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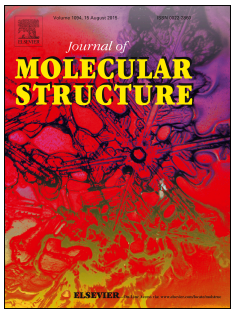


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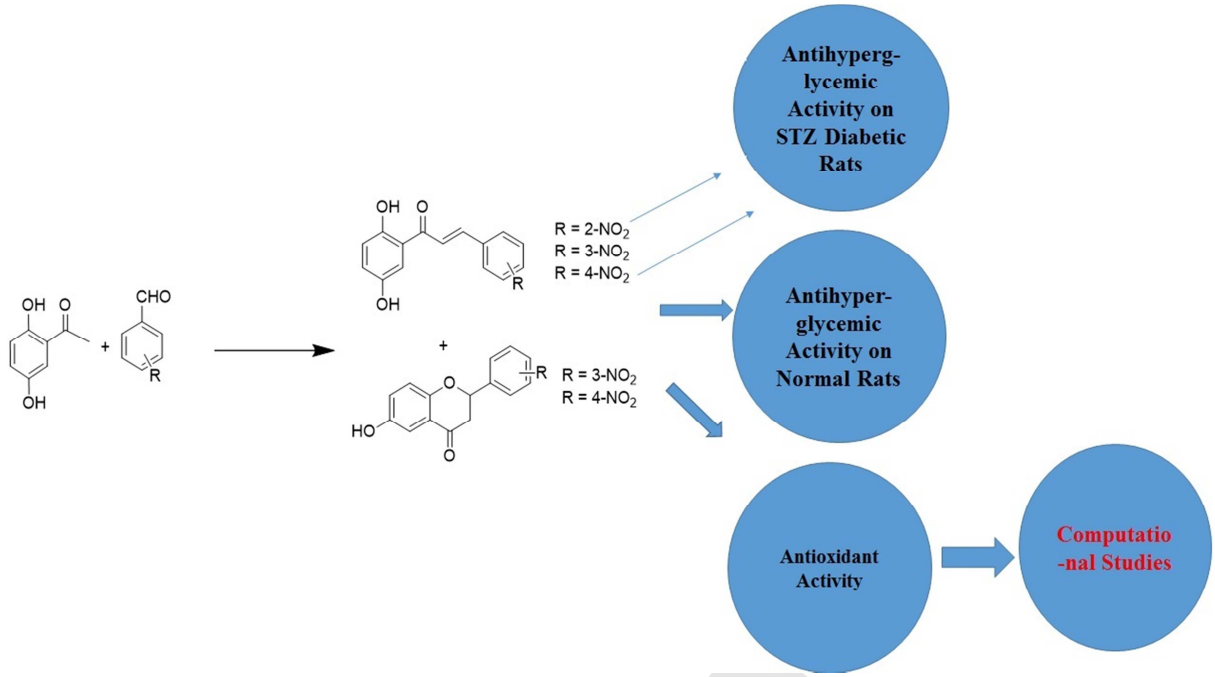

# Synthesis, antihyperglycemic activity and computational studies of antioxidant chalcones and flavanones derived from 2,5 dihydroxyacetophenone

Affifa Tajammal[1], Majda Batool[1], Ayesha Ramzan[1], Malka M Samra[1], Idrees Mahnoor[1], Francis Verpoort[2], Ahmad Irfan[3,4], Abdullah G. Al-Sehemi[3,4], Munawar Ali Munawar[1*], Muhammad Asim R Basra[1*]

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**Running title:** Antihyperglycemic and antioxidant screening of synthesized chalcones and flavanones

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**Abstract**

Chronic exposure of supraphysiologic glucose concentration to cells and tissues resulted in glucose toxicity which causes oxidative stress. Antioxidants have promising effect in suppressing the oxidative stress in the pathogenesis of diabetes mellitus (DM). Condensation of 2,5-dihydroxyacetophenone with different nitrobenzaldehydes was used to synthesize antioxidant nitro substituted chalcones along with nitro substituted flavanones in one step protocol. The compounds were characterized by IR, $^{1}$H NMR and $^{13}$C NMR and then screened for their *in vitro* antioxidant and *in vivo* antihyperglycemic activities. Postulated structures of the synthesized compounds were in agreement with their spectral data. The results indicated that the novel compound (2E)-1-(2,5-Dihydroxyphenyl)-3-(2-nitrophenyl) prop-2-en-1-one (2a) was potent antioxidant because of its lower $IC_{50}$ value compared with trolox and ascorbic acid. Compound 2a also exhibited excellent antihyperglycemic activity in diabetic rats while the compound (E)-1-(2,5-Dihydroxyphenyl)-3-(4-nitrophenyl)prop-2-one (2c) suppressed the hyperglycemia more effectively in normal rats. The radical scavenging activity behavior was elucidated on the basis of hydrogen atom transfer and one-electron transfer mechanisms by density functional theory (DFT). The compound 2a showed the smallest ionization potential and bond dissociation enthalpy. Experimental and computational investigations concluded that compound 2a might be an effective antihyperglycemic agent because of its antioxidative nature and smallest ionization potential.

**Keywords:** Chalcones; Flavanones; Antioxidant; Diabetes; Antihyperglycemia.

## 1. Introduction

Production of free radical is enhanced in the body due to oxidation of glucose, non-enzymatic glycation of proteins and the subsequent oxidative degradation of glycated proteins [1]. In diabetes mellitus (DM), erratically controlled hyperglycemia promotes free radicals accumulation and cause oxidative stress by enhancing the cascade of the oxidative reaction [2]. Antioxidants can be helpful in suppressing the oxidative stress and its related disorder including DM [3].

Chalcones and flavanones are well-known for their wide range of pharmacological activities including antimicrobial [4], anticancer, antioxidant [5], antiangiogenic [6], anti-inflammatory [7], antidiabetic, antihyperlipidemic [8], inhibition of tyrosinase activities [9] etc. Excellent antioxidant activity of chalcones and flavanones is due to the presence of hydroxyl groups on 'A' or 'B' ring of their structure. This antioxidant potential is involved in decreasing the oxidative stress in various diseases including cancer, atherosclerosis, DM, hypertension and heart diseases [10].

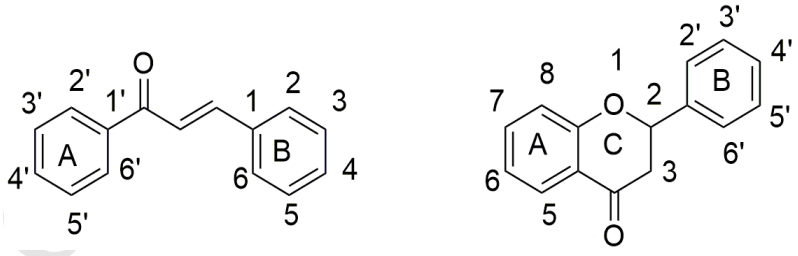


**Figure 1.** Structure of chalcone and flavanone

Both *in vitro* and *in vivo* studies have also demonstrated the effects of hydroxychalcones and hydroxyflavanones, originating from both natural and synthetic sources on the carbohydrate

metabolism and regulation of insulin function [11], [8]. Antioxidant compounds are reported to have potential protective effects on diabetes by improving β-cells function. So enhancing antioxidant defense mechanisms in pancreatic islets may be a potential pharmacological approach to manage the diabetes [12].

Various drugs are on the horizon as well as there is a need to develop a new category of drug with lesser side effects and improve the variety of medications. In recent years, the high therapeutic properties of the chalcones and flavanones related drugs have brought attention of chemists to synthesize various kinds of their derivatives by improving the existing synthetic methodologies. Claisen-Schmidt condensation is usually used to synthesize the chalcones in the presence of basic catalysts such as NaOH/ KOH [13], MgO, BaO, $K_2O$, $Na_2O$ and ZaO [14]. It has been reported previously that the hydroxychalcones are difficult to synthesize in the presence of base due to formation of phenoxide anion. Acidic catalyst such as HCl, $BF_3$, $B_2O_3$ [15], para toluenesulfonic acid [16] and $SOCl_2$/EtOH [17] have been found to be more effective. It is therefore, the present work was designed to synthesize new chalcones and flavanones in the presence of acid without the protection of hydroxyl group. Furthermore, the main objective was to investigate *in vitro* and *in silico* antioxidant activities along with *in vivo* antihyperglycemic activities of synthesized compounds.

## 2. Experimental

### *2.1. Materials and Methods*

Chemicals were purchased from well reputed international suppliers. The chemicals were used mostly as such however when required purified by normal techniques i.e., distillation and recrystallization. Synthesis of 2, 5-dihydroxyacetophenone was done by already reported method

[18]. Silica gel 60 $F_{254}$ TLC plates were used to monitor the reaction. FT-IR, $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on Agilent Technologies 41630, AVANCE AV-400 MHz and AVANCE AV-500 MHz, and Bruker 125 MHz respectively while the EIMS data was taken on JEOL MS 600H-1.

*2.2. General method for the synthesis of chalcones and flavanones*

2,5-Dihydroxyacetophenone (50 mg, 0.3 mmol) and nitrobenzaldehyde (45 mg, 0.3 mmol) were dissolved in hot dry benzene (20 mL). Then p-Toluene sulfonic acid (5 mg, 0.03 mmol) was added. The resulting reaction mixture was refluxed for 48 hours. The reaction was monitored with TLC. After completion of the reaction, benzene was removed under vacuum and residue was purified on silica gel column using hexane-ethyl acetate (4:1) as eluent. In case of 3-nitrobenzaldehyde and 4-nitrobenzaldehyde, corresponding flavanones were also isolated along with chalcones. Whereas with 2-nitrobenzaldehyde only a novel chalcone (2a) was obtained.

*2.3. (2E)-1-(2,5-Dihydroxyphenyl)-3-(2-nitrophenyl) prop-2-en-1-one (2a)*

Orange powder; yield 80 %; IR (ν $cm^{-1}$): 3367; 1648; 1571; 1517. $^{1}H$ NMR (500 MHz / DMSO-$d_6$): δ 11.34 (1H, s, 2′-OH), 9.28 (1H, s, 5′-OH), 8.12 (2H, m, 3-H, 6-H), 8.00 (1H, d, *J=15.5* Hz, β-H), 7.87-7.82 (2H, m, α-H, 5-H), 7.72 (1H, t, *J=7.2* Hz, 4-H), 7.41 (1H, d, *J=3* Hz, 6′-H), 7.05 (1H, dd, *J=8.8* Hz, 3.0 Hz, 4′-H), 6.87 (1H, d, *J=8.8 Hz*, 3′-H). $^{13}C$ NMR: δ 192.83, 154.56, 150.09, 149.22, 138.70, 134.33, 131.65, 130.11, 129.93, 127.67, 125.22, 125.00, 121.87, 118.92, 115.56. MS ($EI^{+}$): m/z 137 (100 %), $M^{+}$ 285 (5), 27 (86), 238 (83).

*2.4. (2E)-1-(2,5-Dihydroxyphenyl)-3-(3-nitrophenyl)prop-2-en-1-one (2b)*

Orange powder**;** yield 53 %; IR (ν $cm^{-1}$): 3430; 1643; 1561; 1530. $^{1}$H NMR (400 MHz / DMSO-$d_6$): δ 11.60 (1H, s, 2′-OH), 9.29 (1H, s, 5′-OH), 8.73 (1H, s, 2-H), 8.31 (1H, d, *j=7.8 Hz*, 4-H), 8.28 (1H, m, 4-H, 6-H), 8.10 (1H, d, *J=15.7 Hz*, β-H), 7.88 (1H, d, *J=15.6 Hz*, α-H), 7.75 (1H, t, *J=7.9 Hz*, 3-H), 7.54 (1H, d, *J=2.8 Hz*, 6′-H), 7.06 (1H, dd, *J=8.8, 2.8 Hz*, 4′-H), 6.87 (1H, d, *J=8.8 Hz*, 3′-H). $^{13}$C NMR: δ 193.41, 154.98, 150.04, 148.89, 142.00, 136.84, 135.64, 130.93, 125.74, 125.30, 125.15, 123.49, 121.54, 118.84, 115.72.

*2.5. 6-hydroxy-2-(3-nitrophenyl)-2,3-dihydro-4H-chromen-4-one (3a)*

Yellow crystals; yield 25 %; IR (ν $cm^{-1}$): 3472, 1670; 1524; 1342. $^{1}$H NMR (500 MHz / DMSO-$d_6$): δ 9.57 (1H, s, 6-OH), 8.40 (1H, s, 2′-H), 8.24 (1H, dd, *J=8.2 Hz*, 2.0 Hz, 4′-H), 8.00 (1H, d, *J=7.8 Hz*, 6′-H), 7.74 (1H, t, *J=8 Hz*, 5′-H), 7.13 (1H, d, *J=3.0 Hz*, 5-H), 7.07 (1H, dd, *J=8.8, 3.0 Hz*, 7-H), 7.02 (1H, d, *J=8.8 Hz*, 8-H), 5.75 (1H, dd, *J=13.1, 2.7 Hz*, 2-H), 3.22 (1H, m, Ha), 2.88 (1H, dd, *J=16.9, 2.9 Hz*, Hb). $^{13}$C NMR: δ 191.72, 154.47, 152.28, 148.35, 141.87, 133.56, 130.72, 125.14, 123.73, 121.63, 121.32, 119.54, 110.46.

*2.6. (E)-1-(2,5-Dihydroxyphenyl)-3-(4-nitrophenyl)prop-2-one (2c)*

Orange powder; yield 65 %; IR (ν $cm^{-1}$): 3355; 1648; 1584; 1522. $^{1}$H NMR (500 MHz / DMSO-$d_6$): δ 11.44 (1H, s, 2′-H), 9.18 (1H, s, 5′-H), 8.28 (2H, d, *J=8.4 Hz*, 3-H, 5-H), 8.13 (2H, d, *J=8.8 Hz*, 2-H, 6-H), 8.07 (1H, d, *J=16 Hz*, β-H), 7.82 (1H, d, *J=15.6 Hz*, α-H), 7.48 (1H, d, *J=2.4 Hz*, 6′-H), 7.04 (1H, dd, *J=8.8, 2.8 Hz*, 4′-H), 6.85 (1H, d, *J=8.8 Hz*, 3′-H). MS ($EI^{+}$): m/z 136 (100 %), $M^{+}$ 285 (91), 266 (9), 238 (8), 163 (45).

*2.7. 6-hydroxy-2-(4-nitrophenyl)-2,3-dihydro-4H-chromen-4-one (3b)*

Light orange powder; yield 20 %; IR (ν $cm^{-1}$): 3355; 1648; 1584; 1522. $^{1}$H NMR (400 MHz / DMSO-$d_6$): δ 9.57 (1H, s, 6-OH), 8.29 (2H, d, *J=8.7 Hz*, 3′-H, 5′-H), 7.82 (2H, d, *J=8.7 Hz*, 2′-H, 6′-H), 7.13 (1H, d, *J=3.0 Hz*, 5-H), 7.07 (1H, dd, *J=8.8, 3.0 Hz*, 7-H), 7.01 (1H, d, *J=8.8*, 8-H), 5.75 (1H, dd, *J=12.9, 2.9 Hz*, 2-H), 3.15 (1H, m, Ha), 2.89 (1H, dd, *J=16.9, 3.0 Hz*, Hb). $^{13}$C NMR: δ 191.56, 154.42, 152.29, 147.78, 146.99, 128.08, 125.16, 124.18, 121.34, 119.54, 110.46, 78.14, 43.92.

*2.8. In vitro Antioxidant Activities*

The synthesized compounds were tested individually against five different *in vitro* antioxidant activities which include 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, iron chelating, $FeCl_3$ reducing power, phosphomolybdenum (PM) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) activity. These activities were carried out for each compound in triplicate while trolox and ascorbic acid were used as standard compounds.

*2.8.1. DPPH free radical scavenging activity*

Free radical scavenging effect of synthesized compounds was determined with DPPH assay. Ethanolic solution of DPPH (0.05 mM, 2.85 mL) and the test compounds (final concentration 2000 µM, 0.15 mL) were mixed and incubated in dark at 37 °C. The absorbance was recorded at 517 nm after 15, 30, 45, 60 and 120 minutes of incubation. The percentage activity of the synthesized compounds was calculated with following equation [19].

Percentage of scavenging activity = (Control absorbance - Sample absorbance/Control absorbance) × 100

*2.8.2. Iron chelating activity*

Different concentrations of methanolic solution of test compounds (final concentration 2000 µM, 2 mL) were mixed with methanolic o-phenanthroline (0.05 % w/v, 1 mL) solution and incubated with methanolic $FeCl_3$ (200 µM, 2 mL) at ambient temperature. The absorbance was determined at 512 nm after 10, 30, 45, 60 and 120 minutes. Finally, the percentage of iron chelating activity was determined with the following formula [20].

Percentage of iron chelating activity = (Test absorbance - Control absorbance/Test absorbance) × 100

*2.8.3. $FeCl_3$ reducing power activity*

In reducing power assay, aqueous solution of potassium ferricyanide (1 %, 2.5 mL) was mixed with ethanolic solution of each test compound (final concentration 2000 µM, 2 mL) and incubated at 50 °C for 20 minutes. After cooling, aqueous trichloroacetic acid (10 %, 2.5 mL) was added and the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant liquid (2.5 mL) was mixed with distilled water (2.5 mL) and freshly prepared aqueous ferric chloride solution (0.1 %, 1 mL). The absorbance was recorded at 700 nm after 10, 30, 45, 60 and 120 minutes of incubation. Percentage increase in reducing power was determined by the following formula [21].

Percentage increase of reducing power = (Test absorbance/Control absorbance - 1) × 100

*2.8.4. Phosphomolybdenum activity*

PM solution was prepared by mixing 100 mL of each sulfuric acid (0.6 M), ammonium molybdate (4 mM) and sodium phosphate (28 mM). Series of dilutions of sample solution were prepared from stock solution of 2000 µM in ethanol. Phosphomolybdenum reagent (3 mL) was mixed with each test compound (0.3 mL) and incubated at 95 °C for 90 minutes. The absorbance was measured at 765 nm after 15, 30 and 45 minutes of incubation against the blank. Percentage increase in reducing power was determined by the following formula [22].

Percentage increase of reducing power = (Test absorbance/Control absorbance - 1) × 100

*2.8.5. ABTS activity*

In ABTS assay, antioxidant activity was performed by preparing aqueous ABTS (7 mM/L). $ABTS^{+}$ was produced by reacting ABTS stock solution with 2.45 mmol/L potassium persulfate. The resulting solution was incubated in dark for 12-16 hours at room temperature (25±2 $^{o}$C). Ethanol was used for diluting the mixture till the absorbance reached to 0.7±0.02 at 734 nm. Various dilutions of each sample solution were prepared from stock solution (2000 µM) in ethanol. $ABTS^{+}$ solution (2.85 mL) was mixed with each test compound solution (0.15 mL) and recorded the absorbance at 734 nm after the intervals of 5, 15, 30, 45, 60 and 120 minutes of incubation against the blank. The activity of each compound was then measured by using the following formula [23].

Percentage of scavenging activity = (Control absorbance - Sample absorbance/Control absorbance) × 100

*2.9. Antihyperglycemic activity*

*2.9.1. Experimental Animals*

Sprague Dawley albino rats (200-250 g) were selected and maintained in animal house at controlled temperature (25±5 °C) and humidity (50±10 %). Animals were provided with free access to autoclaved tap water and pathogen free feed for 24 hours. Animal experiments were approved by Institutional ethical committee (Approval No. D/017/Chem.). International ethical guidelines for the care of laboratory animals were used to maintain rats in animal house.

*2.9.2. Oral glucose tolerance test in normal rats*

The blood glucose level of each rat was inspected by using code free glucometer after 18 hours of starvation. Animals showing blood glucose levels between 80-100 mg/dL were divided into groups of five animals in each. Animals in experimental groups were treated orally with synthesized compounds at single dose of 100 mg/kg body weight after emulsifying in aqueous carboxymethyl cellulose (0.5 %). Animals in control group were given aqueous carboxymethyl cellulose (0.5 %) only. An oral glucose load (2.0 g/kg) was given to each animal exactly after 30 minutes per oral administration of the test sample/vehicle. Blood glucose level of each rat was checked at 1, 3 and 5 hours after the administration of glucose. After checking the results, three out of five compounds were selected and screened for their most effective dose at 50, 100 and 200 mg/kg by using the above mentioned protocol. The dose which improved glucose tolerance significantly was selected for further studies [24].

*2.9.3. Determination of anti-hyperglycemic effects of compounds in streptozotocin induced diabetic rats*

Rats were divided randomly into five groups; Control, Diabetes mellitus (DM), Glibenclamide (GC), 2a and 2c group. Each group contained seven female rats. Rats in DM, GC, 2a and 2c groups were injected with streptozotocin (STZ) (50 mg/Kg) interaperitoneally which was freshly prepared in citrate buffer (0.1 M, pH: 4.5). The elevated blood glucose level (>250 mg/dL) confirmed the hyperglycemic condition in each rats after 48 hours followed by STZ injection. Glucose solution (5 %) was also given to avoid the hypoglycemic effect of the drug. After 48 hours, rats in GC, 2a, and 2c were treated orally with single dose of GC (20 mg/kg), compound 2a (100 mg/kg) and compound 2c (100mg/kg) respectively. Rats in Control and DM groups were treated with vehicle carboxymethyl cellulose (0.5 %). Blood glucose level was determined both before and after the treatment at different intervals (zero time, 1, 3 and 5 hours).

**Statistical analysis**

Statistical analysis was done by using One Way ANOVA followed by Tukey's multiple comparison tests with the help of GraphPad Prism version 7.01. The values were considered statistically significant at $P < 0.05$.

*2.10. Computational details*

The density functional theory (DFT) is being used to shed light on the elector-optical, radical scavenging activity and other descriptors efficiently [25]. The ground state geometries of the neutral and charged species as well as frequency calculations were carried out by DFT at B3LYP/6-31G** level [26]. There are two key mechanisms (a) H-atom transfer (Eq. (1)) and

one-electron transfer (b) for the radical scavenging processes of chain-breaking antioxidant (ArOH) [27], [28], details can be seen in the supporting information.

$$R^{\cdot} + ArOH \longrightarrow RH + ArO^{\cdot} \quad (1)$$

$$R^{\cdot} + ArOH \longrightarrow R^{-} + ArOH^{+\cdot} \quad (2)$$

All calculations were performed by Gaussian 09 software [29].

**3. Results and Discussion**

Chalcones (2a, 2b, 2c) and flavanones (3a, 3b) were isolated from the acid catalyzed condensation reaction of 2,5-dihydroxyacetophenone (1) with nitrobenzaldehydes. Different solvent systems such as toluene, methanol, ethanol, 2-propanol were endeavored, however benzene was found to be the best solvent for these condensation reactions. The synthesized compounds were characterized using, $^{1}$H NMR, $^{13}$C NMR etc. The most prominent indication for the synthesis of chalcones was the appearance of two doublets with $J$ = 15.6 corresponding α and β proton of the ethene linkage. Whereas absence of these signals and appearance of signals at δ 5.75, 3.22, and 2.88 corresponding to 2-H, 3Ha and 3Hb of the hetro ring indicated the formation of flavanones. The synthetic route of flavanones and chalcones from 2,5-dihydroxyacetophenone and the physiochemical data of the synthesized compounds was summarized in detail in figure 2 and Table 1 respectively.

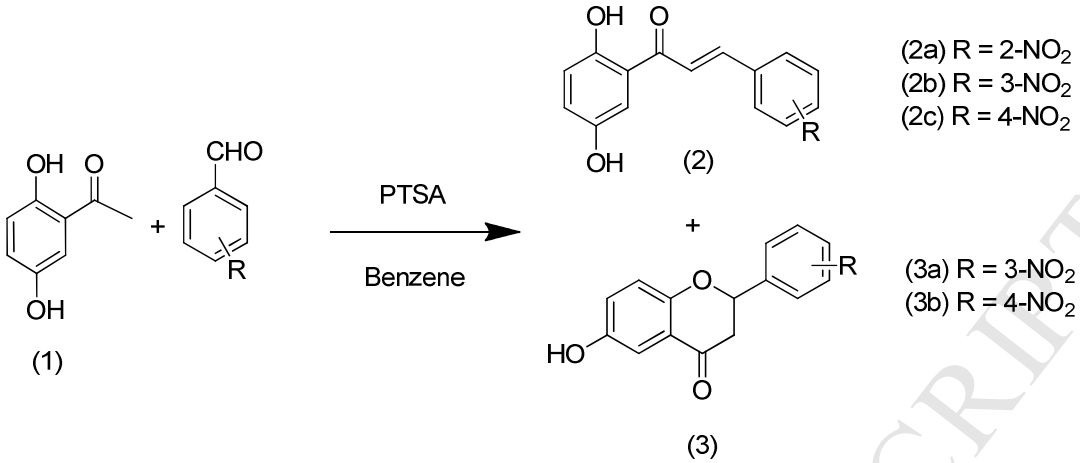


**Figure 2.** Synthesis of nitro substituted hydroxychalcones and hydroxyflavanones

**Table 1.** Physicochemical characterization data of the synthesized compounds

| Comp. no. | R | M.F. | M.W. | M.P. (°C) | Yield (%) |
|---|---|---|---|---|---|
| 2a | 2-nitro | $C_{15}H_{11}NO_5$ | 285.25 | 200 | 80 |
| 2b | 3-nitro | $C_{15}H_{11}NO_5$ | 285.25 | 208 | 53 |
| 2c | 4-nitro | $C_{15}H_{11}NO_5$ | 285.25 | 218 | 65 |
| 3a | 3-nitro | $C_{15}H_{11}NO_5$ | 285.25 | 153 | 25 |
| 3b | 4-nitro | $C_{15}H_{11}NO_5$ | 285.25 | 167 | 20 |

*3.1. In vitro Antioxidant Activities*

*3.1.1. Interaction with the DPPH stable free radical*

The tested compounds interact with the stable free radical DPPH which shows their radical scavenging ability. This interaction was found to be concentration and time dependent (table 2). Among the tested chalcones, compound 2a showed excellent radical scavenging activity where

nitro group is at position 2 of ring 'B'. It exhibited significantly higher activity as compared to the reference compounds trolox and ascorbic acid. The compounds 2b and 2c bearing nitro group at position 3 and 4 of ring 'B' respectively displayed significant good activity when compared with reference compounds but much lower than 2a. It is therefore, DPPH scavenging ability seems to be dependent on the position of nitro group at ring 'B' of the chalcones. The $IC_{50}$ values of tested flavanones 3a and 3b were much higher as compared to all other compounds. So the order of DPPH activity of synthesized compounds was 2a > 2b > 2c >>> 3a, 3b.

**Table 2.** $IC_{50}$ (µM) for DPPH radical scavenging activity

| **Compounds** | **Time (Minutes)** | | | | |
|---|---|---|---|---|---|
| | **15** | **30** | **45** | **60** | **120** |
| Trolox | 225.12±15.12 | 216.25±10.41 | 223.18±15.01 | 214.83±10.43 | 252.73±17.22 |
| Ascorbic acid | 215.50±10.43 | 221.36±14.81 | 234.44±15.71 | 244.46±15.91 | 240.47±15.81 |
| 2a | 65.52±6.21*** | 55.89±5.55*** | 57.78±5.57*** | 53.57±5.21*** | 50.88±5.11*** |
| 2b | 117.19±7.87*** | 112.05±7.85*** | 113.82±7.83*** | 112.59±7.82*** | 111.58±7.81*** |
| 2c | 142.96±8.25*** | 145.47±8.44*** | 144.58±8.43*** | 148.41±8.52*** | 167.71±8.95*** |
| 3a | > 2000 | > 2000 | > 2000 | > 2000 | > 2000 |
| 3b | > 2000 | > 2000 | > 2000 | > 2000 | > 2000 |

Data was shown in Mean ± SD (n = 3) for each compound, ***P < 0.001 showed statistically significant difference when compared with trolox and ascorbic acid.

*3.1.2. Iron chelating activity*

In Iron chelating activity, the synthesized compounds reduced $Fe^{3+}$ to $Fe^{2+}$ which involved in the formation of chelate with o-phenanthroline. This method is used to determine the extent of reduction of ferric ions by synthesized compounds. $IC_{50}$ value for iron chelating activity of each

compound was determined (Table 3). Among the tested chalcones, $IC_{50}$ value of compound 2a was much lower as compared to other chalcones and flavanones showing its greater chelating ability. No significant difference was found between 2a and reference compounds. The $IC_{50}$ value of 2a was found to be very close to ascorbic acid value. The order of increasing activity for synthetic compound was 2a > 2b > 2c > 3b > 3a.

**Table 3.** $IC_{50}$ (µM) for Iron chelating activity

| **Compounds** | **Time (Minutes)** | | | | |
|---|---|---|---|---|---|
| | **10** | **30** | **45** | **60** | **120** |
| Trolox | 0.46±0.23 | 0.49±0.25 | 0.53±0.32 | 0.41±0.24 | 0.28±0.11 |
| Ascorbic acid | 3.33±1.19 | 2.15±1.13 | 0.33±0.67 | 0.23±0.52 | 0.24±0.59 |
| 2a | 4.21±1.14 | 1.85±1.09 | 0.56±0.76 | 0.22±0.98 | 0.31±1.16 |
| 2b | 15.47±5.78 | 11.37±4.61 | 5.09±3.67 | 1.70±1.37 | 0.41±2.33 |
| 2c | 40.61±3.23 | 56.69±3.98 | 46.21±2.31 | 16.82±1.12 | 11.85±1.92 |
| 3a | 185.09±9.33 | 211.89±5.74 | 214.51±4.32 | 210.78±6.54 | 232.21±3.49 |
| 3b | 121.82±10.12 | 126.59±8.81 | 160.70±6.92 | 160.34±8.67 | 224.59±2.37 |

*3.1.3. $FeCl_3$ reducing power activity*

In $FeCl_3$ reducing power assay the chalcones and flavanones reduced potassium ferricyanide ($Fe^{+3}$) to potassium ferrocyanide ($Fe^{+2}$) which then reacted with ferric chloride to form ferric ferrous complex. The reducing capabilities of all compounds were summarized in the form of $IC_{50}$ (Table 4). The $IC_{50}$ of 2a was found to be lower than the reference compounds while 2b and 2c showed satisfactory results. The $IC_{50}$ of chalcones was much lower as compared to

flavanones. The order of $FeCl_3$ reducing power activity of the synthesized compounds was found to be 2a > 2b > 2c > 3b > 3a.

**Table 4.** $IC_{50}$ (µM) for $FeCl_3$ reducing power activity

| **Compounds** | **Time (Minutes)** | | | | |
|---|---|---|---|---|---|
| | **10** | **30** | **45** | **60** | **120** |
| Trolox | 13.75±2.45 | 12.04±3.07 | 11.24±2.74 | 11.43±1.98 | 10.06±2.24 |
| Ascorbic acid | 14.64±3.34 | 14.74±3.29 | 14.02±3.12 | 13.16±3.54 | 12.74±2.87 |
| 2a | 11.28±1.22 | 9.98±2.11 | 9.51±3.45 | 9.21±2.31 | 9.11±3.43 |
| 2b | 15.37±0.24 | 14.49±0.23 | 14.16±0.23 | 13.87±0.26 | 13.43±0.21 |
| 2c | 23.07±5.44 | 21.44±5.21 | 18.32±4.65 | 17.32±4.98 | 16.19±4.26 |
| 3a | 1305.77±18.81 | 673.01±16.42 | 459.85±15.63 | 180.63±15.24 | 138.45±14.35 |
| 3b | 90.01±15.83 | 66.56±12.25 | 53.07±12.66 | 37.12±13.76 | 28.88±12.51 |

*3.1.4. Phosphomolybdenum activity*

This activity is based on the reduction of Mo (VI) to Mo (V) by the test compounds, giving a direct approximation of reducing capacity of the compounds. $IC_{50}$ values for PM activity of all compounds were evaluated (Table 5). In PM activity, the $IC_{50}$ of 2a was found to be lower than all other compounds. The order of activity was 2a > 2b > 2c > 3b > 3a.

**Table 5.** $IC_{50}$ (µM) for Phosphomolybdenum assay

| Compounds | Time (Minutes) | | |
|---|---|---|---|
| | **15** | **30** | **45** |
| Trolox | 16.25±2.35 | 15.89±2.73 | 15.25±3.43 |
| Ascorbic acid | 21.73±1.23 | 17.37±3.61 | 15.82±1.58 |
| 2a | 18.04±0.19 | 19.13±1.47 | 21.65±0.89 |
| 2b | 19.42±2.78 | 20.37±3.77 | 24.73±2.89 |
| 2c | 37.25±1.79 | 35.97±2.37 | 44.96±1.15 |
| 3a | > 2000 | > 2000 | > 2000 |
| 3b | 533.97±11·33 | 270.18±8.98 | 194.30±7.67 |

*3.1.5. ABTS Activity*

The synthesized compounds exhibited potent ABTS activity in a concentration-dependent manner and the $IC_{50}$ values for ABTS activity of all compounds were evaluated (Table 6). Compound 2a exhibited statistical prominent ABTS activity than reference compounds after 5, 15, 30, 45, 60 and 120 minutes. Chalcones 2b and 2c showed significant lower $IC_{50}$ value than standard ascorbic acid after 5, 15, 30 and 45 minutes. The order of activity of synthetic compounds was 2a > 2c > 2b > 3b > 3a.

**Table 6.** $IC_{50}$ (µM) for ABTS activity

| Compounds | Time (Minutes) | | | | | |
|---|---|---|---|---|---|---|
| | **5** | **15** | **30** | **45** | **60** | **120** |
| Trolox | 32.31±0.21 | 51.56±0.32 | 50.38±1.32 | 53.56±1.78 | 31.97±1.43 | 29.33±1.27 |
| Ascorbic acid | 76.79±0.89 | 109.46±1.52 | 97.47±4.36 | 64.19±2.11 | 45.23±1.92 | 37.91±1.74 |
| 2a | 3.19±0.23*** | 2.32±0.87*** | 3.43±1.12*** | 1.65±1.65*** | 0.24±0.54*** | 3.13±2.23*** |
| 2b | 41.44±0.26### | 40.84±1.11### | 52.24±1.96### | 53.99±3.28## | 54.65±3.42 | 46.66±2.64 |
| 2c | 37.79±0.18### | 41.48±0.68### | 43.96±2.54### | 46.21±1.56## | 45.29±2.18 | 37.79±2.43 |
| 3a | 1665.11±11.32 | 601.49±7.97 | 210.69±5.32 | 145.78±0.16 | 121.04±4.91 | 106.03±4.38 |
| 3b | 681.63±8.16 | 307.93±2.23 | 166.98±1.67 | 156.29±2.56 | 145.94±4.22 | 99.31±6.32 |

Data was shown in Mean ± SD (n = 3) for each compound, ***P < 0.001 showed statistically significant difference when compared with trolox and ascorbic acid. ##P < 0.01, ###P < 0.001 showed statistically significant difference when compared synthesized compounds with ascorbic acid.

*3.2. Oral glucose tolerance test in normal rats*

The synthesized compounds were screened for their antihyperglycemic activity in normal rats. Compounds 2b, 2c, and 3b were found to be effective at dose of 100 mg/kg in lowering the hyperglycemia while 2a and 3a appeared to be non-effective in reducing the hyperglycemia in normal rats (Fig 3A). Because 3a and 3b were synthesized in low yield so 2a, 2b and 2c were selected for the selection of minimum dose required for antihyperglycemic effect in normal rats. Three different doses (50 mg/Kg, 100 mg/Kg and 200 mg/Kg) for compounds 2a, 2b and 2c were selected for dose optimization respectively (Fig 3B-D).

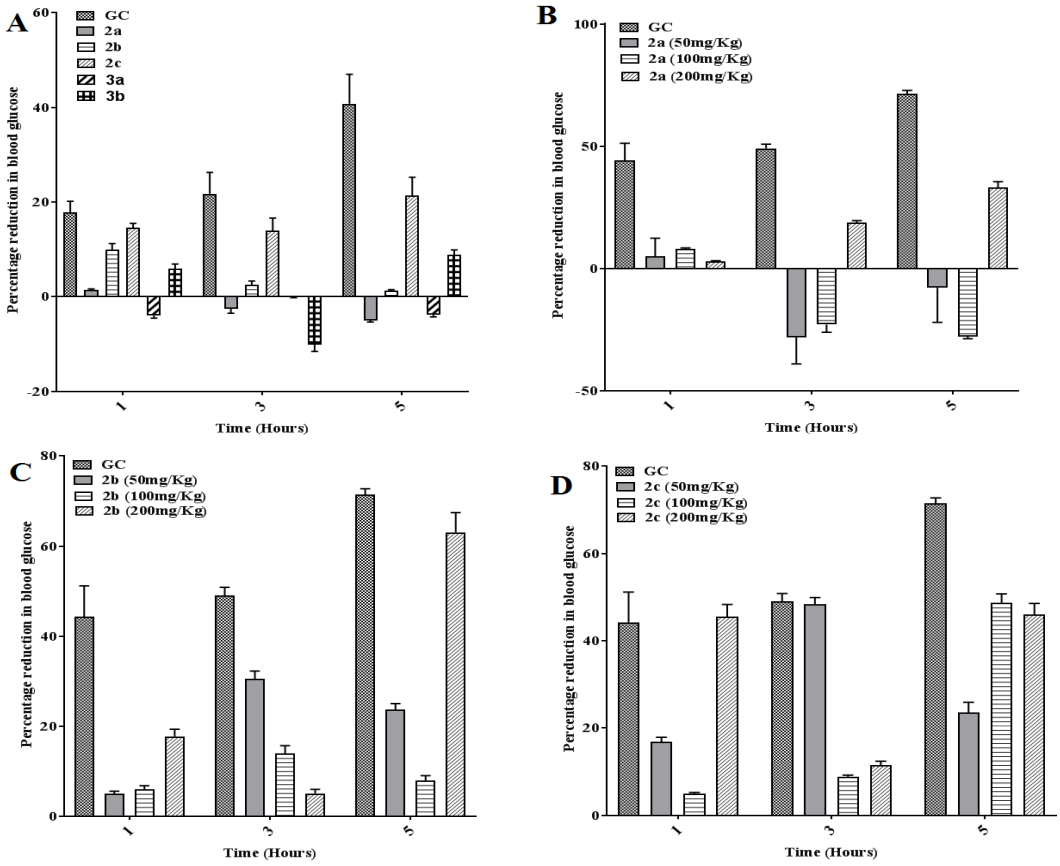


**Figure 3.** Oral glucose tolerance test of synthesized compounds in normal rats and compounds administered to the rats before 30 minutes of glucose load (2 g/kg). Rats administered with compound 2a, 2b, 2c, 3a and 3b at 100 mg/kg each (A), 2a: 50 mg/kg; 100 mg/kg; 200 mg/kg (B), 2b: 50 mg/kg; 100 mg/kg; 200 mg/kg (C), and 2c: 50 mg/kg; 100 mg/kg; 200 mg/kg (D). Data was expressed as percentage reduction in blood glucose level while GC was used as reference drug. Values were expressed as Mean ± SD (n = 5).

The dose optimization study by oral glucose tolerance test (OGTT) in normal rats was revealed that the compound 2c with nitro group at position 4 in ring 'B' of chalcone was more active in lowering the hyperglycemia at 100 mg/kg dose.

*3.3. Anti-hyperglycemic effects of compounds in STZ induced diabetes*

To further evaluate the antihyperglycemic effect of the synthesized compounds, STZ induced type 2 diabetic rats were used to assess the efficacy of compound 2a and 2c at dose of 100 mg/kg**.** The reason for selecting 2a was novelty of the compound and excellent antioxidant abilities than other compounds. In diabetic rats the compound 2a lowered the hyperglycemia more potentially than compound 2c although 2a was less active in normal rats than 2c. Also, 2a being structurally similar to 2c, but with a nitro group at position 2 in ring 'B', more actively produced antihyperglycemic effect in diabetic rats. Thus steric conformation of the compounds is said to be responsible for the anti-hyperglycemic activity of chalcone [30]. It is reported in literature that the antihyperglycemic activity of chalcone derivatives is mediated via stimulation of PPAR-γ [31], [32], [33]. The flavonoids, based on 2-phenylchromone or 2-phenyl benzopyrone, have also shown beneficial effects in the treatment of hyperglycemic disease probably through changes in the activity of intracellular enzymes such as glucosidase enzyme [34]. Presently, compound 2a was found to be excellent antioxidant and have shown effective antihyperglycemic effect in STZ induced diabetic rats. Antioxidants are involved in decreasing the hyperglycemia by minimizing the oxidative stress [35]. Compound 2a significantly reduced the hyperglycemia state in STZ induced rats compared with compound 2c and this effect was in close proximity to the positive control GC (Fig. 4).

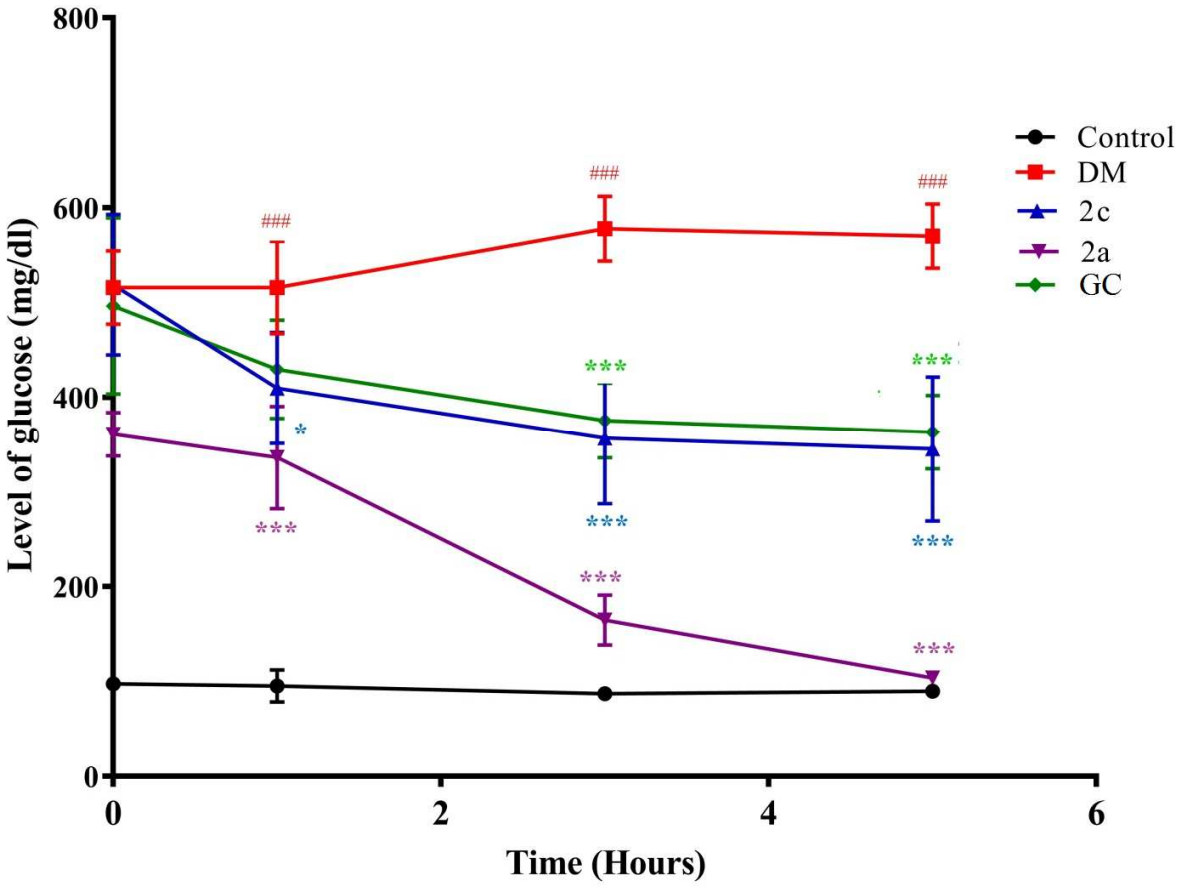


**Figure 4.** Antihyperglycemic effect of compounds in STZ induced diabetic rats. Values were expressed in Mean ± SD (n = 7). *P < 0.05 and ***P < 0.001 statistically significant when compared with diabetic control while $^{###}$P < 0.001 statistically significant when compared with normal Control.

Antibacterial activities were also evaluated for the synthesized compound and only two compounds, 2a and 2c showed potential activity against *Staphylococcus aureus* (data was not shown).

*3.4. Computational investigations*

With the developments of computational chemistry, the research on the theoretical modeling of drug design and functional material design has gained much attention of synthetic chemists to

predict chemical and physical properties of synthetic drugs in biological systems [36]. Previously, it was showed that the density functional theory (DFT) [37], [38] is efficient to limelight on the radical scavenging activity and other physiochemical descriptors of synthetic compounds [39-45]. Energies of the frontier molecular orbitals (FMOs), structure-activity relationship (SAR), and molecular electrostatic potential (MEPs) were systematically explored with the intention to apprehend the biological activity of the studied derivatives.

*3.4.1. Frontier molecular analysis and different electronic properties*

Highest occupied molecular orbitals (HOMO) and the lowest unoccupied molecular orbitals (LUMO) are named as Frontier molecular orbitals (FMOs). Electronic and optical properties of molecules are due to these FMOs. HOMOs and LUMOs were displayed (Figure 5). In chalcone derivatives 2a-2c, the HOMOs are delocalized at the ring A while the LUMOs are localized on the ring 'B' along with the charge distribution on the keto and nitro group.

A comprehensive intra-molecular charge transfer (ICT) was noticed from ring 'A' to 'B'. In flavanone derivatives 3a and 3b, the HOMOs are delocalized on the ring 'A' and partially on ring 'C' along with the –OH group while the LUMOs are localized at the ring 'B' along with the charge distribution on the nitro group. Here, noticeable ICT was perceived from the rings 'A/C' to ring 'B'. The comprehensive ICT from the HOMOs to LUMOs was due to the strong electron withdrawing behavior of the $–NO_2$ group at the ring 'B'. In the reference compound trolox, the HOMO charge density is at conjugated ring (some charge at –OH and $–CH_3$ groups) whereas the LUMO is localized at entire compound illuminating ICT.

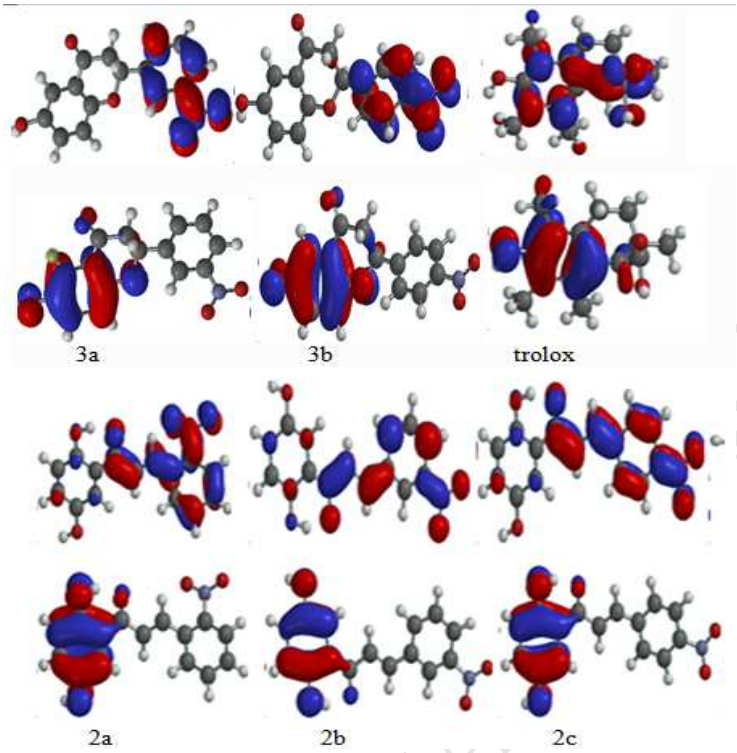


**Figure 5.** The charge density distribution of the HOMOs (bottom) and LUMOs (top) of the chalcone and flavanone derivatives at ground states

The HOMO energies ($E_{HOMO}$), LUMO energies ($E_{LUMO}$), HOMO–LUMO energy gaps ($E_{gap}$), IP and BDE at the B3LYP/6-31G** level of theory were presented. Among the studied chalcone and flavanone derivatives, the highest HOMO and LUMO energy levels were observed for 2a which was also higher than that of phenol. The $E_{gap}$ of all the studied compounds was found to be smaller than the reference compounds, i.e.; phenol and trolox. Hitherto, direct relationship between the HOMO energy levels and the radical scavenging activity of antioxidant compounds was perceived showing that higher HOMO energy levels of studied compounds might lead to the strong electron donating ability as compared to phenol. Additionally, the highest HOMO energy level of 2a would lead better electron donating ability of this derivative among all the studied chalcone and flavanone derivatives (Table 7). The electronegativity (χ), hardness (η),

electrophilicity (ω), softness (S) and electrophilicity index (ωi) at the B3LYP/6-31G** level of theory were presented (Table S1). The smallest χ and ω was observed for the chalcone derivative 2a.

**Table 7.** HOMO energies ($E_{HOMO}$), LUMO energies ($E_{LUMO}$) and HOMO-LUMO gaps ($E_{gap}$) in eV, IP and BDE (Kcal/mol) of chalcone and flavanone derivatives at B3LYP/6-31G** level of theory.

| ***Compounds*** | $E_{HOMO}$ | $E_{LUMO}$ | $E_{gap}$ | **IP** | **BDE** |
|---|---|---|---|---|---|
| Trolox | -5.42 | 0.12 | 5.54 | 690[d] | 381.8[d] |
| phenol | -6.39 | -0.57 | 5.82 | 192[e] | 83[e] |
| 2a | -5.65 | -2.76 | 2.89 | 165 | 82.15[a], 68.17[b], 447.42[c] |
| 2b | -5.74 | -2.81 | 2.93 | 167 | 82.23[a], 68.40[b], 447.62[c] |
| 2c | -5.79 | -3.10 | 2.69 | 168 | 82.11[a], 68.62[b], 447.71[c] |
| 3a | -6.32 | -2.55 | 3.77 | 174 | 71.50 |
| 3b | -6.34 | -2.55 | 3.79 | 176 | 71.60 |

[a] BDE of 2a-x, 2b-x and 2c-x; [b]2a-y, 2b-y and 2c-y; [c]2a-z, 2b-z and 2c-z; [d]The BDE and IP of trolox is 381.8 and 690 Kcal/mol from reference ; [e]The BDE and IP of phenol is 83 and 192 Kcal/mol from reference

*3.4.2. Hydrogen atom transfer mechanism*

The radicals were obtained by hydrogen abstraction, see Figure S1. The vital position for hydrogen atom transfer is hydroxyl. The BDE values presented in Table 7, describes the hydrogen atom donating ability. These values were compared to the trolox [46] and phenol [28]. The BDE values computed at B3LYP/6-31G** level of theory showed that the mono-abstraction of hydrogen usually lowers the BDE values more efficiently. The trend to lower the BDE value in the chalcone derivatives according to the H-abstraction position was observed as y < x < z

(Fig. S1 and Table 7). On the basis of BDE, it was observed that the chalcone derivatives showed better radical scavenging activity as compared to the flavanones. The smallest BDE value was observed for the 2a-y, i.e., 2a chalcone derivative revealing that this compound would be proficient antioxidant contender. Finally, Hydrogen atom transfer mechanism would be favorable in 2a.

*3.4.3. Single electron transfer mechanism*

By single electron donation, the scavenging of free radicals may be attained. Conferring to the one-electron transfer, an electron is removed from the HOMO giving rise to radical cations. The scavenging of free radical can be evaluated by single electron donation. It can be seen from Table 7 that all the derivatives have smaller IP values than the trolox and phenol. Among all the studied derivatives, 2a has the smallest IP value revealing that in aforesaid material electron transfer mechanism might be more inspiring for the scavenging of free radicals. The smaller IP value of 2a as compared to the other counterparts revealed that prior compound would be better antioxidant material (Table 7).

*3.4.4. Molecular electrostatic potential*

The molecular interaction and reactivity sites were assessed by 3-D surface maps of the MEP. In Fig. 6, the MEP surface maps of the chalcone and flavanone derivatives as well as trolox were illustrated to apprehend the positive and negative electrostatic potential (ESP) regions. Negative, positive and zero potential regions were represented by red, blue and green colors, respectively (Fig 6). It is expected that the negative and positive ESP regions would be encouraging for the electrophilic and nucleophilic attack, respectively. In chalcone derivatives, negative ESP (endorsing electrophilic reactive sites) have been realized on the C=O, nitro and -OH oxygen

whereas the nitro of the flavanone derivatives. In trolox, negative ESP at –COOH and hydroxyl oxygen would be promising electrophilic reactive sites.

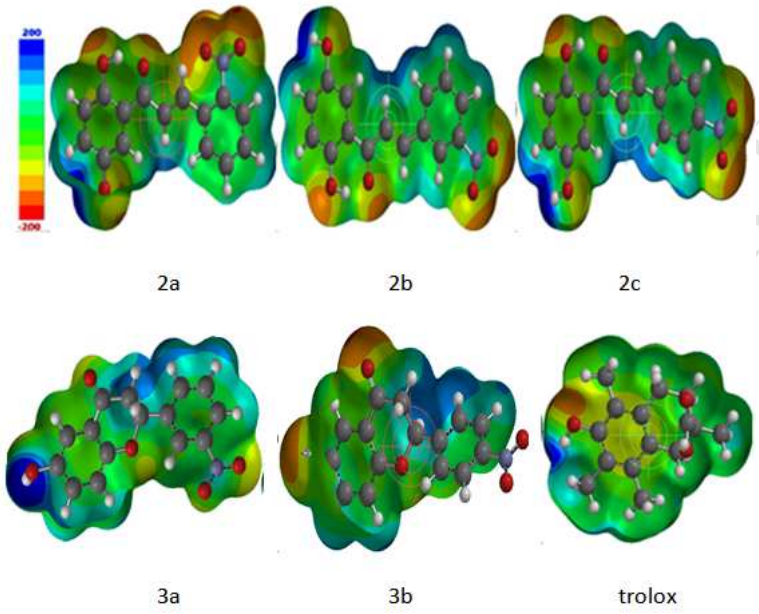


**Figure 6.** The molecular electrostatic potential surfaces of the chalcone and flavanone derivatives

## 4. Conclusion

Acid catalyzed condensation of 2,5-dihydroxyacetophenone with nitrobenzaldehydes leads to the formation of chalcones along with flavanones which showed good antioxidant abilities especially 2a, 2b and 2c. The compound 2a showed excellent antihyperglycemic activity in diabetic rats while 2c exhibited good activity in normal hyperglycemic rats. The determined positions of nitro group at ring 'B' of chalcones exhibited antihyperglycemic activity. The comprehensive intra-molecular charge transfer has been perceived from the HOMOs to the LUMOs. The smaller IP and BDE values for the 2a revealed that this drug would show proficient antioxidant behavior which is in good agreement with the experimental data. In future, compound 2a can be further used to explore its beneficial impacts at molecular level.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## Highlights

- Nitro substituted hydroxychalcones and hydroxyflavanonoes have been synthesized
- Hydroxychalcones (2a, 2b, and 2c) exhibited excellent antioxidant potential
- Compound 2a have shown eminentantihyperglycemic activity on STZ diabetic rats
- Calculations for the structural elucidations by using density functional theory