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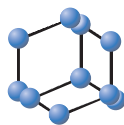
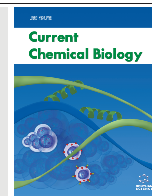
RESEARCH ARTICLE





The Effect of Substituents and Functional Groups on Enhancing the Antioxidant Activity of Benzoin Derivatives

Thanuja Balasundaram[1,*], Kavasseri Ganesan Kripa[2], Thiyagarajan Bhavadharani[2] and Charles Kanagam[3]

[1]*Department of Chemistry, Sri Sairam Engineering College, Tambaram, Chennai 600044, Tamil Nadu, India;* [2]*Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies, Chennai 600 117, Tamil Nadu, India;* [3]*Department of Chemistry, SRM Valliammai Engineering College, Tamil Nadu, India*

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Abstract: ***Background*****:** 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl] -2-4'-methoxyphenyl] ethane and 2-oxime-1-hydroxy, 1-[2-chlorophenyl] -2-4'-methoxyphenyl] ethane derivatives of benzoin have been synthesized from 2'chloro-4-methoxy benzoin by addition reaction. Structural elucidation of the synthesized compounds was carried out through FT-IR, FT-NMR studies. The presence of electron-withdrawing and electron-donating groups enhanced the antioxidant activity, which was analyzed by 2,2-diphenyl-1-picrylhydrazyl assay, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging assay, hydrogen peroxide radical scavenging assay, and Ferric reducing antioxidant power assay methods. The effect of functional groups and substituents in the core structure was studied and compared with its parent compound.

Aim and Objective**:** In this manuscript, two derivatives of benzoin *viz*. 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl] -2-[4'-methoxyphenyl] ethane and 2-oxime-1-hydroxy, 1-[2-chlorophenyl]-2-[4'-methoxyphenyl] ethane (HA) derivatives were synthesized by benzoin condensation and followed by addition reaction to find a potential anti-oxidant agent.

Materials and Methods**:** Qualitative analyses were determined by FT-IR and FT-NMR studies. Antioxidant activities were tested by DPPH assay, ABTS assay, and FRAP assay H2O2 methods.

Results**:** From the obtained results, it is confirmed that the effect of withdrawing and electron releasing groups as a substituent in the core structure of parent compounds enhances the activity of antioxidant. The role of substituents is discussed in detail.

Conclusion**:** The results of the biochemical assay reveal that the synthesized compounds serve as good free radical inhibitors and scavengers, which inhibit the oxidative reactions, and are responsible for cell damage, food spoilage, *etc*. The promising anti-oxidant activities are because of the effective substituents which play a prominent role in the drug industries.

Keywords: FT-IR, FT-NMR, DPPH, ABTS, FRAP assay, benzoin derivatives.

1. INTRODUCTION

Highly reactive oxygen species (ROS) are capable of reacting with membrane lipids, nucleic acids, proteins, and enzymes resulting in cellular damage. These molecules are produced as by-products during the mitochondrial electron transport of aerobic respiration by oxidoreductase and metal-catalyzed oxidation reactions [1]. Various environmental stresses also lead to excessive production of ROS, which leads to aging, high blood pressure, atherosclerosis, *etc*. [2]. Oxidative stress can be reduced by antioxidants which are naturally present in the body [3]. The antioxidants delay or inhibit cellular damage mainly through their free radical scavenging properties [4]. These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquitin, and uric acid, are produced during normal metabolism in the body [5]. Although there are several enzymes and non-enzyme systems within the body that scavenge free radicals, the principle micronutrient antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene [6]. The body cannot manufacture these micronutrients, so they must be supplied in the diet [7].

Antioxidants also constitute a major portion of foodstuffs, which helps to maintain the food taste, color, and prevent aerial oxidation [8]. Synthetic and natural food antioxidants are used routinely in foods and medicine, especially those containing oils and fats to protect the food against oxidation. There are several synthetic phenolic antioxidants,

*Address correspondence to this author at the Department of Chemistry, Sri Sairam Engineering College, Tambaram, Chennai 600044, Tamil Nadu, India; Tel: 9884509338; E-mail: revasuku25@gmail.com

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butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples [9].

These compounds have been widely used as antioxidants in the food, cosmetics, and therapeutic industries [10]. In recent research, it has been found that many potential synthetic compounds which have electron-donating and electron acceptor substituted groups increase the antioxidant activity [11].

In this interest, the present study aims to synthesize 2-phenyl hydrazine-1-hydroxy,1-[2-chlorophenyl]-2-4'-methoxyphenyl] ethane (PH) and 2-oxime-1-hydroxy,1-[2-chlorophenyl]-2-4'-methoxyphenyl] ethane (HA) antioxidant, which has chloro as an electron-withdrawing group, methoxy as an electron-donating group as substituents, and in addition C=NNH group in the core structure which may enhance the antioxidant activity of the compounds.

The above compounds have been synthesized by benzoin condensation followed by an addition reaction. The effect of substituents in increasing the potential of biological activities was studied and compared with reported parent compounds [12].

2. MATERIALS AND METHODS

2.1. Chemicals

Derivatives of benzoin were synthesized by the methods reported in the literature [13]. The reagents used to be of AR grade and have the highest purity. The products obtained were purified initially by PTLC using silica gel. All solvents used in the synthesis were purified by the steam distillation method [14].

2.2. Methods

2.2.1. Preparation of Parent Compound (2C4MB)

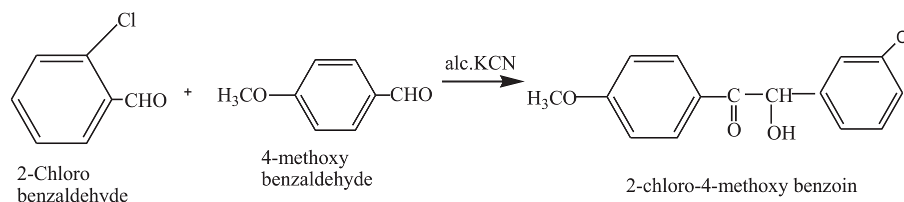
2C4MB compound was synthesized by benzoin condensation using 4 g of KCN dissolved in 75 ccs of water in a one litre flask. About 6.8 g [0.05 mole] of 4-methoxy benzaldehyde, 7 g [0.05 mole] of 2-chloro benzaldehyde, and 75 ccs of 95 % ethanol were added into the flask. The mixture has formed into a solution at the boiling temperature and was refluxed for one and half hours. Steam was then passed through the solution until all the alcohol and nearly all the unreacted aldehyde were removed. The condensed water was decanted from the product and later set aside for crystallization. The product was then pressed as free as possible from oily material on a suction funnel and washed with cold alcohol. In this way, about 14 g [yield was 60 %] of crude product was obtained. The crude mixture was dissolved in hot alcohol and allowed to crystallize slowly (Scheme **1**). The 2-chloro-4′-methoxy benzoin crystallizing out as colorless, hexagonal crystals suitable for X-ray diffraction study was obtained [15]. The melting point of the compound was found to be 84ºC. The yield of pure 2-chloro-4′methoxy benzoin amounts to 60-70 % [16].

2.2.2. Preparation of 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl] -2-[4'-methoxy phenyl] ethane (PH)

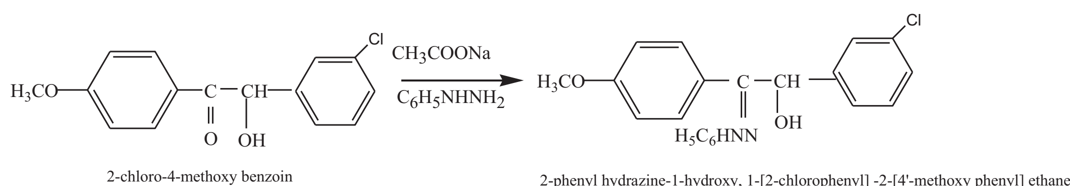
0.5g phenylhydrazine hydrochloride and 0.8g of sodium acetate were taken in a 100 mL beaker and the mixture was dissolved in 5mL of water. 0.2g of 2C4MB dissolved in 5mL of ethanol. The reaction mixture was stirred and shaken well until a clear solution was obtained [17]. The solution was warmed in a water bath for 20 minutes and later was allowed to cool by adding ice and set aside for 15 minutes. The solution was filtered and using ethanol, the filtrate was recrystallized. White crystals were obtained and the purity of the compound was initially confirmed by the melting point. The melting point was found to be 142ºC. The yield of PH is found to be 65-70 % (Scheme **2**).

2.2.3. Preparation of 2-oxime-1-hydroxy, 1-[2-chlorophenyl] -2-[4'-methoxy phenyl] ethane (HA)

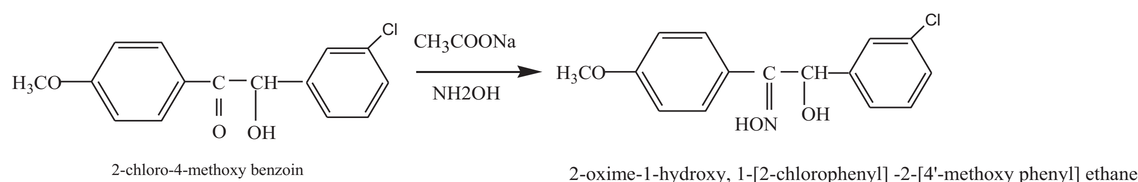
0.5g hydroxylamine and 0.8g of sodium acetate were taken in a 100mL beaker and the mixture was dissolved in 5mL of water. 0.2g of 2C4MB was dissolved in 5 mL ethanol and the reaction mixture was stirred and shaken well until a clear solution was obtained. The solution was warmed in a water bath for 20 minutes and later allowed to cool by adding ice and set aside for 15 minutes [18]. The solution was filtered and the filtrate was recrystallized from ethanol. The melting point of the compound was found to be 123ºC. The yield of HA was found to be60-70 % (Scheme **3**).



Scheme **1**. Synthesis of 2-chloro-4-methoxy benzoin.



Scheme **2**. Synthesis of 2-phenylhydrazine-1-hydroxy, 1-[2-chlorophenyl] -2-4'-methoxy phenyl] ethane.



Scheme **3**. Synthesis of 2-oxime-1-hydroxy, 1-[2-chlorophenyl] -2-[4'-methoxyphenyl] ethane.

The qualitative analysis was initially carried out to confirm the synthesized compounds using Infrared, ^{1}H, and ^{13}C-NMR spectroscopy.

2.2.4. Antioxidant Potential of PH and HA

2.2.4.1. DPPH Assay

The scavenging activity of the synthesized compounds was determined by DPPH scavenging activity [19]. This was found to be a primary screening assay to detect the presence of antioxidant activity and the most widely reported method.

The solution of 0.135mM DPPH was prepared in methanol. Different concentrations of the synthesized compounds (0.5mL) were mixed with 2.5 mL of DPPH solution. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as the reference chemical [20]. DPPH free radical scavenging effect of PH and HA at various concentrations was measured at 517 nm.

The ability of PH and HA to scavenge DPPH radicals was calculated from the following formula:

%DPPH inhibition= [(OD of control - OD of test) / (OD of control)] x100

where $OD_{Control}$ is the absorbance of the blank sample (containing all reagents except the test samples), and OD_{sample} is the absorbance of the samples, discoloration of the solution indicates free radical scavenging activity.

2.2.4.2. ABTS Radical Scavenging Assay

The stock solution of 7mM ABTS was dissolved in 25mL of deionized water and mixed with 140mM of potassium persulfate. The mixture was allowed to stand for 15-16 hr to obtain ABTS radical cations [21].

ABTS radical scavenging activities of different concentrations of PH and HA (0.1mL) compounds were analyzed using ABTS working solution (1.9 mL). The reaction mixture was allowed to stand for 20 min, then the absorbance was measured using a UV-Visible spectrophotometer at 734nm of 0.700 ± 0.02 absorbance [22]. The radical scavenging activity was given as ABTS radical scavenging effect and that was calculated by the equation:

ABTS radical scavenging effect (%) = [(OD of control - OD of test) / (OD of control)] x100.

2.2.4.3. Hydrogen Peroxide Radical Scavenging Assay

The ability of the samples to scavenge hydrogen peroxide was determined [23]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Different concentrations (10 - 320 µg/mL) of samples were added to a hydrogen peroxide solution (0.6 mL, 40mM). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide [24].

OH radicals scavenging assay % = [(OD of control - OD of test) / (OD of control)] x100.

2.2.4.4. FRAP Assay

The antioxidant activity of the synthesized compounds was done using a FRAP assay method. The reaction mixture prepared was analyzed spectrophotometrically [25]. Initially, 300 mM acetate buffer, 10 mL TPTZ in 40 HCL and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37°C were mixed to prepare ferric reducing antioxidant power reagent (FRAP). Various concentrations of synthesized compounds PH and HA were prepared by mixing with freshly prepared FRAP reagent using a 1-5mL variable micropipette (3.995 mL). The reaction progress was analyzed from the changes of the colorless complex to (Fe^{3+}TPTZ) blue colored complex (Fe^{2+}-tripyridyltriazine) which was formed by the action of the electron-donating antioxidants at low pH. The absorbance was recorded at 37°C and was found at 593nm. Results are expressed as mean IC_{50}± SEM of three determinations. Statistical comparisons were made between BHT and PH, BHT and HA, respectively. ANOVA and Tukey tests were used to calculate significance. $^{xx, xxx}$ indicate a significant difference at $p<0.01$, $p<0.001$, respectively [26].

FRAP inhibition % = [(OD of control - OD of test) / (OD of control)] x100.

2.2.4.5. In Vitro Anti-Inflammatory Activity of Benzoin Derivatives

2.2.4.5.1. Inhibition of Albumin Denaturation

5mL of the reaction mixture comprised 0.2 mL of egg albumin, 2.8mL of phosphate-buffered saline (PBS, pH 6.4), and 2 mL of varying concentrations of the synthesized compounds. A similar volume of double distilled water served as a control. Then the mixture was incubated at 37 °C in an incubator for about 15mins and then heated at 70 °C for 5mins. After cooling, their absorbance was measured at 660nm by using a pure blank. Diclofenac sodium was used as a reference chemical and treated as such for the determination of absorbance [27].

Percentage inhibition of protein denaturation = [(OD of control - OD of test) / (OD of control)] x100

3. RESULTS AND DISCUSSION

3.1. Qualitative Analysis

The synthesized compounds were subjected to qualitative analysis using FT-IR spectrum in the frequency region

of 4000 cm^{-1}- 400 cm^{-1}. The study of IR spectra of the synthesized compounds compared with the parent compound is shown in (Table **1**). The peaks at 3398 cm^{-1} and 3475cm^{-1} of PH and HA respectively confirmed the presence of –OH and -NH str, similarly peaks at 1600 $cm^{-1}$and 1666 cm^{-1} correspond to C=O and C=N sym str of PH and HA respectively (Fig. **1a** and **1b**). The ^{1}H NMR and ^{13}C NMR spectra of the title compound are presented in (Fig. **2a-d**), respectively. The chemical shifts are tabulated with the assignments in (Table **2**). In the PMR the spectrum of signals appears at 3.5 and 3.9 ppm of PH and HA, respectively, indicating the presence of -CHOH protons. A singlet at 3.5 and 3.9 ppm indicates the presence of a methoxy group of the synthesized compounds. The signal at 6 ppm corresponds to the –NNH proton, the peak was not observed in the parent compound, confirming the formation of new compounds.

Signals in the range of 6.5 to 7.5 ppm indicate the presence of aromatic protons. In the ^{13}C NMR, the aromatic carbon atoms appear in the range of 120 to 132 ppm.

3.2. Antioxidant Potential of PH and HA

3.2.1. DPPH Assay

Using DPPH biochemical assay, the free radical scavenging effect of the synthesized compounds was analyzed [28]. PH and HA were able to donate electrons to reduce the stable radical DPPH to the yellow-colored nonradical diphenyl-picrylhydrazine (DPPH-H). DPPH is usually used as a reagent to evaluate the free radical scavenging activity of antioxidants based on its absorption change at 517 nm, measured spectrophotometrically. The absorption in the visible region decreases upon receiving an electron or hydrogen from the synthesized compounds. Thus, the radical scavenging capacity of the antioxidants can be obtained based on the absorption change. The degree of discoloration of the solution indicates the free radical scavenging activity. Inhibition percentages with various concentrations of the synthesized compounds are shown in Fig. (**3**).

In the present study, the PH exhibited an IC_{50} of 48.71µg/mL compared to the standard ascorbic acid (19.38 µg/mL) while the HA derivative showed inefficiency to scavenge the free radical, thereby, proving that the PH derivative has an increased potential to annihilate the DPPH radicals (Table **3**). Earlier studies indicate halogen substituent benzilic acid derivatives to show enhanced antioxidant potency in comparison to ascorbic acid [29].

3.2.2. ABTS Radical Scavenging Assay

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+}$) was produced by treating ABTS solution with potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 hours to produce a dark blue solution, which has an absorption at 734 nm. This method has been extensively used to evaluate the antioxidant capacity of compounds, both plant-derived and chemically synthesized. The reaction of the ABTS radicals with synthesized compounds can be easily monitored by the decrease of sample absorbance at 734 nm (Fig. **4**).

The ABTS assay indicated the IC_{50} of PH to be 15.55µg/mL and for HA 29.25 µg/mL, whereas the standard ascorbic acid showed 7.72 (Table **4**) [30].

3.2.3. Hydrogen Peroxide Radical Scavenging Assay

Hydrogen peroxide produces hydroxyl radicals in a biological system, which are harmful and lead to cell damage. The hydroxyl free radical was scavenged by the synthesized compounds, which can donate electrons to the hydroxide radical and thereby neutralize it with water. This assay indicated the standard BHT, PH, and HA to have excellent scavenging activity of 0.734±0.01, 0.479±0.00, and 0.062±0.00 respectively at a concentration of 320 µg (Fig. **5**). The results obtained revealed that the synthesized compounds have potent antioxidant activity.

3.2.4. FRAP Assay

From the results obtained, it was found that a maximum concentration of 320µg/mL of PH showed maximum absorbance of 1.15 and HA showed maximum absorbance of 0.08, while standard BHT had an absorbance value of 0.861 (Fig. **6**). PH value was found to be much higher than the synthetic antioxidant BHT, proving this derivative to be a potent antioxidant comparable to their commercially used synthetic antioxidants.

Table 1. Comparison table of 2C4MB, PH, HA using FT-IR.

Functional Groups	2C4MB (cm^{-1})	PH (cm^{-1})	HA (cm^{-1})
-OH and –NH stretching	3475	3398	3475
Aromatic C-H stretching	3060	3035	3060
Aliphatic C-H stretching	2937	3305	2937
Sym C=O, C=N stretching	1666	1600	1666
Aromatic sym C=C stretching	1569,1602	1505	1569
C-OH in-plane deformation	1268	1247	1268
Presence of Benzene ring deformation	979	1023	979
Disubstituted benzene ring deformation	1087, 1030	760	747

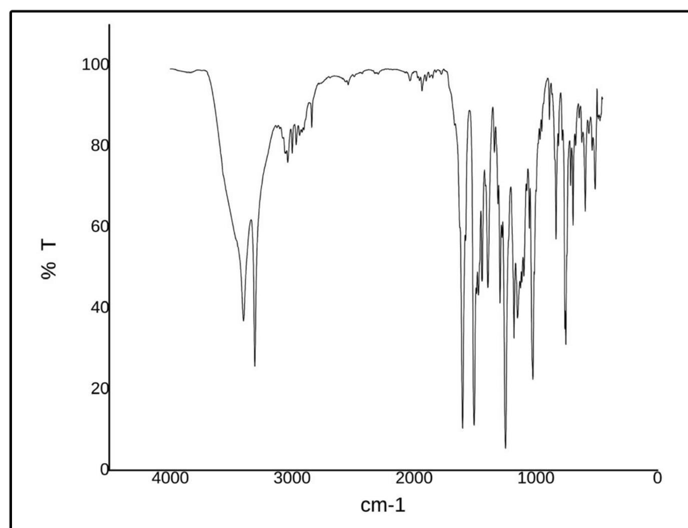


Fig. (1a). FT-IR spectrum of 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl]-2-4'-[methoxyphenyl] ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

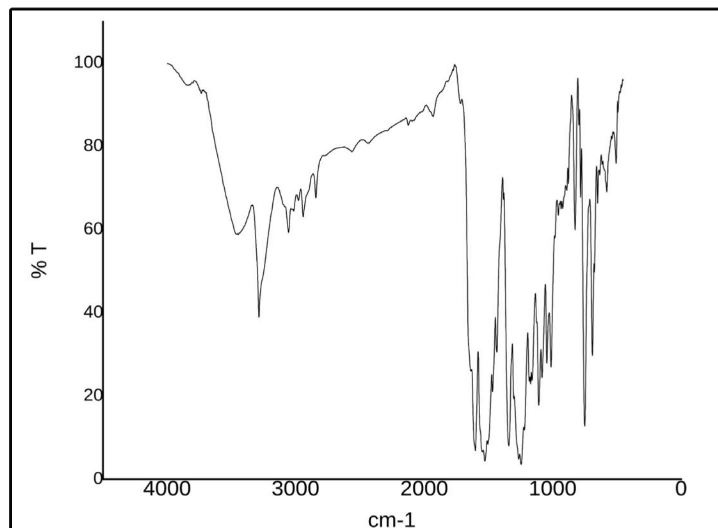


Fig. (1b). FT-IR spectrum of 2-oxime-1-hydroxy, 1-[2-chlorophenyl]-2-4'-methoxyphenyl] ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2.5. Anti-Inflammatory Activity of PH and HA

It has been observed that the denaturation of proteins is one of the main causes of inflammation. Inflammation is the common reaction of living tissue towards injury aided by systemic and local responses. Denaturation of proteins in the cell membrane is a well-established concern for inflammatory disorders as they may result in the production of auto-antigens. The ability of the component to inhibit protein denaturation is a measure of its anti-inflammatory activity.

The anti-inflammatory activities of the synthesized compounds at various concentrations were studied by an albumin denaturation test using a standard drug diclofenac sodium [31]. The results of the present study indicate that the synthesized compounds are found to be effective in inhibiting heat-induced albumin denaturation, as given in (Table **5**). The investigation of the anti-inflammatory activity of the synthesized compounds suggested that HA exhibited maximum protein denaturation inhibition at 70.17% (concentration 100 μg/mL). Diclofenac Sodium, an NSAID standard anti-inflammatory chemical, showed maximum inhibition of 52.61% at 100 μg/mL concentration. The results of the present study indicate that the synthesized compounds PH and HA are found to be effective in inhibiting heat-induced albumin denaturation.

3.2.6. Effect of Substituents and CN Groups on Enhancing the Property of the Antioxidant Activity

The presence of substituents plays a vital role in increasing the potential of antioxidant properties. The presence of the C = NNH group in the core structure may augment the antioxidant potential of the compound. Further C=N group involves hydrogen bonding which is essential for enhancing the property of the drug and the –CN group decreases oxidative metabolism [32]. The presence of electron-donating

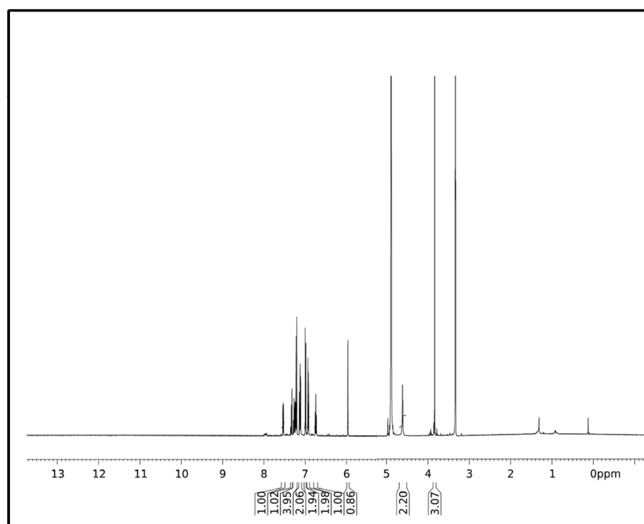


Fig. (2a). FT- H1NMR of 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl]-2-4'-methoxyphenyl]ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

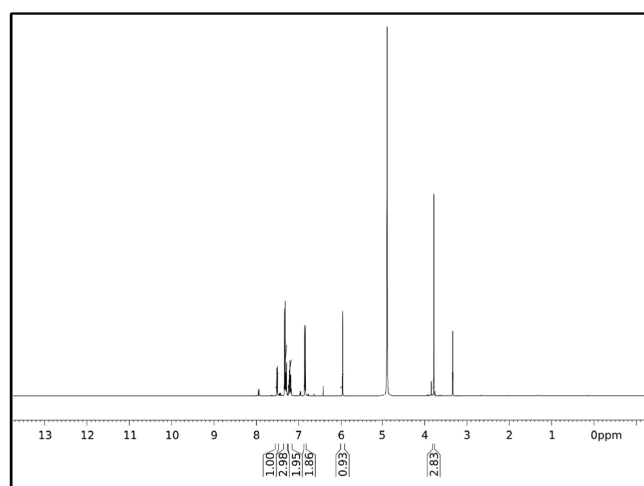


Fig. (2b). FT- H1 NMR of 2-oxime-1-hydroxy, 1-[2-chlorophenyl]-2-4'-methoxy phenyl]ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

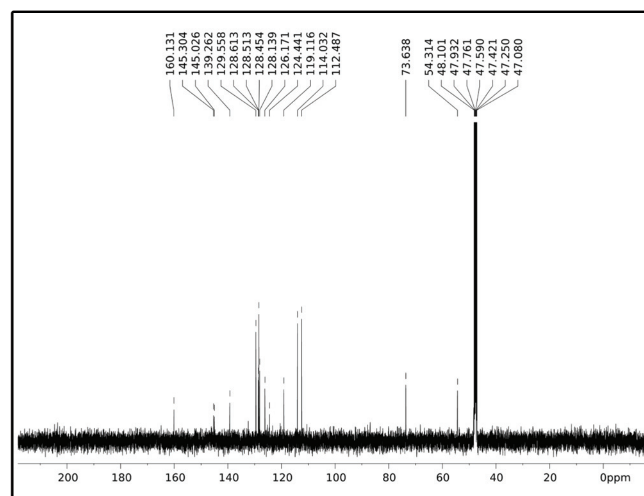


Fig. (2c). FT- C13NMR of 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl]-2-4'-methoxyphenyl]ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

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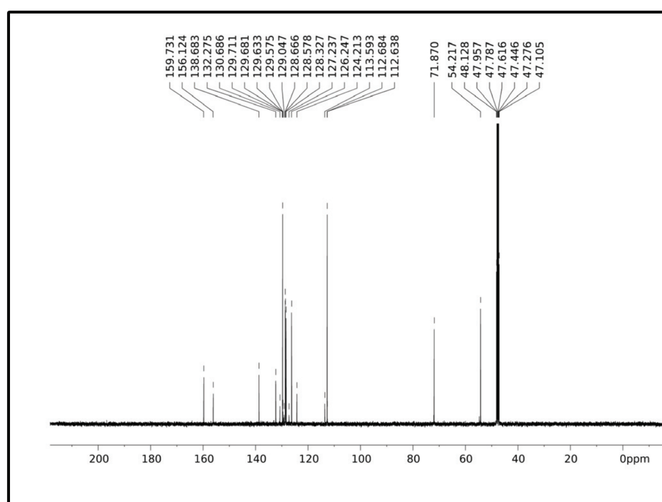
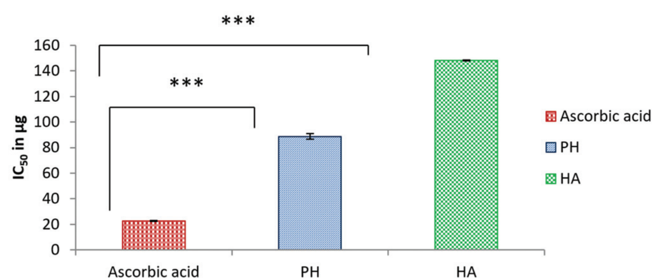


Fig. (2d). FT- H1 NMR of 2-oxime-1-hydroxy, 1-[2-chlorophenyl]-2-4'-methoxy phenyl]ethane. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Comparison table of 2C4MB, PH, and HA using FT-H^1NMR & C^{13} NMR.

Group Identification	2C4MB (ppm)	PH (ppm)	HA (ppm)
-CHOH	3.8	3.5	3.9
Methoxy group of 2C4MB	6.9 -7.5	4	3.3
Aromatic protons	6.9 -7.5	6.5 - 7.5	6 - 7.5
-NNH proton	-	6	6
Aliphatic carbon atoms	54, 72	54, 73	54
Aromatic carbon atoms	127 - 137	129-132	129-132



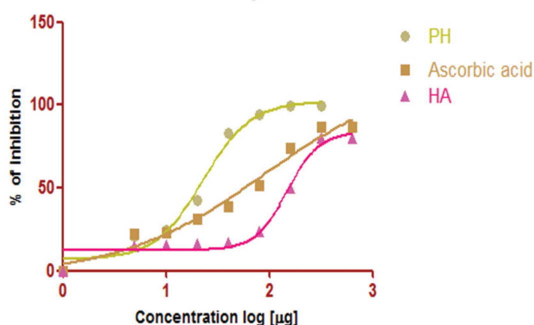


Fig. (3). Percentage inhibitions of the different concentrations of the 2C4MBPH and 2C4MBH by DPPH assay. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

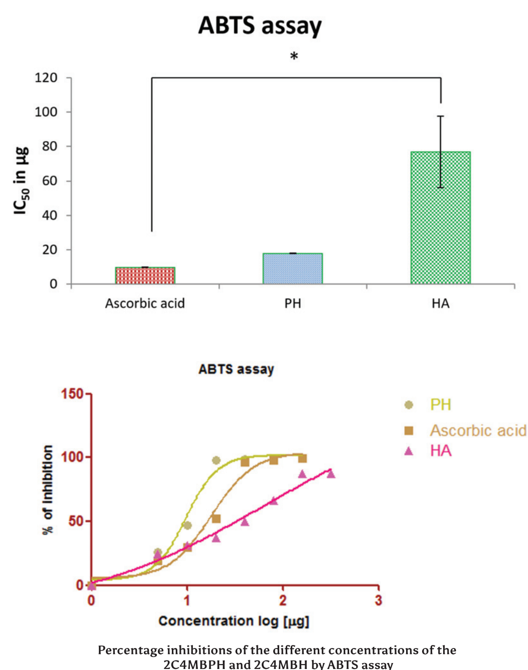


Fig. (4). Percentage inhibitions of the different concentrations of the 2C4MBPH and 2C4MBH by ABTS assay. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

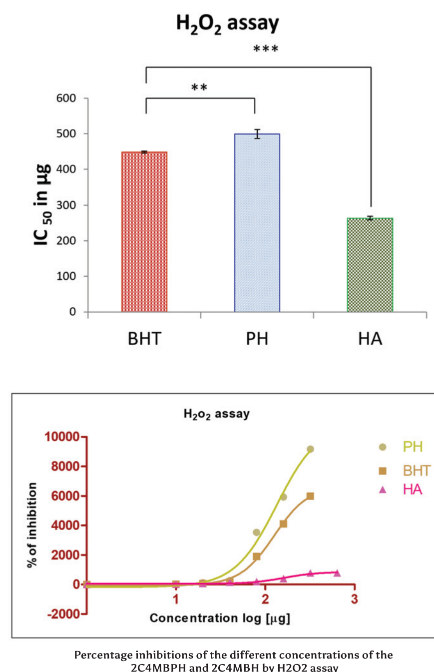


Fig. (5). Percentage inhibitions of the different concentrations of the 2C4MBPH and 2C4MBH by H_2O_2 Assays. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

group ($-OCH_3$) at the ortho position and electron-withdrawing group (Cl) at the para position rampages the free radical generation during the oxidative process which is highly capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. Further, the stability of the compound increases due to the presence of the chloro group at the para position. Although, the chloro group is electronegative which deactivates the aromatic ring by decreasing the electron density on the ring through an inductive withdrawing effect. But as resonance effect controls the regiochem due to the stability of the intermediate carbocation [33]. The molecular design of the synthesized compounds, containing one electron donor and one electron acceptor moiety, provides it with a push-pull configuration, enhancing the stability of the compound, which is essential for drug synthesis [34]. The presence of an additional aromatic ring in PH made the compound more stable through resonance than HA, which was confirmed from the results obtained.

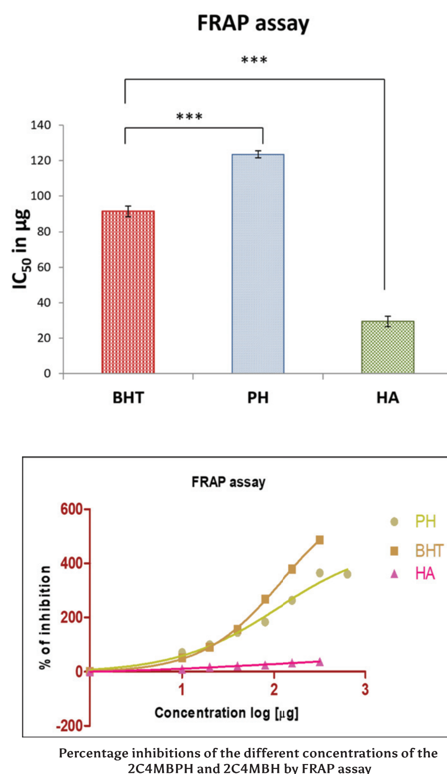


Fig. (6). Percentage inhibitions of the different concentrations of the 2C4MBPH and 2C4MBH by FRAP assay. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 3. IC_{50} of PH and HA by DPPH assay method.

S. No.	Extract Code	IC_{50} (µg/mL)
1	PH	48.71
2	HA	Not active
3	Ascorbic acid	19.38

Table 4. IC_{50} of PH and HA by ABTS assay.

Compounds	ABTS Assay (IC_{50} µg/ml)
PH	15.55
HA	29.25
Ascorbic acid	7.72

Table 5. Anti-inflammatory activities of PH and HA by albumin protein denaturation method.

Concentration (µg/mL)	Diclofenac Sodium	HA	PH
10	37.07	41.51	64.91
20	43.69	36.25	23.94
30	46.31	29.82	16.37
40	48.16	45.61	39.76
100	52.61	70.17	10.16

CONCLUSION

Significant antioxidant activities of the PH and HA compounds were proved by DPPH, ABTS, FRAP, and hydrogen peroxide biochemical assay methods. Inhibition of free radicals with various concentrations of the synthesized compounds, found from the degree of discoloration of the various assays, was performed. The changes in the absorbance were measured spectrophotometrically. Thus, the results of the biochemical assay revealed that the synthesized compounds serve as good free radical inhibitors and scavengers which inhibit the oxidative reactions responsible for cell damage, food spoilage, *etc*. The promising antioxidant activities of PH and HA were due to the presence of methoxy, chloro, and –CNNH groups. Thus, the presence of these functionalized groups in the core structure of benzoin derivatives makes them more suitable for drug stability and antioxidant activity.

LIST OF ABBREVIATIONS

2C4MB	=	2'chloro-4-methoxy benzoin
ABTS	=	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
FRAP	=	Ferric reducing antioxidant power
FT-IR	=	Fourier-transform infrared spectroscopy
FT-NMR	=	Fourier-transform nuclear magnetic resonance
HA	=	2-oxime-1-hydroxy, 1-[2-chlorophenyl]-2-[4'-methoxyphenyl] ethane

PH = 2-phenyl hydrazine-1-hydroxy, 1-[2-chlorophenyl] -2-[4'-methoxy-phenyl] ethane

AUTHORS' CONTRIBUTION

Dr. B. Thanuja: Synthesis & characterization of the compounds, original draft preparation of the manuscript, interpretation of the results and discussion.

Dr. K.G. Kripa. & T. Bhavadharini: Biochemical assay methods analysis.

Dr. Charles Kanagam: Visualization, Investigation, supervision, reviewing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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