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Synthesis and Spectral Studies of R(2),C(4)-Bis(Tert-Butoxycarbonyl)-C(5)-Hydroxy-T(5)-Methyl-T(3)-Phenyl Cyclohexanone and Their Biological Activity

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Abstract

There are three new t(3)-aryl-r(2),c(4)-bis(tert-butoxycarbonyl)-c(5)-hydroxy-t(5)-methyl-cyclohexanones (**1-3)** have been synthesized by condensing tert-butyl acetoacetate with different aromatic aldehydes in the presence of methylamine as a stereospecific catalyst. The structures of the products were confirmed by ^{1}H NMR, ^{13}C NMR, and IR spectroscopic techniques. For compound (**1),** HMBC, HSQC, COSY, NOESY, and Mass spectra of the compounds were studied. Elemental analysis was carried out for all the compounds. In vitro biological applications like anti-inflammatory and antioxidant activities were also performed. The *tert*-butoxycarbonyl cyclohexanones showed good anti-inflammatory and antioxidant activities, and the percentage of inhibitions is nearer to standard drugs.

Keywords: Cyclohexanones; Conformation; Antioxidant; Anti-inflammatory.

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1. Introduction

NMR spectroscopy is a powerful tool for determining the structure and stereochemistry of organic compounds [1-5]. The chemistry of β-keto esters is a wide and interesting area of its theoretical implications, conveniently in synthetic routes and also for their exhibiting as a synthetic reagent to synthesize heterocycles and fused heterocycles [6]. Sabapathy Mohan et al. examined the reaction of alkyl acetoacetate with benzaldehyde in the presence of methylamine as a stereospecific catalyst [7,8]. Continuing our work, we report the analysis of the products obtained by the reaction of acetylacetone with substituted aromatic aldehydes using 1D and 2D NMR spectra. In all cases investigated, only a single product was obtained. In this study, it is of interest to investigate the reaction of alkyl acetoacetate with a substituted aromatic aldehyde in the presence of methylamine (Scheme 1). In all cases they investigated, only a single product was obtained. (prefix 'r' indicates the reference, c- and indicates substituent is *cis* and *trans* to the reference). All the synthesized β-keto esters of **1-3** compounds have been shown to adopt chair

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conformations (1C-3C) with an axial orientation of the OH group and equatorial orientations of all other substituents. For the proof of such information, the ^{1}H NMR, ^{13}C NMR, IR, MASS, HOMOCOSY, NOESY, HSQC, and HMBC spectra have been recorded for compound **1**. Vicinal proton-proton coupling constant can be used for conformational analysis since it depends on the torsional angle between two protons in adjacent carbons [9]. For Compounds (**1-3),** ^{1}H and ^{13}C NMR have been recorded for their conformation analysis. The identification of bioactive molecules and synthesis of natural or synthetic drugs is the starting point in drug discovery research, and also, the synthesized chemical entities must have the drug-likeness and obey the Pfizer rule [10,11]. These are the criteria that followed during the discovery of new drugs. Heterocyclic compounds are an important class and have been found as a crucial structural core in natural and synthetic drugs [12]. In recent years, β-keto esters have displayed a number of important biological properties such as bactericidal, fungicidal, and anti-inflammatory activities [13-15]. Antioxidants have gained a lot of importance because of their potential prophylactic and therapeutic activities against many diseases. The β-keto ester derivatives have also been found to act as cytotoxicity towards various cancer cell lines [16]. In the present study, the anti-inflammatory and antioxidant activities of compounds (**1-3**) have been studied.

2. Experimental

2.1. *Preparation of compounds*

A mixture of tert-butyl acetoacetate and aromatic aldehyde in the mole ratio of 2:1 in the presence of methylamine as a catalyst dissolved in ethanol, and the mixture was heated to boiling. The reaction mixture was kept at room temperature for one day. The separated solid was filtered and purified by recrystallization from ethanol. For all compounds, the purity was checked by TLC. The physical data for compounds **1-3** are given in Table 1.

The β-keto esters **1-3** are colorless crystalline substances that are soluble in DMSO and dioxane, poorly soluble in alkanes, and insoluble in water.

2.2. *Recording of spectra*

The IR spectra for β-keto esters (**1-3**) were recorded on FT/IR-4700 type A instrument. All the NMR measurements were made using 5mm tubes and were recorded on a Bruker III HD Nanobay 400 NMR spectrometer operating at 400.23 MHz for ^{1}H and 100.64 MHz for ^{13}C. For recording ^{1}H NMR spectra, solutions were prepared by dissolving 10 mg of the material in 0.5 mL of $CDCl_3$. For recording ^{13}C NMR spectra, solutions were prepared by dissolving 50 mg of the material in 0.5 mL of $CDCl_3$. The spectra like HOMOCOSY, NOESY, HSQC, and HMBC spectra were recorded on a Bruker 400.23 NMR spectrometer using standard parameters. For recording 2D spectra, solutions were made by dissolving 50 mg of the material in 0.5 mL of $CDCl_3$. For the NOESY spectrum, the

mixing time was one thousand milliseconds. Elemental analysis was performed on a Perkin-Elmer CHNS/O analyzer.

2.3. *Biological studies*

2.3.1. *Anti-inflammatory activity*

BSA denaturation technique: The synthesized compounds and drug standard diclofenac sodium were screened for anti-inflammatory activity by using the inhibition of albumin denaturation technique with minor modification. The standard drug and compounds were dissolved in a minimum quantity of dimethyl formamide (DMF) and diluted with buffer (0.2 M, pH 7.4). The final concentration of DMF in all solutions was less than 2.5 %. The test solution (2.5 mL) containing different concentrations of the drug was mixed with 1 mL of 1mM Bovine serum albumin solution in phosphate buffer and incubated at 37 °C in an incubator for 10 min. After cooling, the turbidity was measured at 660 nm. The percentage of inhibition of denaturation was calculated by using the following formula.

% of Inhibition = 100 x $(A_c-A_t)/A_c$

A_t: Absorbance of test

A_c: Absorbance of control

2.3.2. *Antioxidant activity*

The total antioxidant activities of samples were observed. About 3 mL of antioxidant reagent (0.6 M H_2SO_4, 28 mM Na_3PO_4, and four mM ammonium molybdate) was added to the test samples with various concentrations. The test mixture was accomplished proper diffusion with phosphomolybdenum reagent and was incubated at 95 °C for 90 min in a water bath. The total antioxidant activity of extracts and vitamin C standard drugs were measured, and determined their absorbance at 695 nm using a spectrophotometer. The total antioxidant activities were calculated using the given formula.

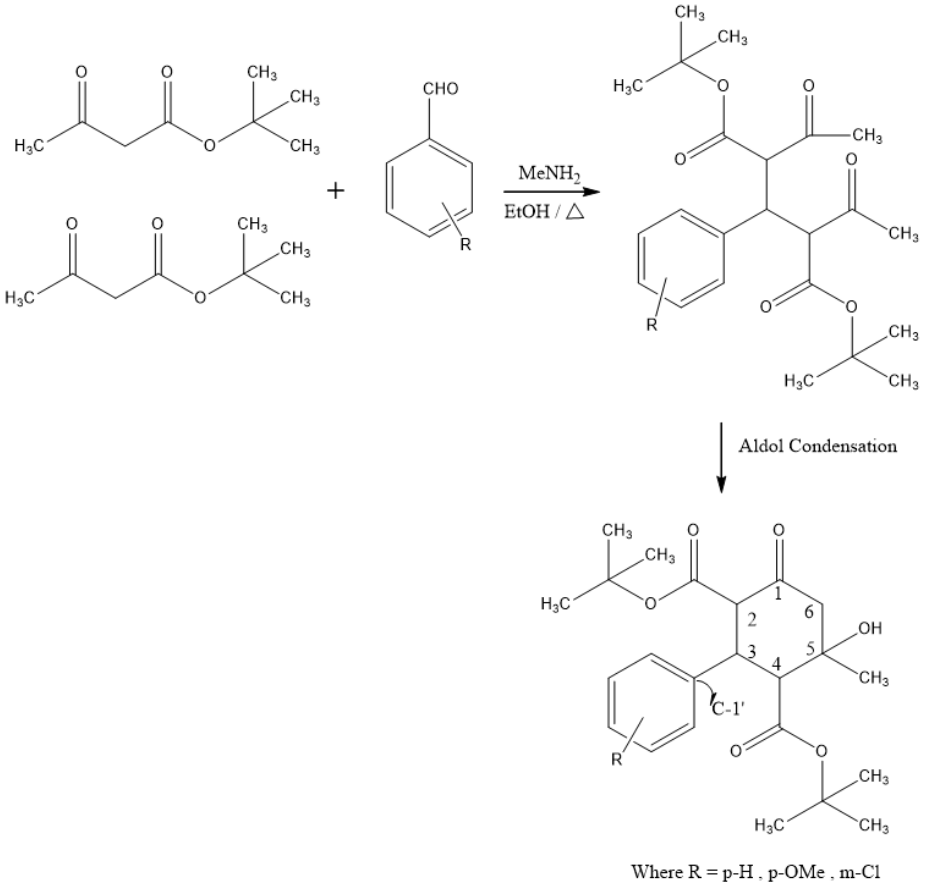
$TOA = [(A_t - A_c) / A_t] X\ 100.$

3. Results and Discussion

3.1. *Synthesis and characterization*

In this study, synthesis of r(2),c(4)-bis(tert-butoxycarbonyl)-c(5)-hydroxy-t(5)-methyl-t(3)-arylcyclohexanones **(1-3)** were synthesized by the condensation reaction of appropriate quantity of tert-butyl acetoacetate with an aromatic aldehyde in the presence of methylamine as shown in Scheme-1. The structure of the products (**1-3)** was confirmed by elemental analysis, IR, Mass spectra, ^{1}H , ^{13}C , 2D spectra, and their biological studies were performed. For naming the compounds, the prefix ‘r’ before the substituent at C-2

indicates that this is the reference substituent, c- and t-indicates that the particular substituent is cis and trans to the reference substituent.



Scheme 1. Synthesis of cyclohexanoes **(1-3).**

All the synthesized compounds (**1-3**) were purified by recrystallization from ethanol. Their homogeneity was checked by TLC. For compound **1,** the percentage of carbon and hydrogen were found as 68.29 and 7.97, respectively. The percentage of carbon and hydrogen, which are calculated for the formula $C_{23}H_{32}O_{6,}$ were 68.32 and 7.96, respectively. The molecular mass of **1** was found as 404, which agrees with the proposed formula, and the data are given in Table 1.

Table 1. Physical data of synthesized compounds (1-3).

Empirical formula	Compound	Yield (%)	MP (°C)	Found		Calculated	
				C (%)	H (%)	C (%)	H (%)
$C_{23}H_{32}O_6$	1	90	125	68.29	7.97	68.32	7.96
$C_{24}H_{34}O_7$	2	88	153	66.32	7.90	66.34	7.89
$C_{23}H_{31}O_6Cl$	3	85	142	62.94	7.15	62.92	7.12

The carbon atoms in the cyclohexanone ring are numbered as shown in Fig. 1. The numbering pattern of the other carbon atoms and oxygen atoms in compound (**1)** is shown in Fig. 2. The hydrogen atoms on the carbons are numbered accordingly.

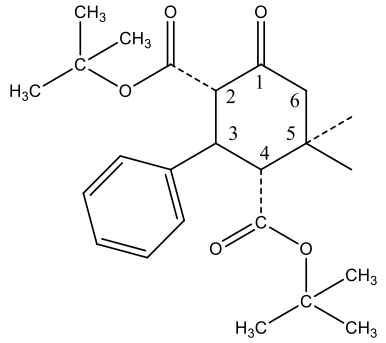


Fig. 1. Numbering of carbon atoms in cyclohexanone ring.

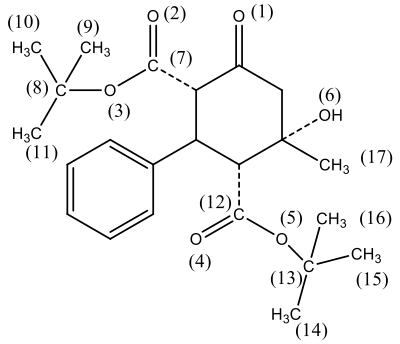


Fig. 2. Numbering of carbons and oxygen in compound 1.

3.1.1. *Analysis of spectra*

The IR spectrum of compound (**1)** showed a medium band at 3491 cm^{-1} due to OH stretching. There were three strong bands observed at 1701, 1711, and 1729 cm^{-1} due to the three kinds of carbonyl groups. The band at 1701cm^{-1} should be due to the keto carbonyl group. Among the two ester carbonyl groups at C-4 should have a lower stretching frequency due to hydrogen bonding with OH. Hence the band at 1711cm^{-1} is due to the ester carbonyl group at C-4. The band at 1729 cm^{-1} should be due to the ester carbonyl group at C-4.

In the ^{1}H NMR spectra of compounds (**1-3)**, we observed that there is a sharp singlet at 1.34-1.35 ppm, corresponding to three protons due to the methyl protons at C-5. There is a triplet at 3.84-3.96 ppm was observed and corresponds to the presence of only one proton. This should be due to the benzylic proton at H-3a. This signal showed to be only as a triplet; this triplet is an overlap of two double-doubles. There are two singlets

appeared at 1.10-1.18 and 1.24-1.25 ppm, with a total integral corresponding to nine protons. These are due to the methyl protons in the tert-butyl group. There is only one singlet is obtained for each of them, and the ^{1}H NMR spectral data is presented in Table 2. For confirming these assignments, the ^{1}H-^{1}H COSY spectrum was recorded for compound (**1)** in $CDCl_3$. The various correlations in the ^{1}H-^{1}H COSY correlations and NOESY spectra are given in Table 3.

From the magnitudes of the COSY spectrum, the following information could be gained. It is seen that the H_{6e} proton shows a correlation with the signal at 2.45 and 2.67 ppm. The H_{6a} proton may couple with only the H_{6e} proton, obviously; the signals at 2.45 and 2.67 ppm are due to the H_{6a} and H_{6e} protons. Also, the signal at 3.49 ppm shows a correlation with the triplets at 3.96 ppm. These correlations suggest that the signal at 3.49 ppm is due to the OH protons. There are long-range coupling exhibited between the OH proton and the methylene proton at 2.45 ppm.

Table 2. Proton chemical shifts in compounds **(1-3).**

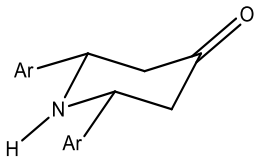
Position	Compound 1	Compound 2	Compound 3
H-2a	3.49	3.46	3.43
H-3a	3.96	3.84	3.84
H-4a	2.94	2.91	2.9
H-6a	2.45	2.43	2.43
H-6e	2.67	2.65	2.65
OH at C-5	3.85	3.86	3.84
CH_3 at C-5	1.35	1.34	1.34
Methyl proton at COOt-But at C-2	1.18	1.10	1.19
Methyl proton at COOt-But at C-4	1.25	1.24	1.25
Aromatic	7.27	7.15	7.32

Since methylene protons at C-5 show NOESY with both the adjacent methylene protons and hence the methyl group should be equatorial. The methyl protons show NOESY with the proton at 2.94 ppm but not with that of 3.49 ppm. Hence the signal at 2.94ppm is due to H-4, and the signal at 3.49 ppm can be assigned to H-2. Aromatic protons show NOESY with H-2a, H-3a, and H-4a. A similar observation has been made in 2,6-diaryl piperidine-4-ones, and the conformation is shown in Fig. 3.

Table 3. Correlations in the COSY and NOESY spectra of compound (1).

Signal	Correlations in the COSY spectrum	Correlations in the NOESY spectrum
7.27 (m,5H)	None	3.96, 3.49, 2.94
3.96 (t,1H)	3.49, 2.94	2.94,3.49
3.88 (d,1H)	None	2.45
3.49 (d,1H)	3.96	2.45, 2.94, 3.96
2.94 (d,1H)	3.96	2.45,3.49, 3.96
2.67 (d,1H)	2.45	2.45
2.45 (d,1H)	3.96, 2.62	3.88, 3.49, 2.94
1.35 (s,3H)	None	2.45, 2.67, 2.94
1.25 (s,9H)	None	1.27
1.18 (s,9H)	None	1.08

In compounds **2** and **3**, individual assignments of aromatic protons were made based on positions, multiplicities, and the integral values of signals.



Ar= p-$OMeC_6H_4$ or m-ClC_6H_4

Fig. 3. Chair conformation of 2,6-diaryl piperidine-4-ones.

3.1.2. *Conformational study*

In compounds **1-3**, the vicinal coupling constants between H(2) and H(3) are found as 12.4 Hz, and that between H(3) and H(4) is found as 12.2 Hz. The observed vicinal coupling constants are consistent with chair conformations 1C-3C for these compounds (**1-3**), as shown in Fig. 4. In the cases of compound **1**, the configuration at C-5 has been proved by the NOESY. The observation of long-range coupling with a reasonable magnitude suggests that the OH proton and axial methylene proton should be in a W-arrangement.

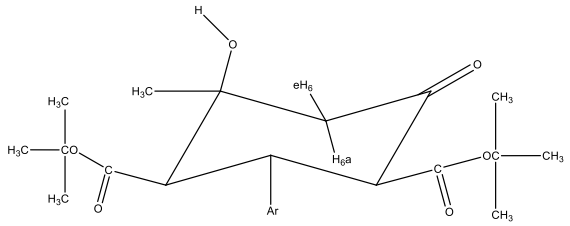


Fig. 4. Chair conformation of compounds (1-3).

3.1.3. *^{13}C NMR spectra*

In order to assign the signals unambiguously, HSQC and HMBC spectra have been recorded for 1. The signals for carbon-containing hydrogen are assigned based on the observed correlations in the HSQC spectrum. Signals showing no correlation in the HSQC spectrum are due to carbonyl carbon containing no proton [17]. The observed correlations in the HSQC and HMBC spectra are given in Table 4. In the HMBC spectrum, the signal 201.7 ppm shows a correlation with H-2a, H-6a, H-6e, and methyl protons at C-5. Hence the signal must be due to the carbonyl carbon C-1. The signal at 174.4 ppm shows a correlation with H-4a and H-6e. Hence, this signal must be due to the CO of COOt-Bu at C-4. The signal at 166.8 ppm shows a correlation with the H-3a. Hence, this signal must be due to the CO of COOt-Bu at C-2. The signal at 138.2 ppm shows a correlation with H-2a, H-3a, and H-4a. Hence, it is due to C-1. The remaining signals at 127.0, 127.7, and 128.7 ppm are due to aromatic carbons. Since in

monosubstituted benzenes, the ortho-carbons have lower chemical shifts than the meta-carbons, the signal at 128.7 ppm may be assigned as C-3,' and C-5', and the signals at 127.7 ppm are due to C-2' and C-6'.

Table 4. Correlations in the HSQC and HMBC spectra of compound 1.

^{13}C chemical shifts (ppm)	Correlations in the HSQC spectrum	Correlations in the HMBC spectrum
201.7	None	$H_{2a,}H_{6a}$, H_{6e}, CH_3 at C-5
174.4	None	H_{4a}, H_{6e}
166.8	None	$H_{2a,}$ H_{3a}
138.2	None	Aromatic, $H_{2a,}$ $H_{3a,}H_{4a}$
128.7, 127.7,127.0	Aromatic protons	Aromatic protons
63.7	H_{2a}	H_{3a}, H_{4a}, H_{6e}
58.8	H_{4a}	H_{2a},H_{3a}, H_{4a}, $H_{6e,}$ CH_3 at C-5
52.9	$H_{6a,}$ H_{6e},	CH_3 at C-5
45.5	H_{3a}	Aromatic, H_{2a}, H_{4a}
28.5	CH_3 proton at C-5	H_{6e}
27.8	CH_3 protons of COOt-Bu at C-4	CO of COOt-Bt at C-4
27.6	CH_3 protons of COOt-Bu at C-2	CO of COOt-Bt at C-2

In the ^{13}C NMR spectrum of compound **1**, for the methyl carbons of the tertiary butyl groups, two signals were observed at 27.6, and 27.8 ppm, and the data are given in Table 5. From the HMBC spectrum, the signals at 27.6 ppm could be assigned to the methyl carbons of the tertiary butyl group at C-2. In compound **3**, the carbon of the methyl group appeared at 55.4 ppm.

Table 5. ^{13}C chemical shifts (ppm) of compounds (1-3).

Carbon position	Compound 1	Compound 2	Compound 3
C-1(C=O)	201.7	201.5	201.8
C-2	63.7	63.4	64.0
C-3	45.5	45.3	44.7
C-4	58.8	56.8	57.1
C-5	73.1	73.1	73.0
C-6	52.9	52.8	52.9
CH_3 at C-5	28.5	28.5	29.7
CO, ipso, and Methyl group of COOt-Bu at C-2	166.8, 82.4, 27.8	166.7, 82.6, 27.9	166.9, 82.3, 28.3
CO, ipso, and Methyl group of COOt-Bu at C-4	174.4, 81.4, 27.6	173.2, 81.6, 28.2	173.4, 81.5, 28.5
Aromatic	127.0, 127.7, 128.7	118.6, 123.3, 129.2	113.7, 129.7
Ipso carbons	138.2(1')	140.4(3'), 147.2(1')	130.4(1'), 159.0(4')
Others	-	-	55.4(CH_3)

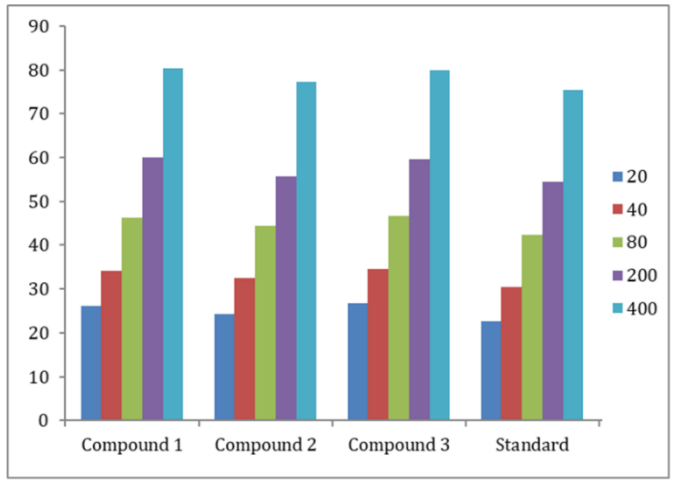
3.1.4. *Analysis of chemical shifts*

It is interesting that the COOt-Bt group at C-2 has larger chemical shifts than the corresponding proton of the COOt-Bu group at C-4. Both the COOt-Bu groups are nearly identical chemical shifts, and also, the protons in the cyclohexanone ring at H-3a has the

largest chemical shifts, and among all the carbons in the cyclohexanone ring at C-3 have the lowest chemical shift values because of the H-3a proton, which is deshielded by the magnetic anisotropic effect of the aromatic ring at C-3.

3.2. *In vitro anti-inflammatory activity (BSA denaturation assay)*

Compounds **1**, **2,** and **3** were screened for in-vitro anti-inflammatory activity by using pro*tein denaturation techniques* [18]. Two different protein techniques, like Bovine serum albumin (BSA) techniques, were studied. In these activities, the absorbance changes at 660nm were identified by UV-Visible spectrophotometric techniques, and Diclofenac sodium was used as a reference drug. The results were compared with the reference drug at various concentrations, such as 20, 40, 80, 200, and 800 mM. The various concentrations of compound **1**, compound **2,** and compound **3** showed a more potent anti-inflammatory activity than standard diclofenac sodium drug in the assays of BSA. The percentage inhibition of both the denaturation techniques was calculated by using the following formula, and the % inhibition is represented in Fig. 5. The compounds **1**, **2,** and **3** showed very good percentage inhibition than standard diclofenac sodium.



Standard: Diclofenac sodium / Concentration (mg/mL).

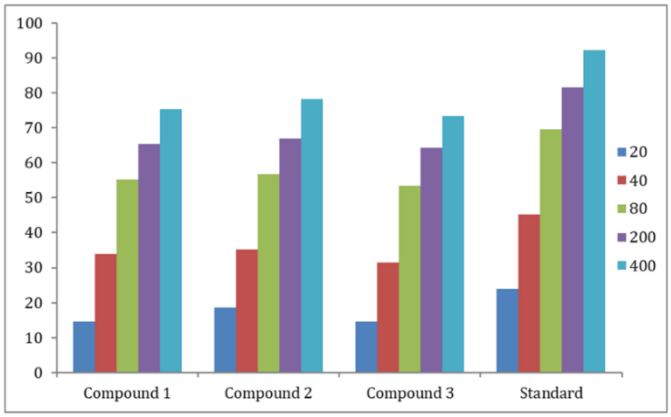
Fig. 5. Chart of Anti-inflammatory activities of compounds (1-3).

3.3. *In vitro antioxidant activity*

The total antioxidant activity of compounds **1, 2,** and **3** were evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al.* [19]. The total antioxidant activity was performed based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acid pH.

The total antioxidant activity of the cyclohexanones **(1-3)** was tested in different concentrations (10-400 µm). The results of cyclohexanones **(1-3)** and reference are given

in Fig. 6. The phosphomolybdenum also showed a nearer performance to the standard drug. These results suggested that cyclohexanones **(1-3)** have shown good antioxidant activity. The percentage inhibition was calculated from the control and test- results.



Standard: Vitamin C / Concentration(mg/mL)

Fig. 6. Chart of Antioxidant activities of compounds (1-3).

4. Conclusion

In the present study, three numbers of r(2), c(4)-bis(tert-butoxycarbonyl)-c(5)-hydroxy-t(5)-methyl-t(3)-phenylcyclohexanones **(1-3)** were synthesized and characterized by using the NMR spectroscopy technique. The NMR spectral data of cyclohexanones **(1, 2 & 3)** suggests that these compounds exist in chair conformation with an axial orientation of the hydroxyl group and equatorial orientation of all other substituents. Further, they are screened for their anti-inflammatory and antioxidant oxidant activities. All three compounds have shown good activity as compared with the reference drug used.

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