The Influence of Halogens(CL) at Meta-positions of Aromatic Rings InChalcones on Their *In Vitro* Anti-inflammatory Activity

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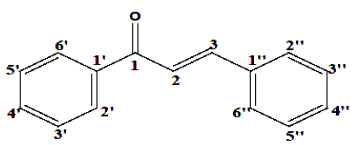
ABSTRACT

The discovery of new therapeutic agents for inflammatory disorders has attracted more attention in recent years. Chalcone term is given to the flavonoid compounds bearing the 1,3-diphenyl-2-propen-1-one framework. Generally, chalcones are precursors of flavonoids with two aromatic rings joined together through three carbons, α, β-unsaturated carbonyl system. In plants, chalcones are converted to the respective (2S)-flavanones by enzymatic reaction of chalcone isomerase. Based on the close chemical and biogenetic relationship between flavanones and chalcones, they are considered as natural products. For anti-inflammatory activity of chalcones, activated macrophages play an important role and compounds with that inhibit nitro oxide production by macrophages have been found potential for the prevention and treatment of inflammatory disorders. Some functional groups such as dimethylamine, methoxy and butoxy groups increase the electron density of the B-ringresulting in significant loss of anti-inflammatory activity. Therefore, in this project three compounds were synthesised for chalcones containing halogens (-Cl) at meta-positions on aromatic rings in chalcones and tested for their anti-inflammatory activity. The synthesized compounds were purified by column chromatography and characterised by ^{1}H-NMR, ^{13}C-NMR, FTIR, Mass and UV spectra. Further evaluation of their*in vitro* anti-inflammatory activity were carried out using RAW 264.7 mouse macrophages. The test dose of chalcones were determined was cytotoxicity (MTT) assay on RAW264.7 mouse macrophages. The results showed that the halogen substitution at meta-positions on aromatic rings improved the anti-inflammatory activity for the compound (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III*)* shows the best activity.

Keywords: Chalcones, m-chlorobenzaldehyde, m-chloroacetophenone, Ethyl alcohol, Anti-inflammatory activity, RAW 264.7 cells

Introduction

In Chalcone term is given to the flavonoid compounds containing 1,3-diphenyl-2-propen-1-one skeleton (1-3). Generally, chalcones are open-chain flavonoids with two aromatic rings joined by a three carbon, α, β-unsaturated carbonyl system. By a stereoselective enzymatic reaction catalysed by enzyme, chalcone isomerase in plants and are converted to the corresponding (2S)-flavanones. Based on the close chemical and biogenetic relationship between flavanones and chalcones, these often considered as natural products. The general structure and numbering scheme of chalcones is presented in Fig 1.1.



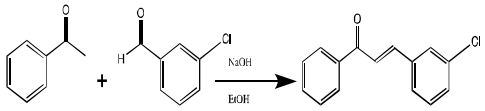
Materials and Methods

1.Chemicals

All the chemicals including ketones (acetophenone, 3-chloro acetophenone, 3-floro acetophenone), aldehydes (benzaldehyde, 3-chloro benzaldehyde and 3-floro benzaldehyde), and sodium hydroxide were of analytical grade and bought from Sigma Aldrich and used without further purification. Deionized double-distilled water was used throughout the experiments. Absolute ethanol from Sigma Aldrich was also used without further distillation. The resulting (E)-chalcone, (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one, (E)-1,3-bis(3-chlorophenyl)prop-2-en-1-one, (E)-1-(3-fluorophenyl)-3-phenylprop-2-en-1-one, (E)-1,3-bis(3-fluorophenyl)prop-2-en-1-one products were synthesized and purified based on previously reported literatures (86-88)

2. Synthetic routes

The (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one was prepared by stirring the mixture of 3-chloro acetophenone (8.57 mMol, 1.3 g) and benzaldehyde (8.57 mMol, 0.9 g) in minimum amount of ethanol at room temperature for 8 hours. After completion of reaction using then thin layer chromatography (TLC) (ehtylacetate (10): hexane (90))and a solution of sodium hydroxide 40%, was added drop wise. Then, the reaction mixture was stirred at room temperature for 2-3 hours until the residue was formed. The solid was collected by filtration and washed with cold ethanol to remove the unreacted starting materials. The product, (E)-chalcone, was then recrystallized from ethanol and dried for 1 h by vacuum filtration and vacuum oven for 1-day Yellow powder was obtained in ca 66% yield; Melting point:(91-93 ℃) (Scheme 3.1).



Scheme 1 Synthesis (E)-3-chlorophenyl)-1-phenylprop-2-en-1-on

3.Instrumentation

The synthesized chalcones were kept in a desiccator and related melting points were determined by Electro Thermal Digital Melting point apparatus model IA 9100 (0-400) $^{\circ}C$. The IR of the products were recorded by using Perkin Elmer GX spectrophotometer in the range of 400-4000 cm^{-1} and the spectrophotometer is attached with Attenuated Total Reflectance (ATR) sample holder. Nuclear Magnetic Resonance (NMR) for ^{1}H and ^{13}C experiments were performed with Joel- ECP 400 MHz and bench top NMR (50 MHz) spectrometer using $CDCl_3$ and DMSO-d_6 as solvents. UV-visible absorption spectrum of the compounds were recorded using UV-VIS spectrophotometer using quartz

cuvette. Multi-Analyte ELIS Array Kits from Qiagen were used for anti-inflammatory tests.

Results and Discussion

1.Preparation of meta halogen chalcones by the reaction of halogen substitution of acetophenone and benzaldehydes

The condensation reaction of substituted acetophenones with different benzaldehydes in the presence of catalysts have been already reported in the literature [91-93]. In this work, we have synthesized several chalcones bearing halogens (Cl and F) on their benzene rings from condensation reaction of meta halogen acetophenones and meta halogen benzaldehydes in the presence of sodium hydroxide. The influence of halogen substituents at meta position of aromatic rings on the inflammatory activity of prepared chalcones has been further evaluated.

1. The characterization of the compound of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (I)

IR spectrum of synthesized (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (Figure 4.1) showed the absorption bands of (C-H) $_{stretching}$, C=O, C=C, (=C-H) $_{bending}$ and C-Cl functional groups at 3043, 1661, 1568, 758 and 743 cm^{-1}, respectively as shown in Fig 1.

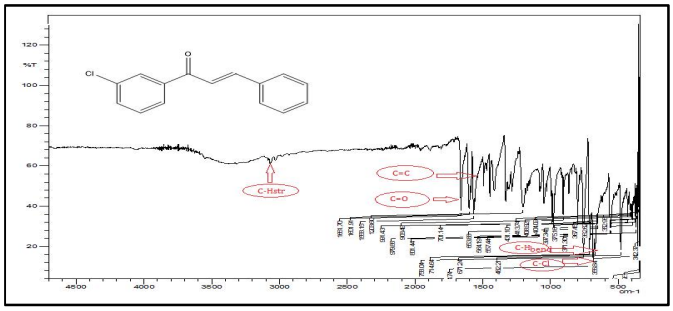


Figure 2 Infra-red (IR) spectrum of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (I)

The ^{1}H NMR and ^{13}C NMR of product I, were in agreement with the structure of this compound. The ^{1}H NMR spectrum showed the vinyl protons as two doublets at chemical shifts of 8.15 and 7.70 ppm, respectively with coupling constant. The aromatic ring protons were also observed as doublets, triplets and multiples at chemical shifts of about ppm8.16 (1H, d, *J* = 2.0 MHz), 8.15(1H, s), 7.87(1H, s), 7.78 (2H, s), 7.744(1H, s), 7.69(1H, t, *J* = 1.5 MHz), 7.67 (1H, d, *J*= 1 MHz), 7.44 (3H, t, *J* = 1.5 MHz).The ^{13}C NMR–spectrum of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (I) showed carbonyl group at 188.3 δ ppm and the carbons of vinyl group at chemical shifts of 145.2, 122 δ ppm. The benzene ring carbons were also appeared at corresponding chemical shifts of 145, 139, 134, 133, 131, 129 δ ppm

The LC-MS fraction pattern of product I, also showed the (M+Na)+ and

(M+H+CH3CN)+ clusters at m/z of 265.11 and 283.14, respectively (Figure 5).

The UV-visible spectrum showed the electron transaction of π π* at maximum UV band of 245 nm (Figure 3).

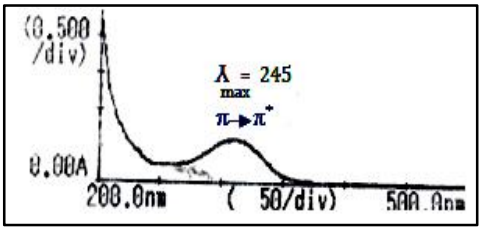


Figure 3. UV-visible spectrum of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (I)

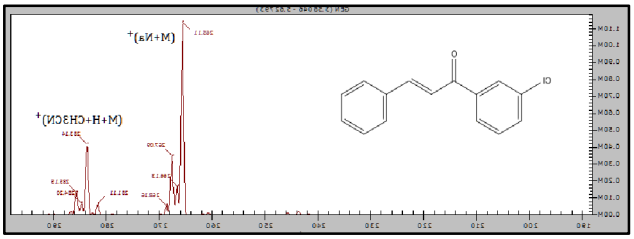


Figure 4. LC-MS spectrum of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one

2 The characterization of the compound (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II):

In infra-red (IR) spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II), the absorption bands of (C-H) stretching, C=O, C=C, (=C-H) bending and C-Cl functional groups were observed at 3066, 1656, 1562, 703 and 681 cm-1, respectively as shown in Fig 5.

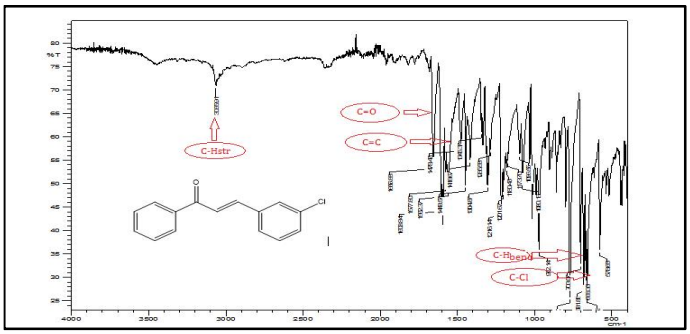


Figure 5 Infra-red (IR) spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one

The 1H NMR spectrum of product (II) showed the vinyl protons as two doublets at chemical shifts of 8.17 and 8.00 ppm, respectively. The aromatic ring protons were also observed as doublets, triplet and multiples at chemical shifts of about 8.18(1H, d, J=5 MHz),8.17(1H, d, J =1.5 MHz),8.16(1H,s) 8.06(1H, s), 8.60(1H, s), 7.80 (1H, s), 7.73(1H,s)7.68 (1H, s), 7.58 (1H, d),7.47 (2H, s).ppm as shown in Fig 4.8. The broad peak

appeared at 3.38 ppm, is representing the presence of solvent impurity (DMSO).

The 13C NMR–spectrum of product (II) showed the carbon of carbonyl The benzene ring carbons were also appeared at corresponding chemical shifts of 137, 134, 134, 133,130, 129, and 128 δ ppm

group at 189.5 δ ppm and the carbons of vinyl group at chemical shifts of 142.7, 124 δ ppm.

The LC-MS fraction pattern of product I, also showed the (M+Na)+ and (M+H+CH3CN)+ clusters at m/z of 265.11 and 283.14, respectively (Figure 6).

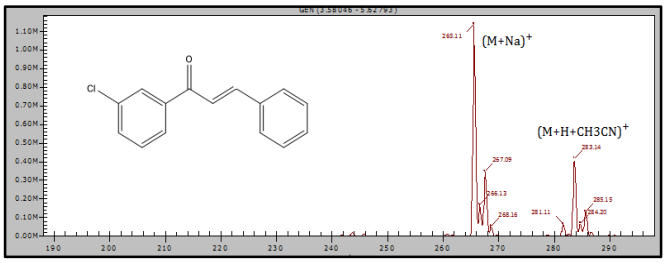


Figure 6 LC-MS spectrum of (E)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one

The characterization of the compound (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II):

In infra-red (IR) spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II), the absorption bands of (C-H) $_{stretching}$, C=O, C=C, (=C-H) $_{bending}$ and C-Cl functional groups were observed at 3066, 1656, 1562, 703 and 681 cm^{-1}, respectively as shown in Fig 7.

The UV-visible spectrum of compound (II) also showed the electron

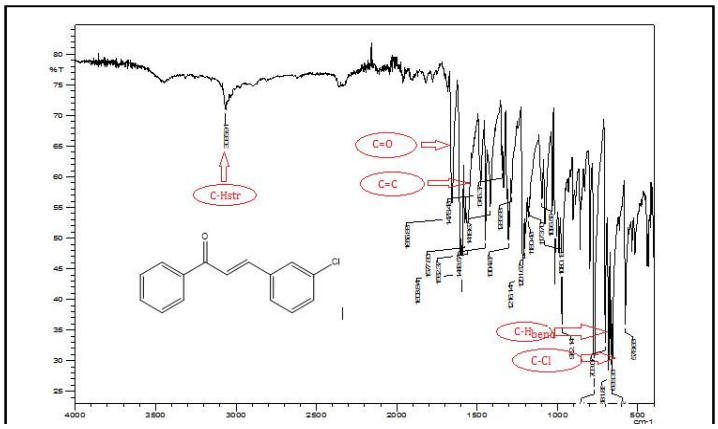


Figure 7 Infra-red (IR) spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one

The ^{1}H NMR spectrum of product (II) showed the vinyl protons as two doublets at chemical shifts of 8.17 and 8.00 ppm, respectively. The aromatic

ring protons were also observed as doublets, triplet and multiples at chemical shifts of about 8.18(1H, d, J=5 MHz),8.17(1H, d, J =1.5 MHz),8.16(1H,s) 8.06(1H, s), 8.60(1H, s), 7.80 (1H, s), 7.73(1H,s)7.68 (1H, s), **The ^{13}C NMR**–spectrum of product (II) showed the carbon of carbonyl group at 189.5 δ ppm and the carbons of vinyl group at chemical shifts of 142.7, 124 δ ppm. The benzene ring carbons were also appeared at corresponding chemical shifts of 137, 134, 134, 133,130, 129, and 128 δ ppm 7.58 (1H, d),7.47 (2H, s).ppm as shown in Fig 4.8. The broad peak appeared at 3.38 ppm, is representing the presence of solvent impurity (DMSO).

The UV-visible spectrum of compound (II) also showed the electrontransaction of $\pi \longrightarrow \pi^*$ at maximum UV band of 240 nm (Figure 8).

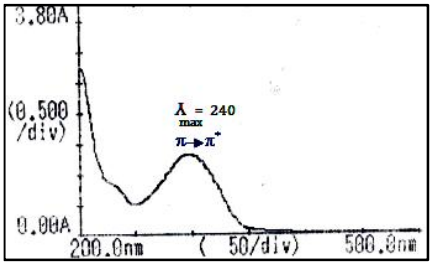
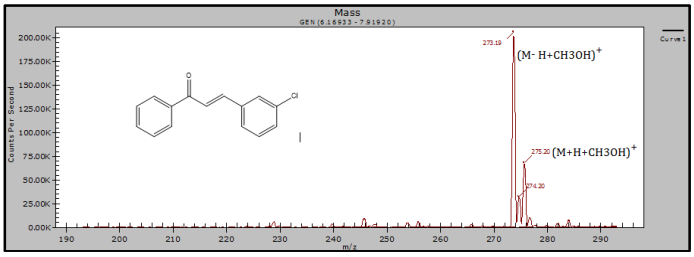


Figure 8 UV-visible spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II)
The LC-MS fraction pattern of product II, represented (M-H+CH3OH) + and (M+H+CH3OH) + clusters at m/z of 273.19 and 275.20, respectively (Figure 9).



Figure(9) LC-MS spectrum of (E)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one (II)

The characterization of the compound (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III) The IR spectrum of product (III) also showed the adsorption bands of corresponding functional groups of (C-H) $_{\text{stretching}}$, C=O, C=C, (=C-H) $_{\text{bending}}$ and C-Cl at 3070, 1669, 1565, 773, 696 cm^{-1} as shown in Table 4.1 in comparison with product (I) and (II). The ^{1}H NMR of (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III) showed the vinyl protons as

two doublets at chemical shifts of 7.67 and 7.10 ppm, respectively. The aromatic ring proto answered also observed in overlap to each other at chemical shifts of about 7.53, 7.35, 7.23, 7.01, and 6.85 ppm asppm

The UV-visible spectrum of compound (III) showed the electron transaction of π π* at maximum UV band of 250 nm (Figure 10).

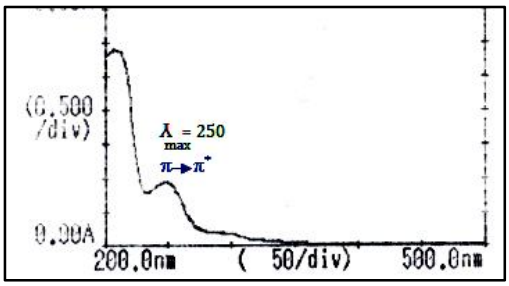
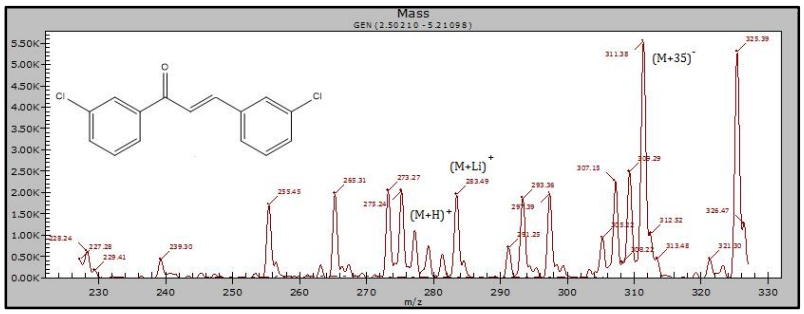


Figure (10)UV-visible spectrum of (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III)

The LC-MS fraction pattern of product III, showed and (M+H)+, (M+Li)+, and (M+35)- clusters at m/z of 273.27 ,283.49 and 311.38, respectively (Figure 11).



Figure(11) LC-MS spectrum of (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III)

2 In vitro anti-inflammatory activity of chalcones

2.1The chalcones were tested for their effect on viability of RAW 264.7 cells using MTT assay.

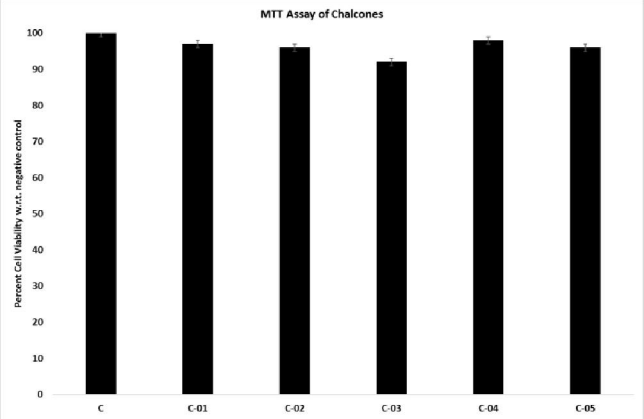


Figure (12)Cytotoxic effect of chalcones onRAW 264.7 cell line. RAW 264.7 cells were treated with chalcones at a concentration of 100 μM. Cell viability was determined using MTT assay. All the compounds were found to be non-toxic on RAW 264.7 cells.

For all *in vitro* experiments, the activities of halogen substituted chalcones (C-I to C-III) were compared with unsubstituted simple chalcone (C). The MTT assay results revealed that all the chalcones at 100 μM (the highest concentration used in subsequent studies) do not decrease the cell viability of RAW 264.7 cells indicating that all the chalcones were non-toxic.

Nitro oxide inhibiting test

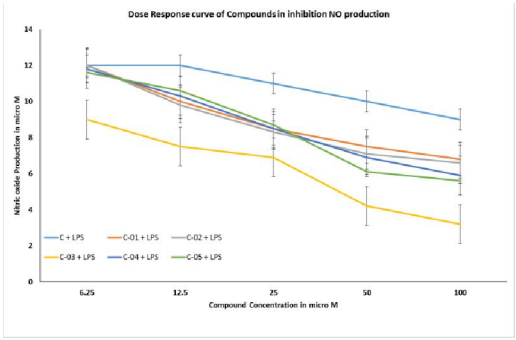


Figure (13)*In vitro* anti-inflammatory activity chalcones in inhibiting NO production in RAW 264.7 cell line.

The chalcones (C-I to -III) were found to more active than simple chalcone (C) in reducing nitric oxide production in RAW 264.7 cells. These results indicate that halogens at meta-position on aromatic rings of chalcones have positive influence on their anti-inflammatory activity. Among mono-substituted chalcones, fluorine showed positive influence than chlorine in inhibiting NO production in RAW 264.7 cells. Disubstitutedchalcone (C-III) was found to be more potent than mono-substituted chalcones. The IC50 values of chalcones were shown in the following Table 2

Chalcone	**IC_{50} value**

C	> 100 μM
C-01	> 100 μM
C-02	> 100 μM
C-03	29.7 μM

Table.2: IC50 values of chalcones in inhibiting NO production by RAW 264.7 cells

From the Table 4.2, it is clearly evident that chalcones substituted with mono-substituted and disubstituted chlorine were found to be more potent than simple chalcone (C). Since di-chlorsubstitutedchalcone (C-III) was found to be more potent, it's influence on the expression cytokines were determined using multi-analytie ELISA kit from Qiagen whose results were shown in Fig 14.

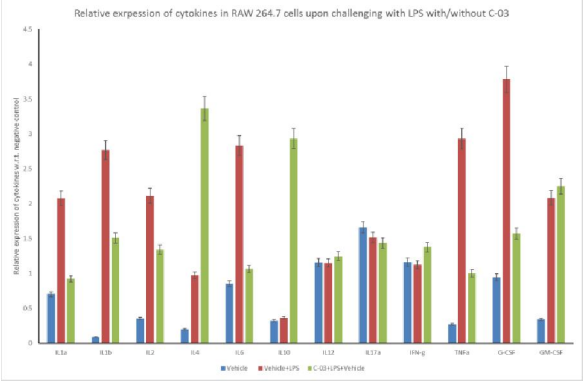


Figure (14)Relative expression of cytokines in RAW 264.7 cells treated with LPS with and without disubstitutedchalcone (C-III)

Interleukin (IL)-1α, IL-1β, IL-2, IL-6, TNF-α, G-CSF and GM-CSF are pro-inflammatory cytokines. IL-4, IL-10, IFN-γ and anti-inflammatory cytokines. The remaining cytokines IL-12 and IL-17α could be either pro- or anti-inflammatory cytokines. From the figure 4.24; it is clearly evident that C-03 at a concentration of 25μM reduced the expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6, TNF-α and G-CSF). The chalcone C-III at a concentration also increased expression of anti-inflmmatory cytokines such as IL-4 and IL-10. The chalcone C-III does not possess any effect on expression of cytikines, IL-12, IL-17α and IFN-γ.

Determination of *in vitro* anti-inflammatory activity of chalcones

In this project, RAW 264.7 were used to determine the anti-inflammatory activity of the targeted compounds.

Cell Culture

Cell culture is type of the basic *in vitro* determination used in the biological assays, giving a good knowledge for studying the physiology of cells, determine the toxicity as well as mutagenic and carcinogenesis potential of the new compounds. Cell culture or *in*

vitro analyses play an important role in drug screening and development (82).

RAW 264.7 mouse macrophage cells are the most widely used in-vitro model to test the anti-inflammatory activity of compounds (83-85). Expression of cytokines in RAW 264.7 cells upon treatment with Chalcone (E)-1,3-bis(3-chlorophenyl) prop-2-en-1-one (III) From previous studies, it was found that dichloro-substituted chalcone (C-III) was found to be the most potent chalcone. Therefore, its effect on expression of cytokines profiles was determined at a concentration, 25 μM (closest to IC_{50} values). In accordance with previous procedures, the RAW 264.7 cells were pretreated with C-III for 4 hours followed by challenging with LPS for 20 hours. After incubation period, the cell supernatants (50 μL) were placed in multi-analyte ELISA array plate and the relative expression of cytokines (either pro- or anti-) were determined whose results were shown in Fig 4.24. In general, the chalcone C-III suppressed the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines

CONCLUSION

In the present study, five chalcones were synthesized in which the halogens (Cl and F) were substituted at meta positions on aromatic rings. All the compounds were purified well by using column chromatography technique, and then characterised using physical and spectral data such as ^{1}H and ^{13}C NMR, Mass and FTIR spectra. Furthermore, *in vitro* anti-inflammatory activity of chalcones were determined in RAW 264.7 mouse macrophage cells using Greiss reagent. The in vitro anti-inflammatory activity of chalcones were compared with simple chalcone to determine the influence of halogens on anti-inflammatory activity. The results showed that the halogen substitution at meta-positions on aromatic rings improved the anti-inflammatory activity of chalcones, the order of activity being di-chloro substituted >monofluoro substituted >monochloro substituted > simple chalcone. The toxicity of chalcones were determined using MTT assay and the chalcones were found to be non-toxic against RAW264.7 cells at the concentration of 100 μM, the highest concentration used in *in vitro* assays. The dichloro-substituted chalcone (C-III) was found to be the most potent and tested for its influence on expression of cytokines using multianalyte ELISA array kit. The chalcone, C-III (E)-1,3-bis(3-chlorophenyl)prop2-en-1one, reduced the expression of a few pro-inflammatory cytokines, increased the expression of a few anti-inflammatory cytokines and few more cytokines were unaffected.

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