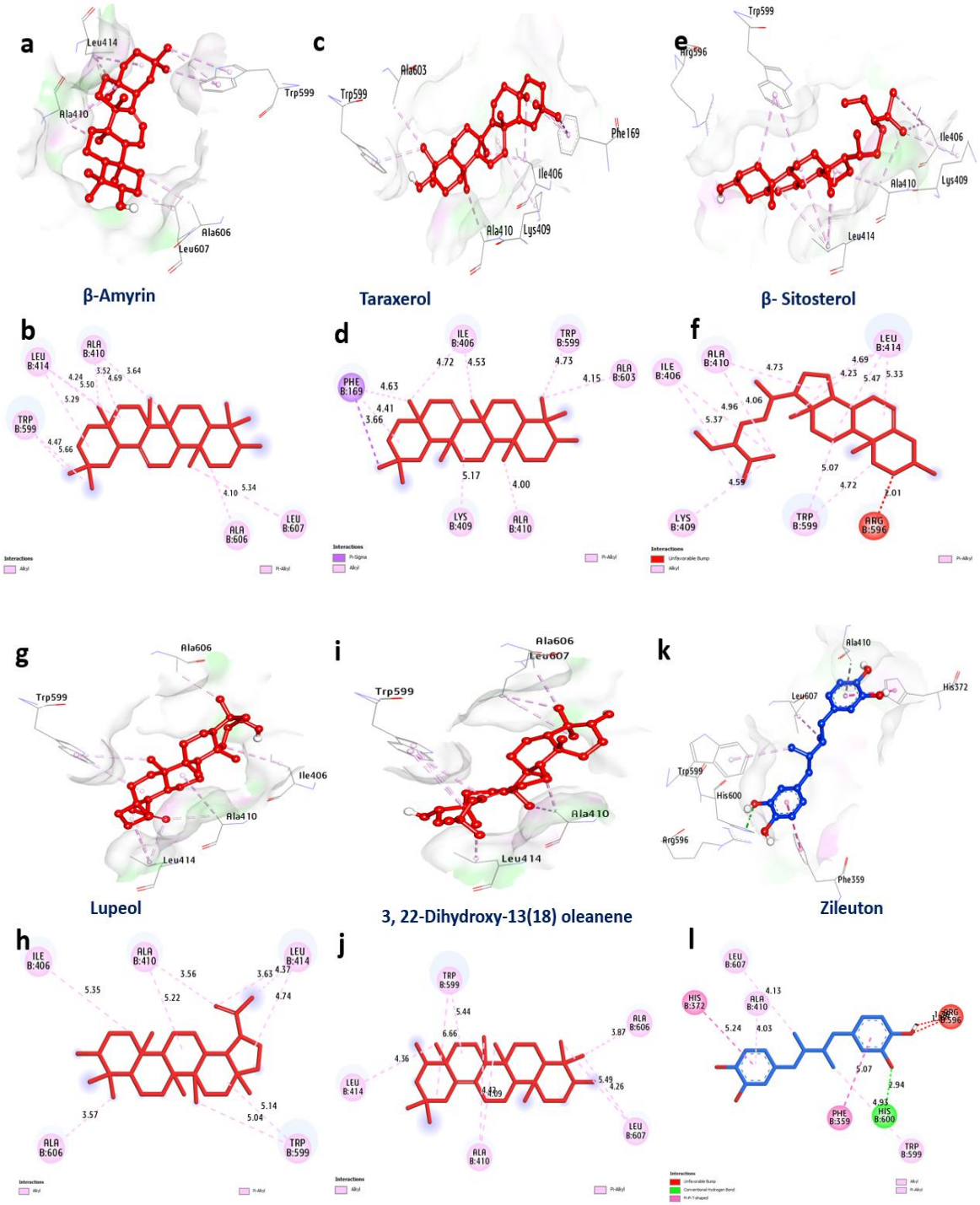

Fig. 5. 3D Interaction of compounds at the activity site of 5-LOX (PDB: ID 6N2W): (a and b) β-amyrin, (c and d) taraxerol, (e and f) β- sitosterol, (g and h) lupeol, (I and j) methyl linoleate and (k and l) zileuton

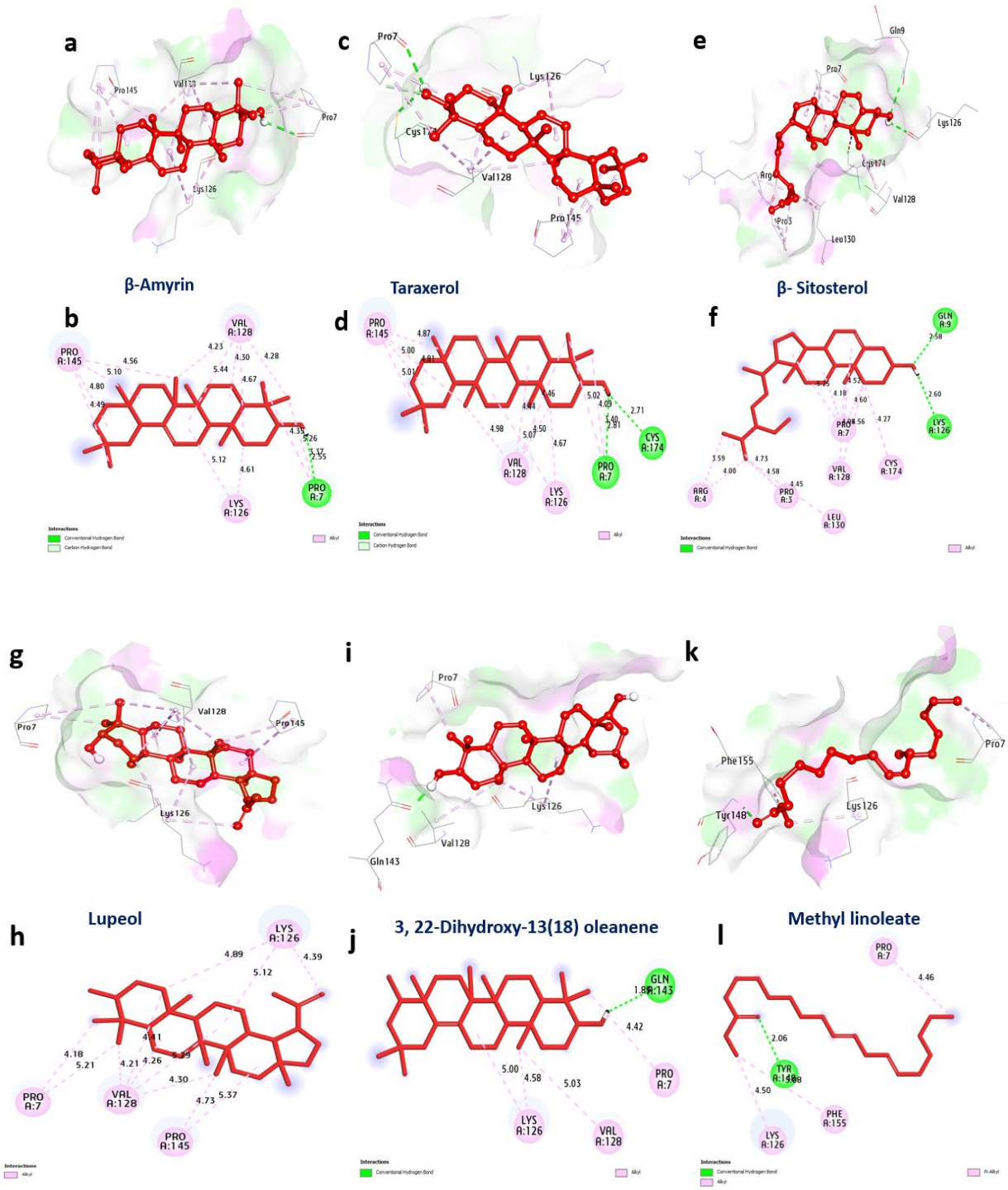

Fig.6. 3D Interaction of compounds at the activity site of IL-6 (PDB:ID 1N26): (a and b) β-amyrin, (c and d) taraxerol, (e and f) β- sitosterol, (g and h) lupeol, (I and j) methyl linoleate and (k and l) methyl linoleate.

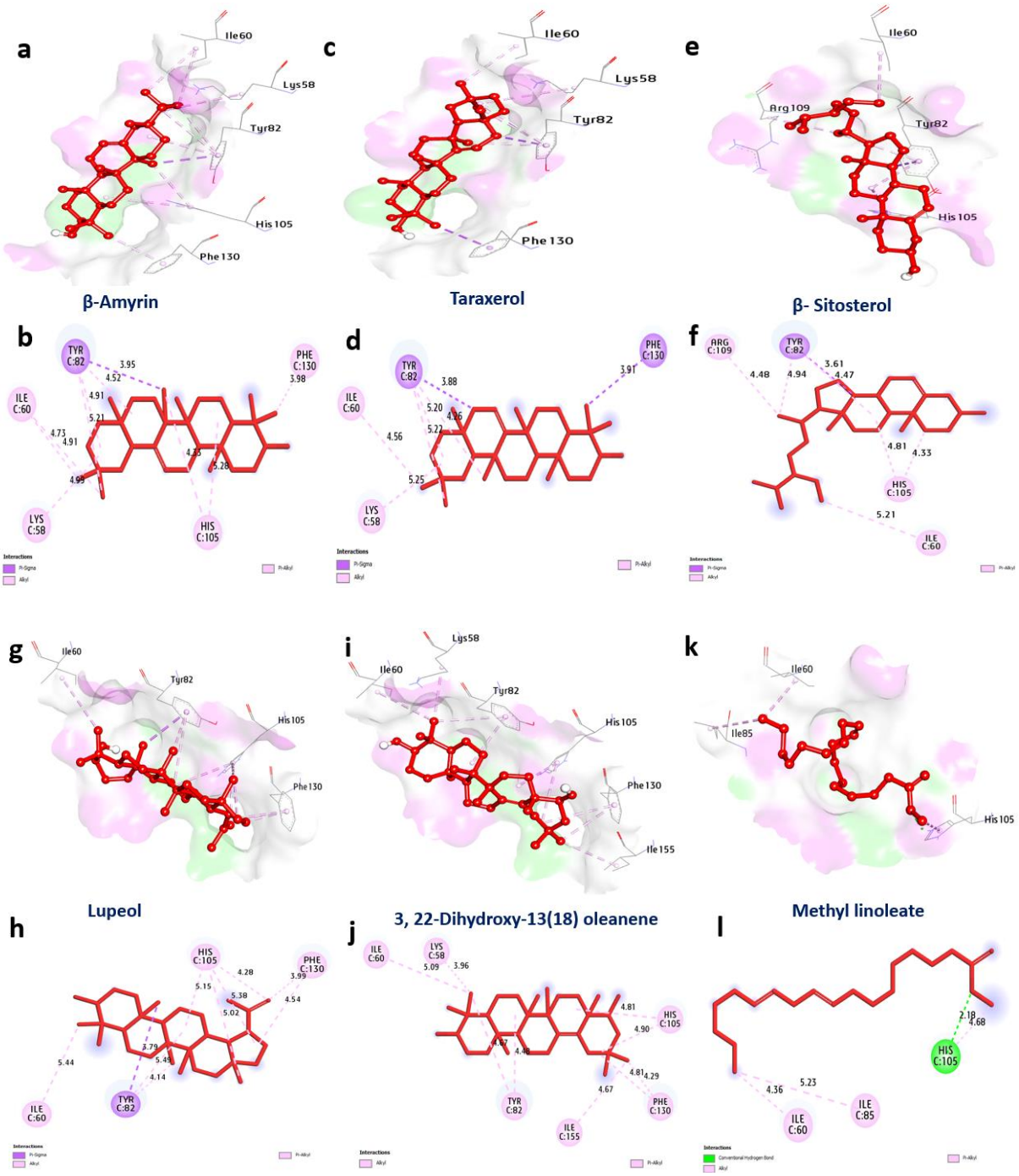

Fig.7 3D Interaction of compounds at the activity site of (TSHR) (PDB:ID 2XWT) (a and b) β-amyrin, (c and d) taraxerol, (e and f) β- sitosterol, (g and h) lupeol, (I and j) 3, 22-dihydroxy-13(18) oleanene and (k and l) methyl linoleate.

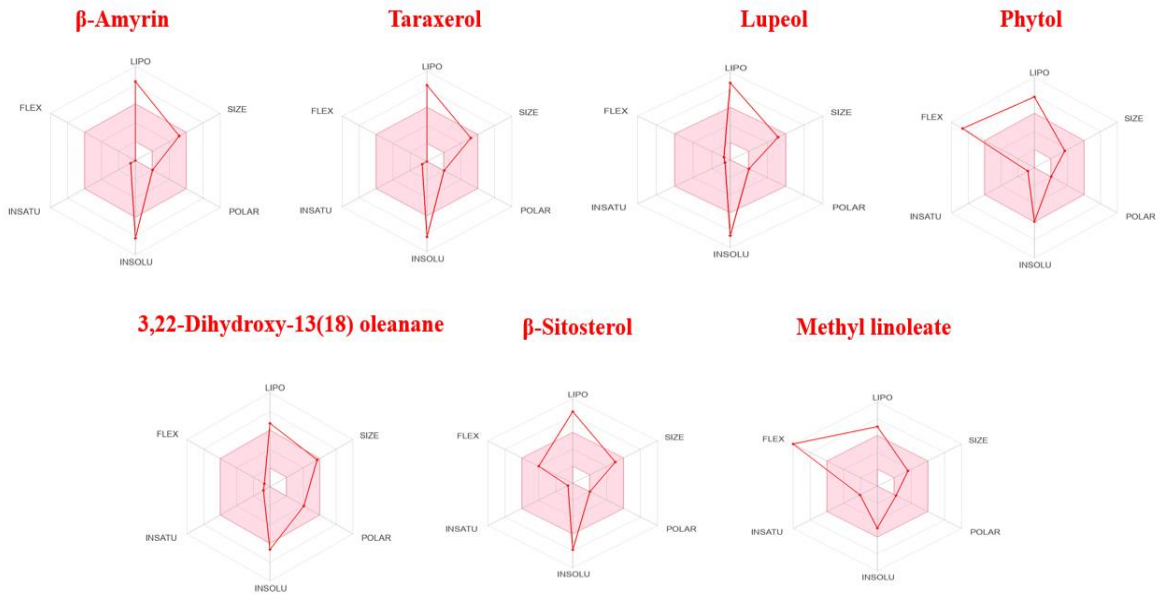

Fig.8. Chemicals' oral bioavailability calculated by the Swiss ADME approach

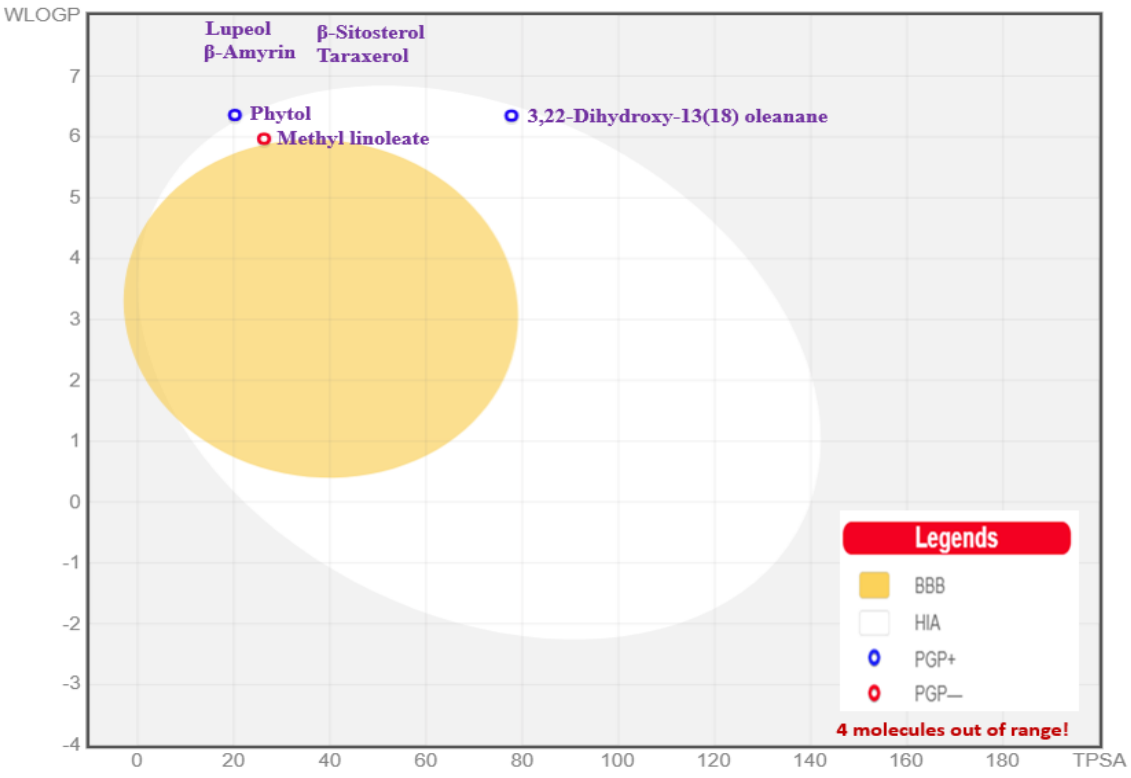

Fig.9. The boiled egg model for selected compounds.

However, correlating the remarkable hypothyroidism activity implied by the pet. ether extract of *F. carica* leaves and its various phytochemical components, the following could be noted: Regarding fatty acids influence on thyroid activity, a recent study stated that T2 and T3 both inverted the fatty acid-induced response to insulin *via* diverse models, depending on the degree of saturation of the fatty acids. The mechanism of action of mitochondrial respiration decreased in the order palmitate-oleate-linoleate [46]. Prolonged alterations of thyroid hormones levels can result in physiological variations distressing many proteins, where polyunsaturated fatty acids supplementation can help prevent the adverse cardiac tissue remodeling accompanied by arrhythmia [47].

On the other hand, triterpe
nes are very common compounds in *Ficus* plants where numerous studies proved their anti-inflammatory effects. Each triterpene skeleton possesses its action on certain inflammatory markers, e.g. ursane- type inhibits 5-1ipoxygenase activity, while the endogenous corticoid is prohibited by oleanane-derivatives (in our study isolated compounds 1, 2 and 4 namely: α-amyrin, taraxerol, and 3, 22 dihydroxy-13 (18) oleanene, respectively). Moreover, lanostanes are specific for phospholipase while unsaturated triterpenes guard against peroxidation [48].

A recent study was underdone on F. *carica* leaves detected the presence of phytol as a major compound in the unsaponifiable fraction, in addition to lupeol, α –amyrin and β-sitosterol while, linoleic was detected from the saponifiable fraction of the leaves. These findings supported our GC/MS data [35]. It is noteworthy that the isolated compounds (1-5) were all formerly detected from *F. carica* different parts. Only β-sitosterol has been previously isolated from the leaves [45].

α -Amyrin and its esters have been evaluated for COX-1 and 5-LOX activities. They showed considerable effect on both indices through carrageenan paw edema test in rats. The study suggested similarity of the anti-inflammatory effect of the compounds to that of methotrexate on neutrophils. It was found that their mechanism was implied by stabilization of plasma membranes [48]. Taraxerol, a pentacylic triterpene, attained many pharmacological activities; antioxidant, anti-inflammatory besides its effect on oxidative and inflammatory markers in induced cardiotoxicity rats [49]**.** It was previously detected in the plant aerial parts but isolated for the first time in our study [50].

Lupeol, a bioactive triterpene isolated from *F.carica* extract in our study, displays a wide range of bioactivities being antioxidant, anti-inflammatory and cytotoxic. It can be used as chemopreventive to avoid several diseases, in addition to its wound healing role in diabetic patients [51, 52]. The hypothesized mechanism by which lupeol possessed its action was through PI3K/Akt Wnt pathways prohibition. Additionally, Siddique et al., proved the competitive androgen receptor antagonist effect of lupeol through DNA inhibition to binding sites of Ca P cells, and so weakening of their responsive genes. Hence, lupeol was proven to be potent anti- prostate cancer agent [53]. Additionally, β-sitosterol acted as a 5-α-reductase inhibitor and it acts as anti-inflammatory agent due to blocking of 5-lipoxygenase pathways of arachidonic acid [54, 55].

Phytol is an aromatic diterpene widely used fragrance and cosmetic industry, while it medicinally possessed antioxidant, antinociceptive, anti-inflammatory, and anti-allergic effects as well as excellent immunostimulant and antimicrobial activity against many bacterial strains [56].

Thyroid gland is an endocrine organ that is liable for the creation, stockpiling, and arrival of thyroid chemicals triiodothyronine (T3) and thyroxin (T4). These chemicals are fundamental for cell development and improvement [57]. One of the thyroid problems is hypothyroidism which is characterized as a lack in thyroid hormones creation because of thyroid gland dysfunction prompting disturbance in the synthesis and secretion of hormones [31]. The ongoing examination planned to assess the hormonal and histological changes in hypothyroid-model, likewise to evaluate whether the extract has expected enhancing impacts against hypothyroidism**.**

In the present investigation, the thyroid gland's histological results, which included signs of hyperplasia, cellular cytoplasmic vacuolation, dilated congested blood capillaries, and an increase in follicular cell height in addition to an increase in thyroid follicle diameter, validated the hypothyroid status. Furthermore, vacuolated and degraded thyroglobulin aggregates were observed in both the inter-follicular and follicular cells. Comparable outcomes were reported by [58]. It is commonly known that thyroid hormones regulate a wide range of bodily processes, including the metabolism of fats and carbohydrates, the intake of oxygen, and several physiological processes like growth, development, and reproduction. Furthermore, a number of factors, such as stress and circadian rhythm, influence the TSH level rather than thyroid function [59]. Some biochemical and clinical disorders, such as hyperthyroidism and hypothyroidism, are brought on by changes in the normal amounts of T3 and T4. Reactive oxygen species (ROS) production is thought to be directly associated with thyroid dysfunction. Thyroid hormone-induced tissue damage may be explained by oxidative stress, which primarily targets mitochondria within cells [60]. Because they produce ROS, mitochondria have a significant impact on how cells

function. Numerous physiological symptoms are linked to thyroid dysfunction. Lipid peroxidation is one of these that arises from either excessive or insufficient thyroid hormone output. According to Yang et al. [61]**,** hypothyroidism is therefore known to cause metabolic suppression, a decrease in respiration rate, a decrease in the production of free radicals, and a decrease in peroxide levels. It has been well documented that thyroid dysfunctions increase lipid peroxidation reactions and reactive oxygen species [61]. Zhou et al. [62] found that cytokines have a significant impact in the development of hyperthyroidism and that IL-6, in particular, is critical in vascular inflammation due to its diverse biological actions in distinct target cells. TSH, IL-1, and maybe TSH receptor antibodies cause the thyroid gland to create IL-6 [63]. As a result, it influences B lymphocyte function, causes the body to manufacture autoantibodies, and raises the risk of hyperthyroidism [64].

Conclusion

Ficus carica leaves pet. ether extract had demonstrated both protective and curative effects against hypothyroidism disorder. In addition, it possessed significant antioxidant and anti-inflammatory properties. The exerted effects could be attributed to the diversity and synergy of its lipoidal content. The results were emphasized by molecular docking studies of compounds exhibited high binding interactions towards the specific tested biomarkers receptors.

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Conflict of interest

The authors declare no conflict of interest achieving this research study.

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