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Anti-inflammatory and Antioxidant Activity Screening for New Steroidal Schiff Bases

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Abstract: In this study, two aldehyde derivatives were synthesized from cholesterol and ergosterol, respectively, and subsequently reacted with three different primary amines (2-amino 6-fluoro benzathiazole, thiosemicarbazide, carbohydrazide) to form the corresponding Schiff's bases. The synthesized compounds were characterized using various spectroscopic techniques, including IR, ^{1}H-NMR, and GC Mass. Furthermore, the compounds were evaluated for their antioxidant and anti-inflammatory properties. Among the synthesized compounds, three to four compounds exhibited noteworthy anti-inflammatory and antioxidant activities.

Keywords: *Steroidal derivatives; Cholesterol; Ergosterol; Anti-inflammatory; Antioxidant; Schiff bases*

1 Introduction

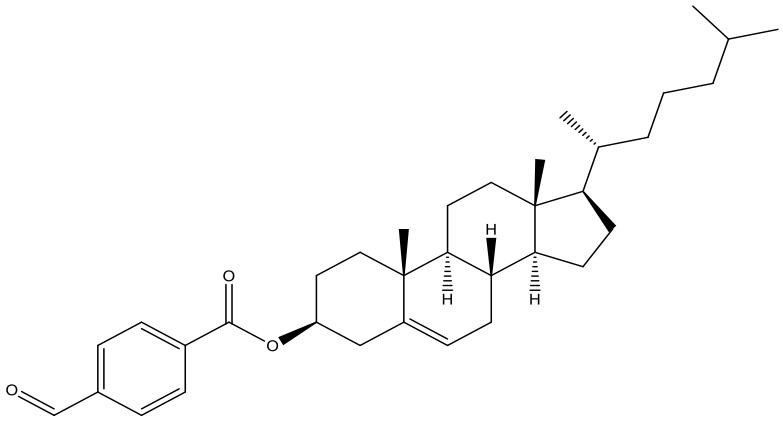
Steroids are polycyclic substances with a variety of biological functions. They are highly lipophilic and easily penetrate most cells to interact with intracellular receptors since they are bio-synthesized from cholesterol through a sequence of enzyme-mediated transformations, making them the perfect agents for treating a wide variety of illnesses (Tantawy, Nafie, Elmegeed, & Ali, 2017). Cholesterol is implicated in drug binding to G protein-coupled receptor (GPCR) targets, which is controlled by the membrane environment and external functions, according to a wealth of experimental and computational evidence (Kiriakidi et al., 2019). Ergosterol has long been recognized as a target for the action of antifungals and as a factor in the regulation of membrane fluidity and structure. Ergosterol, however, is an immunologically active lipid that triggers pyro ptosis, and two recent investigations have shown that practically all of its biosynthesis processes are viable therapeutic targets (Rodrigues, 2018). Natural plants are also sources of steroid compounds as described in several studies (El bastrioui *et al.,* 2022; Oussaid *et al.,* 2020). Schiff initially described Schiff bases in 1864, which are condensation byproducts of primary amines with carbonyl compounds. Numerous investigations revealed the significant chemical and biological significance of a lone pair of electrons in the sp2 hybridized orbital of the nitrogen atom of the azomethine group (Ashraf *et al.,* 2011). Schiff base of sulphanilamide has been developed, the condensation of 4-aminobenzene-1-sulfonamide

(sulphanilamide drug) with 4-Hydroxy-3-methoxybenzaldehyde (vanillin), yielded derivative of Schiff base in good yield and giving less toxic effect than sulphanilamide drug itself (Al-Halfi *et al.,* 2020). The Steglich esterification of cholesterol and ergosterol to an aldehyde is a variation of an esterification with dicyclohexylcarbodiimide as a coupling reagent and 4-dimethylamino pyridine as a catalyst (Neises & Steglich, 1978). Since the production of ester functional groups is crucial for the synthesis of many commercially accessible medications and building blocks, esterification is commonly considered as a key transition within organic and medicinal chemistry (Jordan *et al.,* 2021). Sweah & Auribie, 2020 created a collection of Schiff bases made from variously substituted aniline compounds and both aliphatic (Glyoxal) and aromatic (Vanillin) aldehydes. They were also created in complexes with Iron (III). Escherichia coli and Staphylococcus aureus, Gram-positive and Gram-negative strains of two different kinds of human pathogenic bacteria were utilized to test the Schiff base library and its complexes. The antibacterial properties of the Schiff bases and their complexes on Staphylococcus aureus varied (Sweah & Auribie, 2020). Four novel triazole Schiff Bases were produced by Aghaward SA *et al.* using the green condensation method. The findings demonstrated that the substances had a strong binding energy to block the receptors 5EKN and 3PP0, demonstrating their anticancer efficacy (Azad Aghaward *et al.,* 2023). In the current study, ergosterol-cholesterol aldehydes were used to synthesis a novel series of steroidal derivatives that were then tested for their anti-inflammatory and antioxidant properties.

2 Method And Materials

The synthesized compounds were first purified by silica column gravity chromatography using appropriate solvents with eluent ratio (10-50% ethyl acetate in chloroform). The IR spectra were captured using an FT-IR Affinity-1 spectrophotometer by Bruker at Thi Qar University's College of Education for Pure Sciences. At the Chemistry Department of the University of Basrah's College of Education for Pure Sciences, nuclear magnetic resonance Spectra appeared conducted. ^{1}H NMR was recorded on a NEO 400 (400 MHz) Bruker Avance spectrometer making use of the leftover solvent as the internal reference in all cases in deuterated chloroform using TMS as an internal standard. Chemical shift is given in δ ppm. The mass spectra at Samarah University were captured by the use of a Shimadzu GCMS-QP2010 plus model. (**Scheme 1-4**)

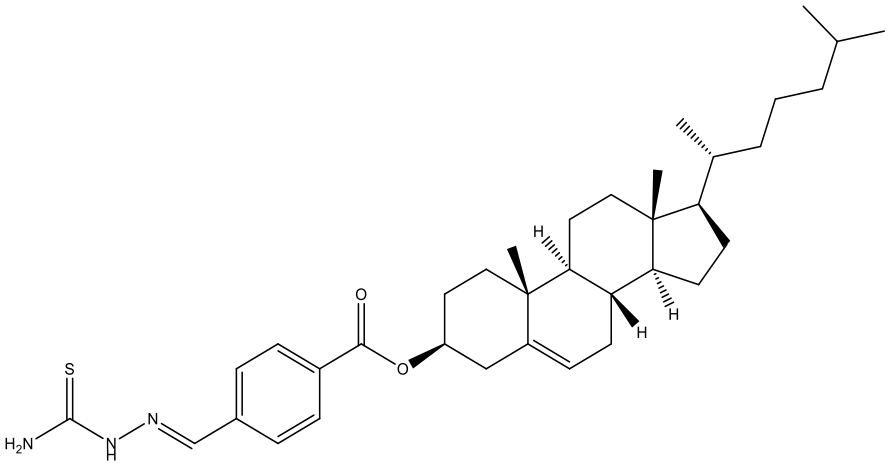
(1) 3S,8S,9S,10R,13R,14S, and 17R-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl4-formylbenzoate (ald.1)



Chemical Formula: $C_{35}H_{50}O_3$
Molecular Weight: 518.78

In round bottomed flask, cholesterol (3 gm, 7.759 mmol), as well as Four-carboxy benzaldehyde (7.759 mmol in 1.2 gm), 4-dimethylamino pyridine (DMAP) (0.24 gm, 1.939 mmol), dicyclohexylmethanediimine (DCC) (1.61 gm, 7.76 mmol) were placed then the round was evacuated, and Dry dichloromethane (DCM) 30 mL was added. For seven hours, the reaction was agitated at 0° C. TLC (thin layer chromatography) kept an eye on the reaction (5 % methanol in DCM). 50 mL DCM was added to the reaction product once the reaction was finished. The product was filtered in Buchner funnel. Another 50 mL of DCM was added, and the product was filtered again. The product was left to evaporate (the solvent) at room temperature. It was chromatographed on silica gel in 5% ethyl acetate in DCM to obtain cholesterol aldehyde as light white crystals, (3.8 gm pure product, 69%, 7.375 mmol). TLC R_f = 0.87 (5% methanol in DCM). ^{1}H NMR (400 MHz, $CDCl_3$ deuterated chloroform) δ 10.1 (s, 1H), 8.2 (d, 2H), 7.9 (d, 2H), 5.4 (t, 1H), 4.9 (m, 1H), ppm. (KBr disk, cm^{-1}) IR: 3010.44(stretching of Aromatic C-H), 1711(stretching of ArC=O), 2862-2948(stretching of Aliphatic or cyclic C-H, M^+ ion peak (519).

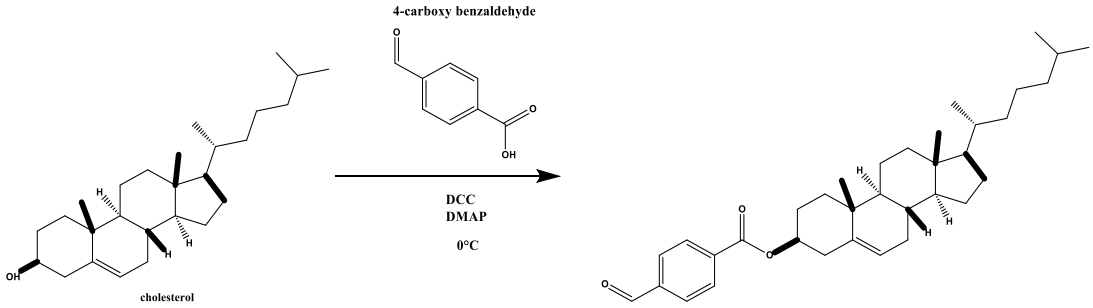
(2) Synthesis of (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl4-((E)-(2-carbamothioylhydrazineylidene) methyl) benzoate (t1)



Chemical Formula: $C_{36}H_{53}N_3O_2S$
Molecular Weight: 591.90

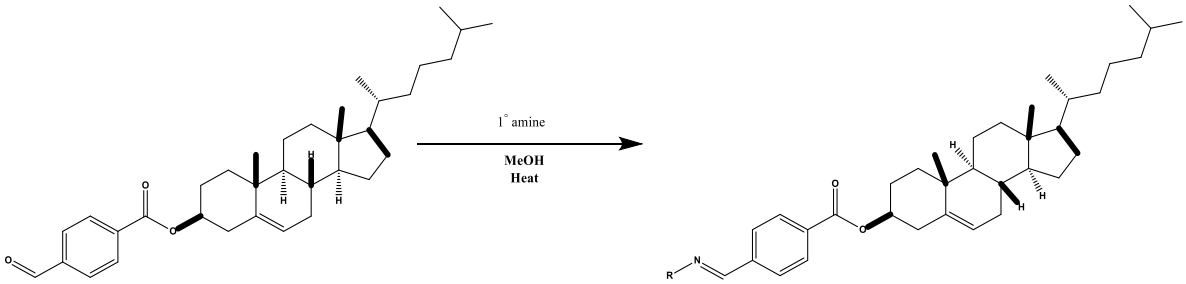
In round bottomed flask, thiosemicarbazide (0.035 gm, 0.385 mmol) was solubilized within 5 mL of methanol and placed under reflex in thermal circulator for 15 minutes for complete solubility. Cholesterol aldehyde derivative (ald.1) (0.2 gm, 0,385 mmol) was then added to the flask and the mixture is heated under reflex with stirring for 5 hrs. TLC kept an eye on the reaction (10 % ethyl acetate in DCM). It was chromatographed in 20 % ethyl acetate in DCM on silica gel for obtaining product as light-yellow crystals, (0.1 gm pure product, 50%, 0.169 mmol). TLC R_f = 0.62 (10% ethyl acetate in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.8 (s, 1H), 7.9 (s, 1H), 8.1 (d, 2H), 7.7 (d, 2H), 6.5 (s, 1H), 5.5 (d, 1H), 4.8 (m, 1H) ppm. IR (KBr disk, cm^{-1}): 3430(stretching of Secondary N-H), 3146, 3235(stretching of Primary NH_2), 1696(stretching of C=N), 1711(C=O), 2849-2935(stretching of Aliphatic or cyclic C-H), 3047(stretching of Aromatic C-H), M^+ ion peak (591). Thiosemicarbazides have promising antitubercular, antibacterial and herbicidal properties. Also, complexes of thiosemicarbazides with metals ions can hugely increase their activity (Rogalewicz *et al.,* 2022; Al-Mutabagani *et al.,* 2021; Muhammad *et al.,* 2020; Bahojb Noruzi *et al.,* 2019).

Scheme 1



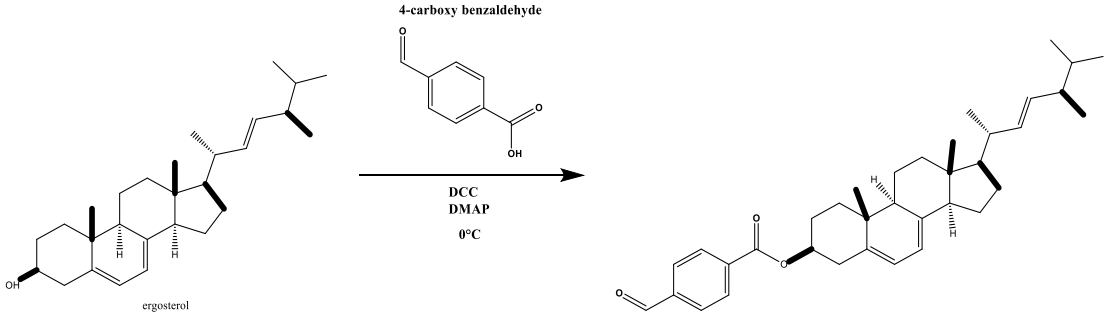
Scheme 1. General pathway for the synthesis of cholesterol aldehyde derivative

Scheme 2



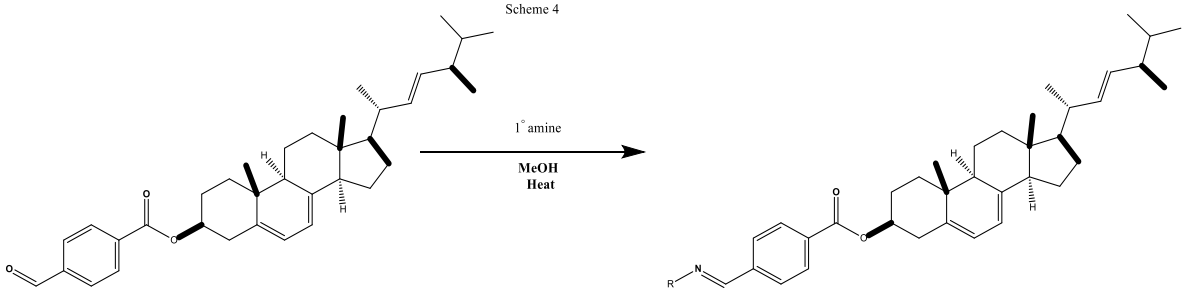
Scheme2. General pathway for the synthesis of cholesterol Schiff base derivatives

Scheme 3



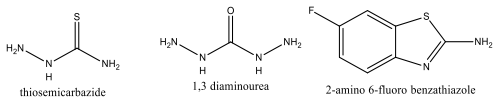
Scheme 3. General pathway for the synthesis of ergosterol aldehyde derivative

Scheme 4



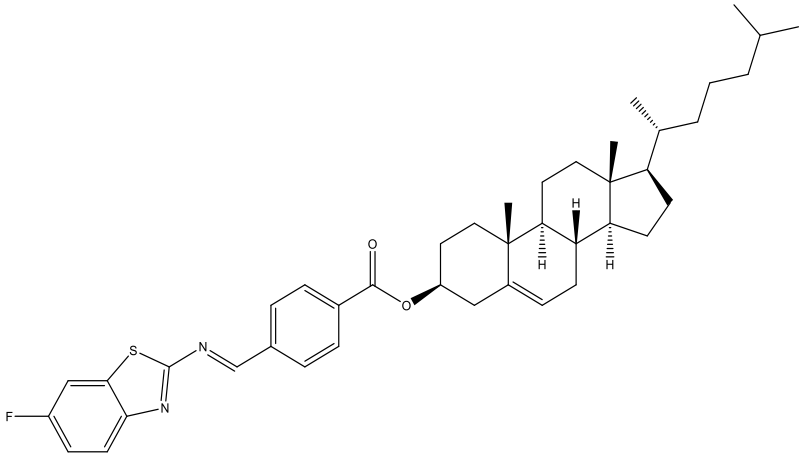
Scheme 4. General pathway for the synthesis of ergosterol Schiff base derivatives

Note: If R is thiosemicarbazide, the resulting compound either t1 or t2
If R carbohydrazide, the resulting compound is either c1 or c2
If R is 2-amino 6-fluoro benzathiazole, the resulting compound is either a1 or a2



Scheme 1-4. general pathways for synthesis of schiff base derivatives

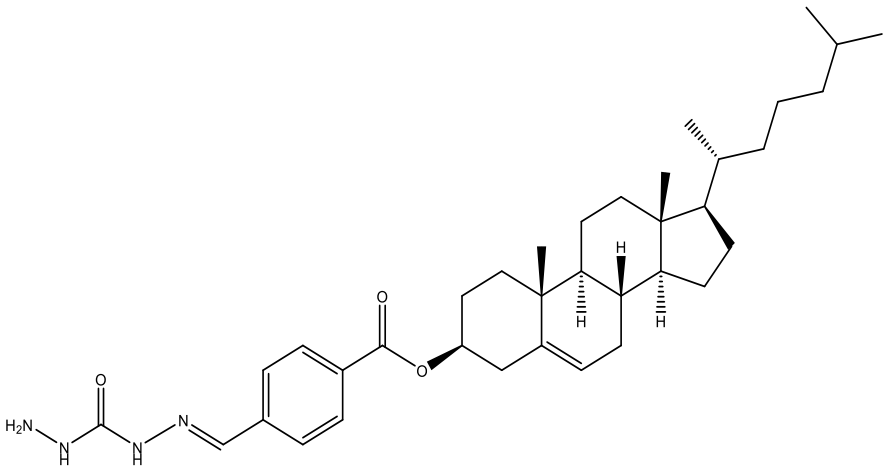
(3) Synthesis of (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3 -yl4-((E)-((6-fluorobenzo[d]thiazol-2-yl) imino) methyl) benzoate (a1)



Chemical Formula: $C_{42}H_{53}FN_2O_2S$
Molecular Weight: 668.96

In round bottomed flask, 2-amino 6-fluoro benzothiazole (0.13 gm, 0.77 mmol) was completely dissolved after being solubilized in 3 mL of methanol and placed under reflex in a thermal circulator for 15 minutes. Cholesterol aldehyde derivative (ald.1) (0.4 gm, 0.385 mmol) was then added to the flask and the mixture is heated under reflex at 150°C while stirring for 5 hours. Yellow appeared immediately after the addition of aldehyde. TLC kept an eye on the reaction (10 % ethyl acetate in chloroform). To obtain the product as light-yellow crystals, on silica gel, it underwent chromatography in ten % (Acetate of ethyl in DCM), (0.025 gm pure product, 5%, 0.037 mmol). TLC R_f = 0.5 (10% Acetate of ethyl in chloroform). ^{1}HNMR (400 MHz and $CDCl_3$) δ 9.1 (s, 1H), 8.21 (d, 2H), 8.1 (d, 1H), 7.9 (d, 1H), 7.5 (d, 1H), 7.1 (t, 1H), 5.5 (t, 1H), 4.9 (m, 1H) ppm. IR (KBr disk, cm^{-1}): 1706(stretching of C=O), 1641(stretching of C=N), 3093(stretching of Ar-CH), 2859-2933(stretching of Cyclic or aliphatic C-H), M^+ ion peak (668).

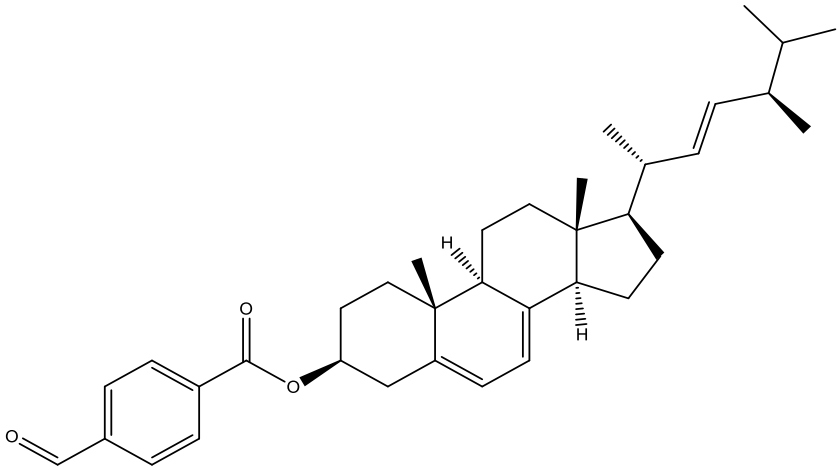
(4) Synthesis of (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 4-((E)-(2- (hydrazine carbonyl) hydrazinylidene) methyl) benzoate (c1)



Chemical Formula: $C_{36}H_{54}N_4O_3$
Molecular Weight: 590.85

In round bottomed flask, carbohydrazide (0.112 gm, 1.23 mmol) was solubilized in 5 mL of methanol and placed under reflex in thermal circulator for 15 minutes for complete solubility. Cholesterol aldehyde derivative (ald.1) (0.641 gm, 1.23 mmol) was then added to the flask and the mixture is heated under reflex with stirring for 5 hrs. TLC kept an eye on the reaction (10 % ethyl acetate in chloroform). It was chromatographed on silica gel in 10% ethyl acetate/DCM to obtain product as light-yellow crystals, (0.056 gm pure product, 8%, 0.095 mmol). TLC R_f = 0.5 (10 % ethyl acetate in chloroform).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.7 (s, 1H), 8.3 (s, 1H), 8.2 (d, 2H), 8 (d, 2H), 5.5 (t, 1H), 5.3 (m, 1H), 4.9 (s, 2H) ppm. IR (KBr disk, cm^{-1}): 1714 (stretching of C=O), 1653 (stretching of C=N), 2851-2942 (stretching of Cyclic or aliphatic C-H) M^+ ion peak (591). Survey literature indicated that carbohydrazide complexes enhance the structural therapeutic effects and variety of its pharmaceutical activities like: antitumor, antifungal and anti-HIV (Abdel-Rahman *et al.,* 2023; Kumar *et al.,* 2023; Al-Zakri *et al.,* 2020; Warad *et al.,* 2013).

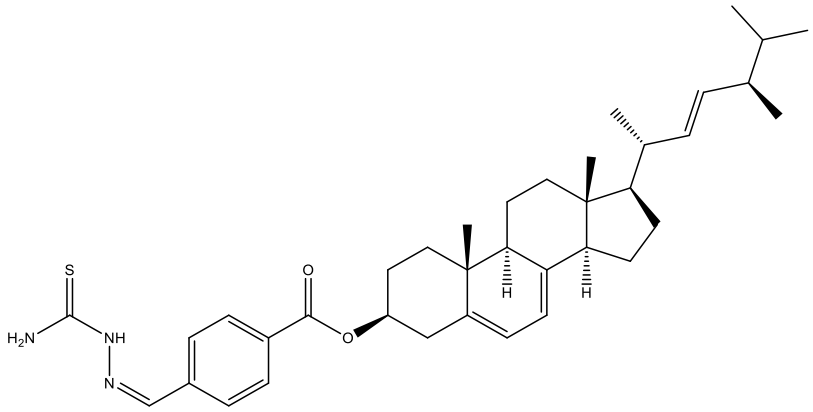
(5) Synthesis of (3S,9S,10R,13R,14R,17R)-17-((2R,5R, E)-5,6 dimethylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta [a] phenanthren-3-yl 4-formylbenzoate (ald.2)



Chemical Formula: $C_{36}H_{48}O_3$
Molecular Weight: 528.78

In round bottomed flask, provitamin D2 (3 gm, 7.563 mmol) and 4-carboxy benzaldehyde (1.135 gm, 7.563 milli moles), 4-dimethylamino pyridine (DMAP) (0.231gm, 1.891mmol), dicyclohexylmethanediimine (DCC) (1.56 gm, 7.563 mmol) were placed then the round was evacuated, and 30 mL of dry dichloromethane was added. The reaction was stirred at 0° C for 7 hours. The reaction was monitored by TLC (5% ethyl acetate in DCM). After completion of reaction, 50 mL DCM was added to reaction product. The product was filtered in Buchner funnel. Another 50 mL DCM was added, and the product was filtered again. The product was left to evaporate (the solvent) at room temperature. It was chromatographed on silica gel in 10% ethyl acetate in DCM to obtain Ergosterol (provit D2) aldehyde as light brown crystals, (0.825 gm pure product, 15%, 1.56 mmol). TLC R_f = 0.9 (10% methanol in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.1 (s, 1H), 8.3 (d, 2H), 8.2 (d, 2H), 8 (d, 2H), 5.4 (s, 2H), 5.2 (d, 2H), 4 (m, 1H) ppm. IR (KBr disk, cm^{-1}): 1710(stretching of aldehydic C=O), 2729(stretching of Aldehydic C-H), 2872-2959(stretching of Cyclic or aliphatic C-H), M^+ ion peak (528).

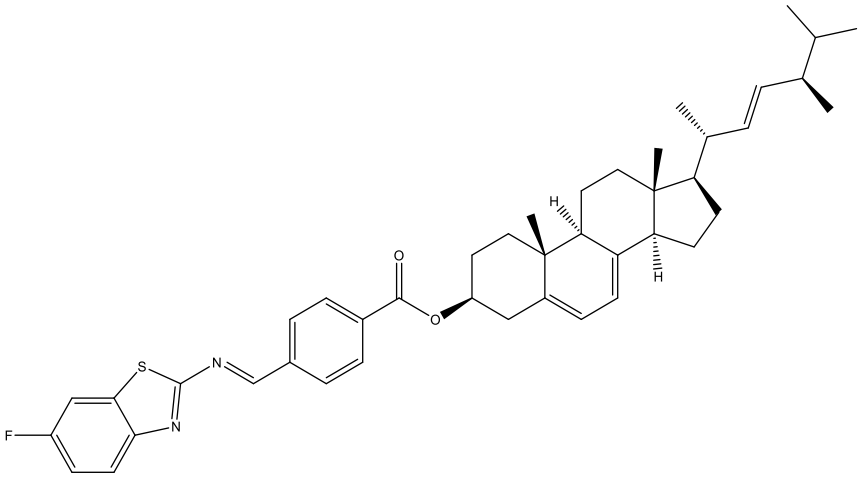
(6) Synthesis of (3S, 9S, 10R, 13R, 14R, 17R)-17-((2R, 5R, E)-5,6-di methyl hept-3 -en-2-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta [a]phenanthren-3-yl4-((Z)-(2-carbamothioylhydrazineylidene) methyl) benzoate (t2)



Chemical Formula: $C_{37}H_{51}N_3O_2S$
Molecular Weight: 601.89

In a round bottomed flask, thiosemicarbazide (0.052 gm, 0.567 mmol) dissolved in three milliliters of methanol with 3 mL of ethanol and placed under reflex in thermal circulator for 30 minutes for complete solubility. Ergosterol aldehyde derivative (ald.2) (0.3 gm, 0,567 mmol) was then added to the flask and the mixture is heated under reflex while stirring at 150 °C for 5 hours. A dark brown color appeared immediately after addition of aldehyde. TLC kept an eye on the reaction (10 % ethyl acetate in chloroform). To produce the product as dark-brown crystals, 10 % ethyl acetate in DCM was used to chromatograph it on silica gel, (0.05 gm pure product, 18%, 0.083 mmol). TLC R_f = 0.75 (10 % ethyl acetate in chloroform). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.5 (s, 1 H), 8.11 (d, 2H), 7.9 (s, 1 H), 7.7 (d, 2H), 6.5 (s, 1H), 5.3 (d, 2H), 5.2 (d, 2 H), 4 (m, 1 H) ppm. IR (KBr disk, cm^{-1}): 3420(stretching of primary N-H), 3162-3263(stretching of Secondary N-H), 2869-2956 (stretching of Cyclic or aliphatic C-H), 1698(stretching of C=C), 1716(stretching of C=O), M^+ ion peak (601)

(7) Synthesis of (3S, 9S, 10R, 13R, 14R, 17R)-17-((2R, 5R, E)-5,6-di methyl hept-3-en-2-yl)-10,13-dimethyl-2,3,4, 9,10, 11, 12, 13, 14, 15, 16, 17-dodeca hydro-1H cyclopenta [a]phenanthren-3-yl4-((E)-((6-fluorobenzo[d]thiazol-2-yl)imino)methyl)benzoate (a2)

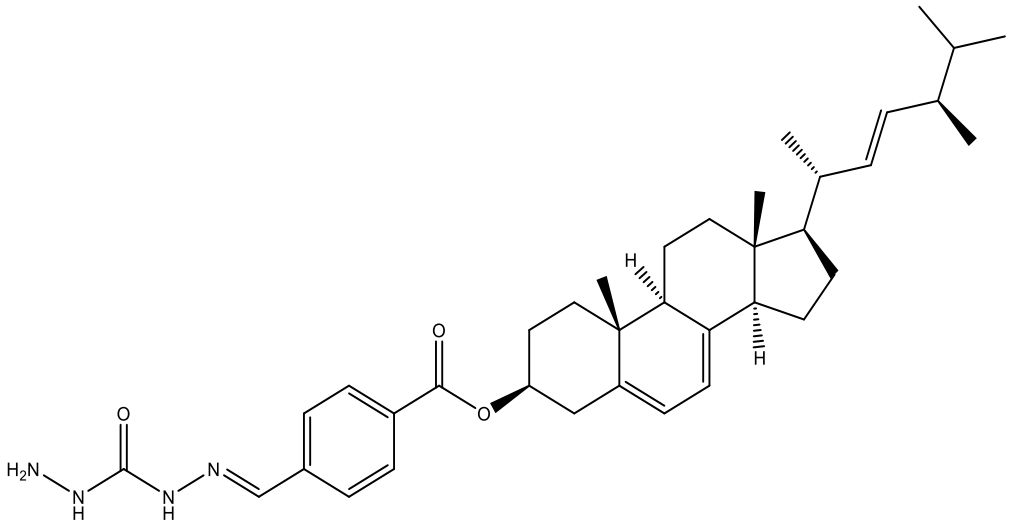


Chemical Formula: $C_{43}H_{51}FN_2O_2S$
Molecular Weight: 678.95

In round bottomed flask, 2-amino 6-fluoro benzothiazole (0.064 gm, 0.378 mmol) dissolved within three milliliters of methanol then placed under reflex in thermal circulator for 15 minutes for

complete solubility. Ergosterol aldehyde derivative (ald.2) (0.2 gm, 0.378 mmol) was then added to the flask with 3 mL of chloroform and the mixture is heated under reflex at 150°C while stirring for 4 hours. Yellow appeared immediately after the addition of aldehyde. TLC kept an eye on the reaction (Ethyl acetate in chloroform at 10%). To obtain the product as light-yellow crystals, it underwent chromatographic analysis with silica gel (ten % ethyl acetate in DCM), (0.05 gm pure product, 20%, 0.076 mmol). TLC R_f = 0.375 (10 % Acetate of ethyl with chloroform). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.1 (s, 1H), 7.7 (d, 1H), 7.5(t, 1H), 7.4 (t, 1H), 7.3 (d, 2H), 7.1 (d, 2H), 4.9 (d, 1H), 4.6 (s, 1H), 4.1 (m, 1H) ppm. IR (KBr disk, cm^{-1}): 2830-3000(stretching of cyclic or aliphatic C-H), 1728(stretching of C=O), 1629(stretching of C=N), 1573(stretching of C=C), M^+ ion peak (678).

(8) Synthesis of (3S, 9S, 10R, 13R, 14R, 17R)-17-((2R, 5R, E)-5, 6-di methyl hept-3-en-2-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenanthren-3-yl4-((E)-(2-(hydrazinecarbonyl)hydrazinylidene)methyl)benzoate (c2)



Chemical Formula: $C_{37}H_{52}N_4O_3$
Molecular Weight: 600.85

In round bottomed flask, carbohydrazide (0.017 gm, 0.189 mmol) dissolved within 4 milliliters of methanol then placed under reflex in thermal circulator for 15 minutes for complete solubility. Ergosterol aldehyde derivative (ald.2) (0.1 gm, 0.189 mmol) was then added to the flask with 4 mL of chloroform and the mixture is heated under reflex with stirring for 5 hrs. TLC kept an eye on the reaction (10 % ethyl acetate in chloroform). To produce the product as light-yellow crystals, 10 % ethyl acetate in DCM was used to chromatograph it on silica gel, (0.024 gm pure product, 20%, 0.04 mmol). TLC R_f = 0.385 (10% Acetate of ethyl in chloroform). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.4 (s, 1H), 8.1 (s, 1H), 8.05 (d, 2H), 8 (d, 2H), 7.7 (s, 1H), 6.4 (d, 2H), 5.3 (m, 1H), 5.1 (s, 1H), 4.6 (d, 2H) ppm. IR (KBr disk, cm^{-1}): 1719(stretching of C=O), 1604(stretching of C=N), 2870-2958(stretching of Cyclic or aliphatic C-H), 3264(stretching of Aromatic C-H), M^+ ion peak (601).

3 Anti-inflammatory In-Vitro Screening (Sathe *et al.*, 2011)

Protein Denaturation as a Method, the chemicals created were assessed for in-vitro anti-inflammatory efficacy (ref.). The investigated substances and the normal drug were dissolved in a modest amount of dimethyl sulfoxide (DMSO) and then diluted with phosphate buffer (0.2 M, pH 7.4). Less than 2.0 % of DMSO was present at the final concentration in each solution. The tested solution (1 mL) was combined with 1 mL of 0.2 M Bovine albumin solution in phosphate buffer, and all solutions were

then diluted with 4 mL of 0.2 M phosphate buffer saline. The tested solution was then placed in an incubator and kept at 27°C for 15 minutes. Denaturation was induced by holding the reaction mixture in a water bath at 60°C for 10 minutes. Then measurement of turbidity was carried out at 660 nm after cooling (U V-1100 Spectro photometer, E-Chrom Tech Co, Ltd.). The standard medication was dexamethasone. **Table 1** and **Figures 1 & 2** provided the results. control solution was solvent + reagent (denatured albumin) while blank solution was solvent + test or standard compounds:

$$\text{percentage of inhibition} = \frac{[\text{Abs660 (Control)} - \text{Abs660 (Test)}]}{[\text{Abs660 (control)}]} * 100$$

Table 1. Screening of synthetic compounds for anti-inflammatory properties

Tested Compound	Value of absorbance at 1000 µg/mL	% of denaturation inhibition	Absorbance value at 500 µg/mL	% of denaturation inhibition	Absorbance value at 250 µg/mL	% of denaturation inhibition
Control	0.050	-	0.050	-	0.050	-
Dexamethasone	0.215	330	0.170	240	0.060	20
ald.1	0.070	40	0.060	20	0.060	20
ald.2	0.060	20	0.056	12	0.056	12
c1	0.129	158	0.088	76	0.070	40
c2	0.086	72	0.075	50	0.073	46
t2	0.056	12	0.060	20	0.057	14
t1	0.186	272	0.124	148	0.085	70
a1	0.800	1500	0.290	480	0.260	420
a2	0.110	120	0.060	20	0.060	20
Ergosterol	0.070	40	0.060	20	0.056	12
cholesterol	0.170	240	0.150	200	0.130	160

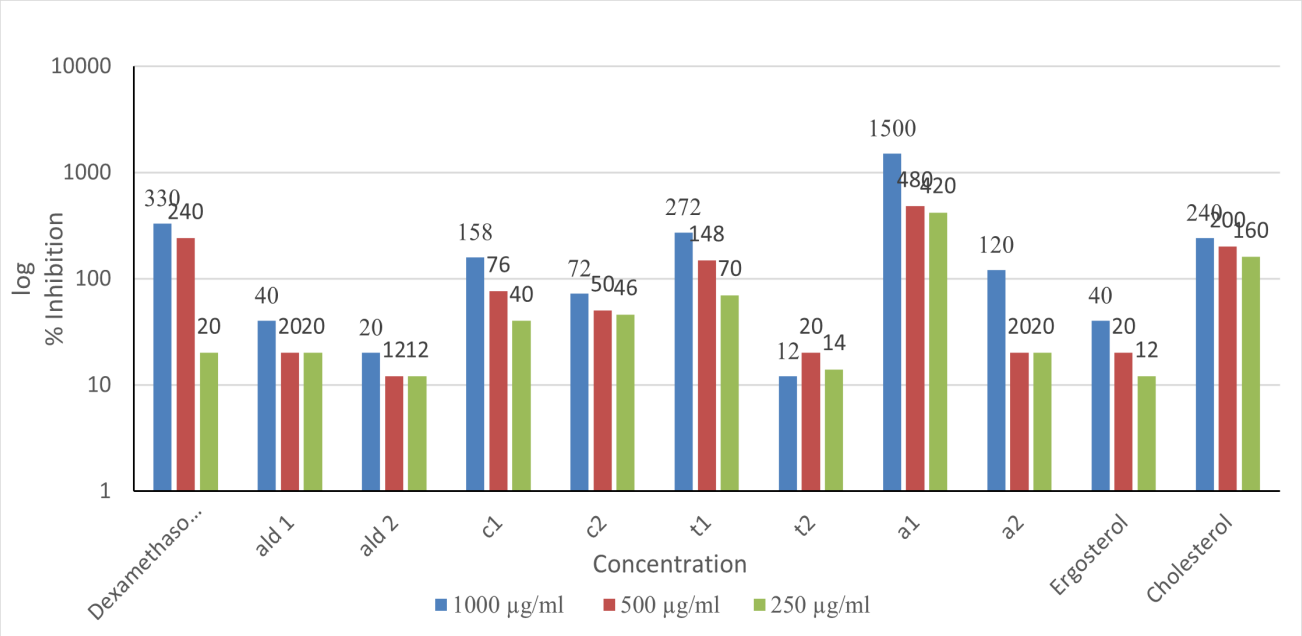


Figure 1. Comparing synthetic substances to Dexamethasone, the percentage of protein denaturation inhibition.

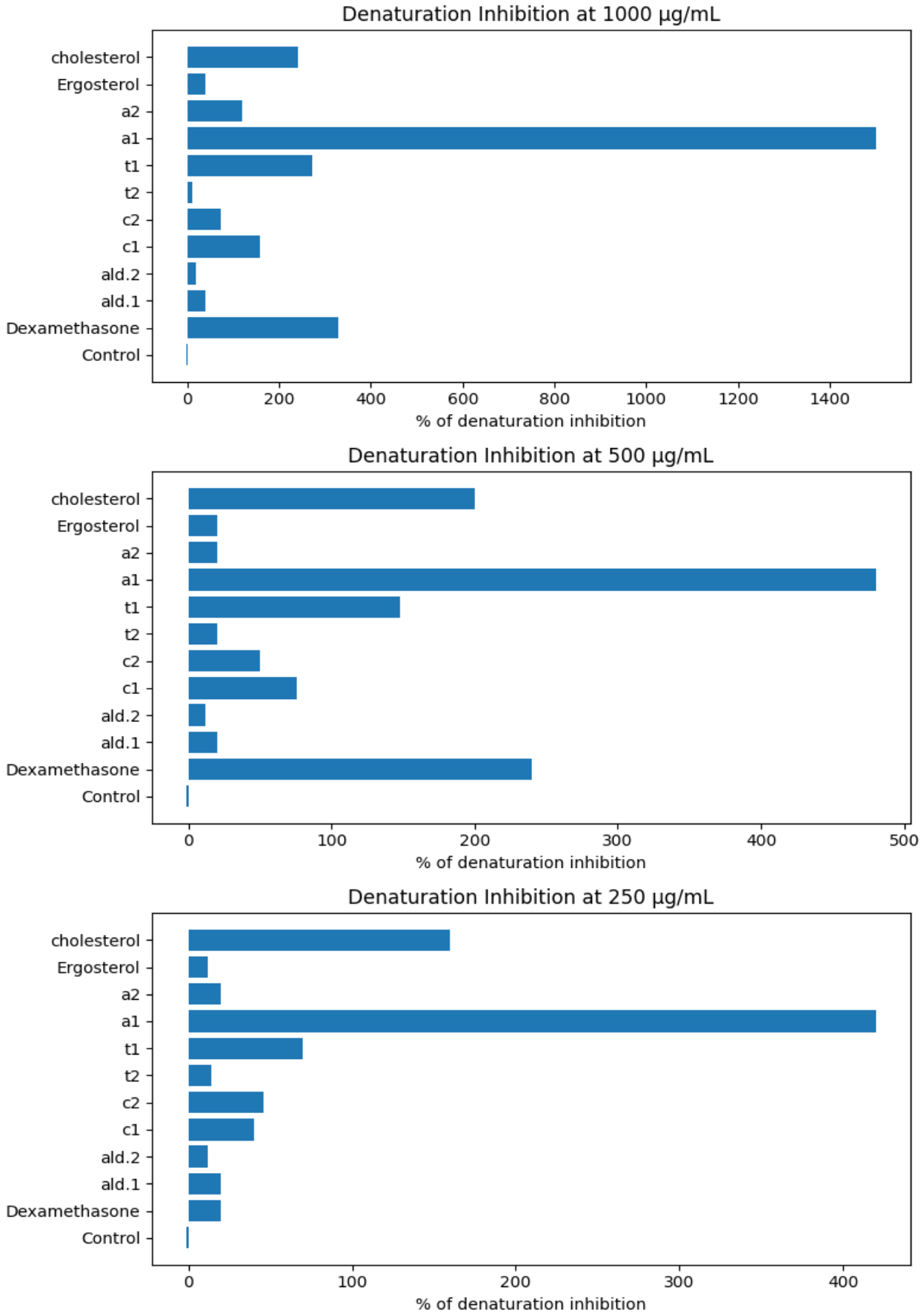


Figure 2. Another representation of anti-inflammatory activity of Schiff base derivatives (without log.).

4 In-vitro Antioxidant Screening (Subhashini *et al.*, 2011)

Using a hydrogen peroxide (H_2O_2) scavenging method, the produced compounds were tested for in-vitro antioxidant activity. The Standard (Ascorbic acid) and all the compounds were dissolved in DMSO as a solvent to create a stock solution (500 µg/mL), from which different concentrations (two-fold dilutions) of 125 and 250 µg/mL were generated in separate volumetric flasks. To 0.5 mL of each solution, the volume was raised to 2 mL with phosphate buffer saline 0.2M after 0.5 mL hydrogen peroxide was added (pH was 7.4). A drug-free control solution made with DMSO in phosphate buffered saline was created (0.5 mL DMSO +1 mL 0.2 M phosphate buffer saline + 0.5 mL H_2O_2 solution). The absorbance at 230 nm was recorded using a U.V spectrophotometer (UV-1100 Spectrophotometer, E-Chrom Tech Co, Ltd.) opposed to blank (Phosphate buffer saline). Using the following formula, the % inhibition by hydrogen peroxide scavenging activity was determined:

$$\text{percentage of inhibition} = \frac{[\text{Abs230 (Control)} - \text{Abs230 (Test)}]}{[\text{Abs230 (control)}]} * 100$$

control solution was solvent + reagent (hydrogen peroxide) while blank solution was solvent + test or standard compounds. The results were showed in **Table 2**, **Figure 3**

Table 2. Antioxidant Screening of synthesized compounds against H_2O_2

Compound	% Inhibition at 250 µg/mL	% Inhibition at 125 µg/mL
Control	Abs.= 0.45	Abs.= 0.45
t2	80	30
t1	97	33
c2	31	37
c1	2	33
ald.1	53	49
ald.2	75	53
a1	91	62
a2	42	42
Cholesterol	2	18
Ergosterol	84	59
Vitamin C	75	62

5 Assay for scavenging DPPH radicals (Rahman *et al.*, 2015)

The methanol-based 2,2-diphenyl-1-picrylhydrazyl solution was decolored to test the steroidal derivatives' capacity to donate hydrogen atoms (DPPH). When dissolved in methanol, DPPH produces a violet or purple hue that, in the presence of antioxidants, fades to a range of yellow hues. Samples (samples of investigated substances and vitamin C were produced with DMSO 1:1 0.2M phosphate buffer saline) at various concentrations (125,250 µg/mL) were combined with 0.5 mL of a 0.2 mg/mL DPPH in methanol solution. After fully vortexing, the reaction mixture was kept at room temperature for 30 minutes in the dark. After diluting the mixture with 1mL of 0.2 M phosphate buffer saline, at 517 nm, the mixture's absorbance was spectrophotometrically determined. Reference was made using control solution (DMSO + phosphate buffer saline + DPPH). The formula below was employed to compute the % of activity to scavenge DPPH radicals:

$$\text{The proportion of radical scavenging activity in DPPH} = \frac{(\text{A0} - \text{A1})}{\text{A0}} * 100$$

where A1 is the test/standard absorbance and A0 is the absorbance of the control. Three times at each concentration, the experiment was repeated, as shown in **Table 3** and **Figure 4**

control solution was solvent + reagent (DPPH reagent) while blank solution was solvent + test or standard compounds

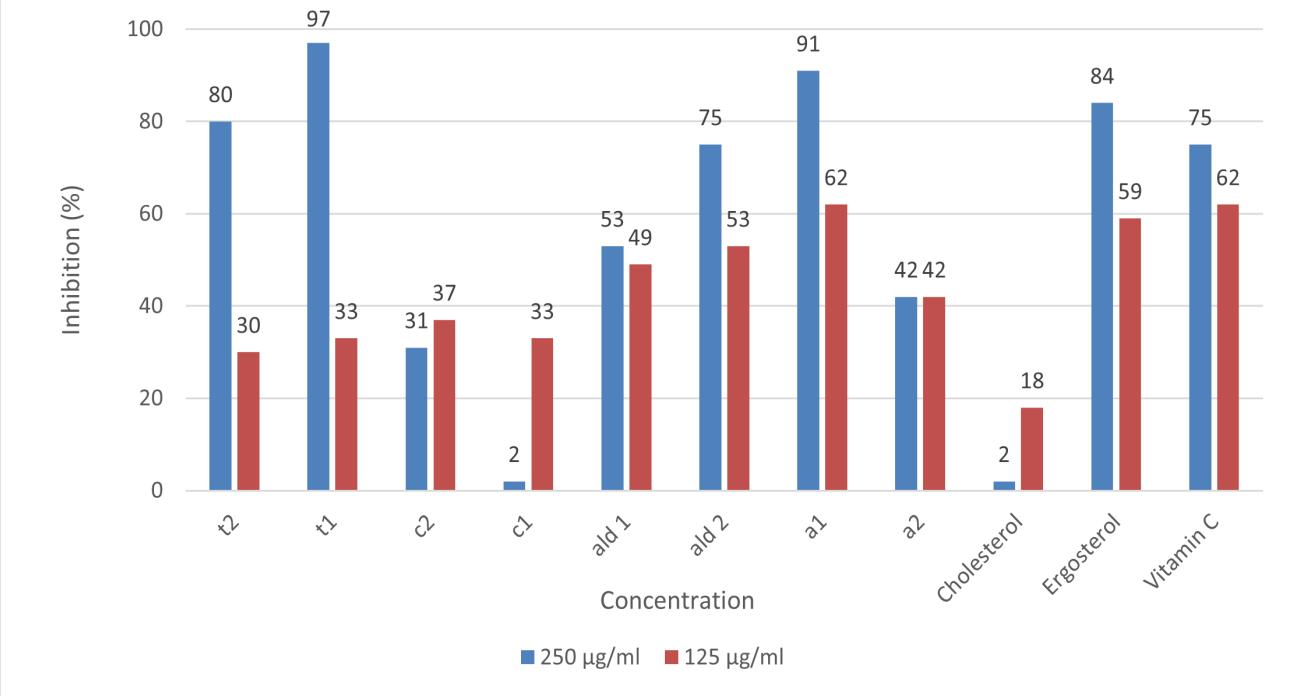


Figure 3. shows the percentage of synthetic chemicals' ability to scavenge hydrogen peroxide in contrast to vitamin C.

Table 3. Antioxidant screening of synthetic compounds against DPPH

Tested Compound	% Inhibition at 125 µg/mL	% Inhibition at 250 µg/mL
Control	Abs.= 0.26	Abs.= 0.26
t2	0	15
t1	0	19
c2	8	42
c1	19	31
ald.1	0	0
ald.2	42	58
a1	12	31
a2	31	58
Cholesterol	0	0
Ergosterol	0	0
Vitamin C	50	69

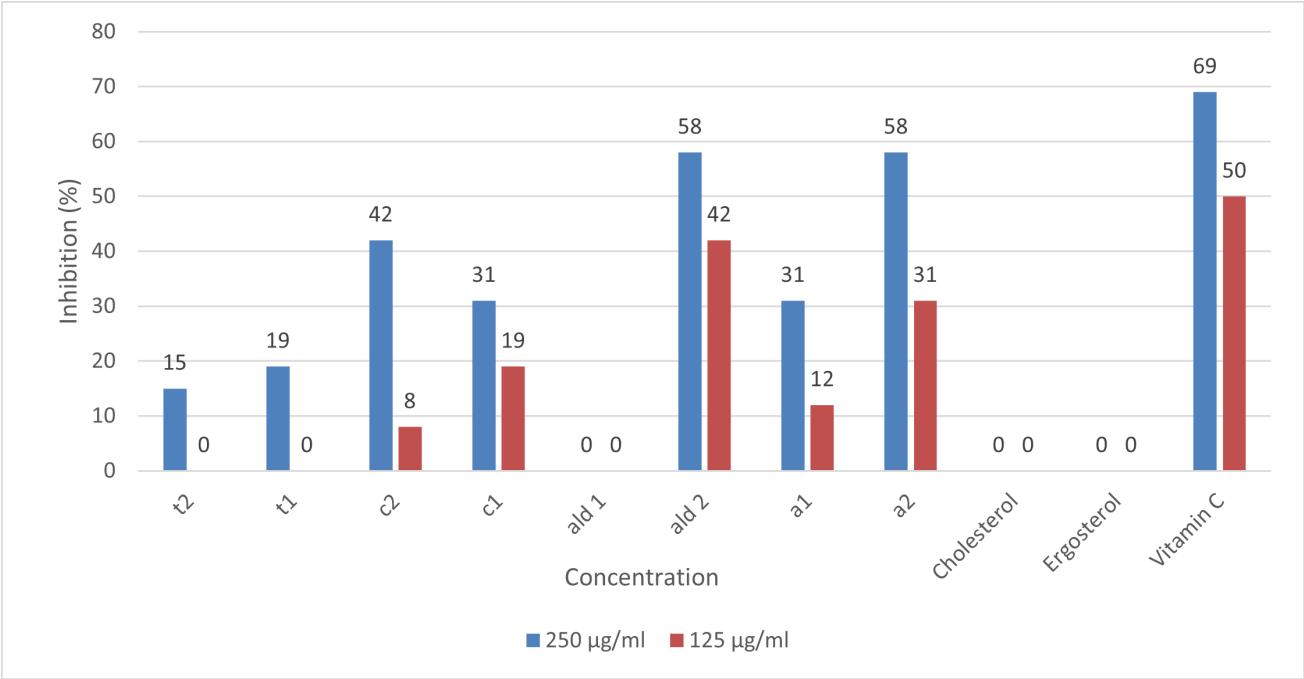


Figure 4. percentage inhibition of DPPH reagent scavenging activity of synthesized compounds in comparison to Vitamin C

6 Results And Discussion

6.1 Protein denaturation activity

The absence of the aldehyde band, which typically appeared around 10 ppm in the ^{1}H-NMR, and the appearance of the imine (HC=N) signal at roughly 9 ppm were the key changes to the NMR chart that occurred and that gave an explanation for what transpired (Pavia *et al.,* 2001). Schiff base steroidal derivatives' anti-inflammatory effects were assessed using the denaturation of bovine serum albumin technique (Dharmadeva *et al.,* 2018). At a concentration of 1000 g/mL, the concentration-dependent maximum inhibition rate was seen with a1, a2, c1, t1, and c2. The level of inhibition of a1 compound at 1000 µg/mL was significantly higher than dexamethasone. At concentration of 250 µg/mL, compound a2 inhibition rate was higher than dexamethasone while at concentration of 500 µg/mL, compound a1 inhibition rate was strongly higher than dexamethasone. It was also found that cholesterol and ergosterol had some anti-inflammatory effect. Protein denaturation involves changes to electrostatic hydrogen, hydrophobic, and disulfide bonds, and its mechanism is unexpected (Sen *et al.,* 2015). In inflammatory diseases such rheumatoid arthritis, cancer, and diabetes, protein denaturation results in the generation of autoantigens. So, it is possible to decrease inflammatory activity by preventing protein denaturation (Sangeetha & Vidhya, 2016). One particular class of medication (a steroid) served as the study's standard of reference. Dexamethasone, a synthetic glucocorticoid, inhibits the migration of immune cells and vasodilation, two causes of inflammation, and has been used in the past to treat autoimmune diseases such arthritis, asthma, and allergic reactions. Invading the host cell membrane, dexamethasone binds to glucocorticoid receptors in the cytoplasm. Due to a series of immune cell reactions brought on by this, pro-inflammatory cytokines like IL-1, IL-2, IL-6, IL-8, TNF, and IFN- are suppressed by lessening gene transcription (Patel *et al.,* 2020). In the present study, it was found that the inhibition rate of all tested compounds and dexamethasone gradually increases with the increase in concentration. Compounds a1, a2 are 2-amino 6-fluoro benzathiazole derivatives of

cholesterol, ergosterol respectively. 2-Amino benzothiazole derivatives are bicyclic ring system with multiple applications literature review anti-inflammatory properties (Evers *et al.,* 2002). **Figure 1**.

6.2 Antioxidant assays

The antioxidant capacity of endogenous compounds cannot be assessed using a single assay technique. The experimental design and assay idea used in various antioxidant tests also differ (Jadon *et al.,* 2017; Šeregelj *et al.,* 2020). As an illustration, various methods such as metal ions for oxidation, such as the FRAC assay procedure, while others use organic radical generators, such as DPPH (Gulcin, 2020). The time component involved in their chemical processes that result in free radicals through an oxidation reaction is distinct from one another. According to their rate and duration of scavenging, as a control, several antioxidants are thought of for various assay processes as different methodologies require distinct experimental setups and procedures. Anti-oxidants can either be polar or non-polar, such as vitamin E, phenolics, flavonoids, etc. They have the ability to neutralize free radicals by either giving electrons or hydrogen (Rahman et al., 2015).

6.2.1 Hydrogen Peroxide Scavenging Activity

By donating hydrogen, the extra electron that causes radical reactivity would be eliminated (Kadhum, Al-Amiery, Musa, & Mohamad, 2011). In the preceding ten years, researchers have been intensely interested in the topic of free radicals. Numerous professionals are interested in the variety of effects that free radicals can have on biological systems. Free radicals have been shown to play a critical part in the onset of some diseases and the aging process (Kadhum et al., 2011; Naama et al., 2013). Eight synthesized steroidal derivatives were tested for hydrogen peroxide-based in vitro scavenging efficacy. These tested compounds showed high scavenging activity (**Table 2**). The eight synthetic compounds displayed a potent scavenging action against H_2O_2 in **Table 2.** Except for c1, c2, a concentration-dependent decline in H_2O_2 activity with a relatively low concentration of 125 µg/mL was noticed. Compound c1 and cholesterol were shown to have very minimal inhibitory effect. The 250 µg/mL concentration was the highest found (**Table 2**). Compounds t1, t2, a1, and ergosterol demonstrated the strongest percentage scavenging activity, followed by ald2, ald1, and a2. Standard medications like vitamin C were employed, and its percentage of inhibition was 75.00 ± 2.00. Results indicated that new tested compounds like a1, t1, t2 have more scavenging power than vitamin C. The hydrogen-donating activity showed a high correlation between concentration of the Schiff base derivatives and rate of inhibition, with hydrogen peroxide radicals serving as the hydrogen acceptor (Al-Amiery, Al-Majedy, Ibrahim, & Al-Tamimi, 2012). These steroidal derivatives showed their capacity to reduce the stable radical using the hydrogen peroxide test. It should be noted that because this molecule's free radical intermediates are stable, benzothiazole derivatives have a stronger scavenging activity (Djuidje et al., 2022; Yadav et al., 2012). **Figure 3.**

6.2.2 DPPH radical scavenging activity

Figure 4 shows the free radical scavenging activity of the eight synthesized compounds and standard vitamin C. Starting with a2 tested compound, it had the highest activity of the compounds. As compared to conventional vitamin C, which had a scavenging activity of 69 % at a concentration of 250 µg/mL, a2, ald.2, c2, and c1 were each 58, 58, 42, and 31, respectively, **Figure 4**. The following chemicals and vitamin C were in order of their ability to scavenge free radicals: vitamin C > a2, ald.2 > c2 > a1, c1 > t1. It is believed that antioxidants' ability to donate hydrogen is what causes them to affect DPPH (Kedare & Singh, 2011). It's crucial to engage in radical scavenging actions to stop free

radicals from damaging many ailments, including inflammation. The ability of a substance to scavenge DPPH free radicals is a well-known test for antioxidant capability. In the DPPH assay, the chemical's addition transforms the violet color of the DPPH solution into diphenylpicryl hydrazine, a result that is yellow in hue. This method has been routinely used to forecast antioxidant activity since it can be completed quickly (Rahman et al., 2015). According to our findings, normal vitamin C and the DMSO extract of a2, ald.2 showed nearly identical levels of free radical scavenging action (**Figure 4**).

Conclusion

New steroidal derivatives were successfully synthesized using chemical methods. Steroidal Schiff base derivatives have substantially more potent anti-inflammatory properties than reference medicines (dexamethasone) when used to the bovine albumin denaturation procedure. Biologically active pure steroidal Schiff base compound is better than cholesterol or ergosterol itself. To prove the anti-inflammatory action of steroidal Schiff base derivatives, additional in vivo and in vitro experiments should be carried out. Also, different steroidal Schiff base derivatives from cholesterol and ergosterol were examined against free radicals like DPPH reagent and hydrogen peroxide, The maximum antioxidant and free radical scavenging activity was shown by these substances. Therefore, additional research is required to isolate and pinpoint the antioxidant activity of compounds related to steroids.

Additional Information

A pdf document file including copies of synthetic Schiff base's FT-IR, ^{1}H NMR (400 MHz, $CDCl_3$), and mass spectra.

Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest.
Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects

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