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Structure-radical scavenging activity relationships of hydroxytoluene derivatives

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Abstract

Research works proposed that radical scavenging activity of flavonoids is due to ring B, and the remaining part of the molecule can be disregarded. Thus the objective of this work is to observe whether hydroxytoluenes account the radical scavenging activity of flavonoid and to establish structural requirements for their activity (as they showed appreciable activity) and elucidate a comprehensive mechanism that can explain their activity and termination. Thus, the radical-scavenging activity of nine hydroxytoluene derivatives against 2,2-diphenyl-1-picrylhydrazyl, DPPH was determined. The relative change in energy (ΔH_f) associated with the formation of phenolic radicals and the spin distributions in these radicals were determined using computational programs (Density function theory and Hartree Fock). By correlating experimental data with ΔH_f, the most active compounds and structural features that are responsible for their activities were identified. Reaction product of 4-methyl catechol with 2,2-diphenyl-1-picrylhydrazyl, DPPH was isolated and characterized in order to unravel the mechanism of termination of most active hydroxytoluenes. Termination enthalpy (ΔH_2) of methyl-catechols and methyl-hydroquinone, once the termination mechanism explained, was calculated to understand its role in the radical scavenging activity.

Keywords: Hydroxytoluenes; Radical scavenging activity; Enthalpy; Spin distribution; ΔH_2.

1. Introduction

Free radicals are chemical compounds with unpaired electrons [1, 2]. Free radicals, because of their reactivity, can participate in unwanted side reactions resulting in cell damage. Thus, free radicals contribute to more than one hundred disorders in humans [3, 4]. As a result, much interest has been focused on finding antioxidants to prevent the radical induced impairments in chemical, food and pharmaceutical industries [1].

Flavonoids are common polyphenolic radical scavengers that commonly occur in plants. This class of compounds includes flavones, isoflavones, and the 2, 3-dihydro derivatives of flavones, namely flavanones, which are inter convertible with the isomeric chalcones.

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Flavanones undergo a series of transformations affecting the heterocyclic C ring to give rise to other family members, including anthocyanins and catechin [5].

Many research works have been conducted to unravel the mechanism and structure-antioxidant activity relationships of flavonoids and it was observed that ortho-dihydroxy (catechol), pyrogallol or 3-OH substitutions impart high activity [6-8]. In flavonoids two different pharmacophores are found, i.e., on the B-ring and the AC-ring. In AC-ring the hydroxyl group at position 3 is the most reactive one and its activity is enhanced by the electron donating effect of the hydroxyl groups at positions 5 and 7. In ring B, it is the catechol moiety that plays vital role and the activity of one of the hydroxyl groups is enhanced by the electron donating effect of the other one [9-11].

Although 1, 4-pyrone ring enlarges the conjugation system of flavonoids, it is not beneficial to reduce the O-H BDE, due to its electron-withdrawing property, and thus, it is unlikely to enhance the free radical scavenging activity of flavonoids [12]. The radical scavenging activity of flavonoids is due to ring B [5, 6, 13], while the 2–3 double bond, the 4-oxo or 3-OH group or any of their combinations are not essential and the rest of the molecule can be disregarded [14].Thus hydroxytoluene derivatives could successfully account for the radical scavenging activity of flavonoids due to ring B and also appreciably could do so for activities due to ring A. This has indicated the possibility of synthesizing simplified phenolic structures that may retain an appreciable radical scavenging activity [14].

2. Experimental

2.1. Materials

2.1.1. Chemicals

DPPH, ethylacetate, hydroxytoluene derivatives (2-hydroxytoluene, 3-hydroxytoluene, 4-hydroxytoluene 3,4-dihydroxytoluene, 2,5-dihydroxy-toluene, 2, 6-dihydroxytoluene, 2,4,6-trihydroxytoluene and 4-hydroxy-3-methoxyltoluene) (all from Sigma Aldrich), hexane and spectroscopic grade methanol (BDH, England**),** Silica gel F_{254} (Merck, England).

2.1.2. Instruments

UV cabinet and UV/ Vis spectrometer, NMR charts (^{1}HNMR and ^{13}CNMR) were recorded using a Brüker 400 MHz Advance NMR spectrometer.

2.2. Methods

2.2.1. Free radical scavenging activity test

The free radical scavenging activity of hydroxytoluene derivatives was determined with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) using the method described by Burits *et* al. [15] and modified by Seyoum e*t al.* [14]. Methanol solutions of the hydroxytoluene derivatives were added to 0.004% (w/v) DPPH solution in methanol. After incubation of the mixture at room temperature in the dark for 30 min, the UV/Vis absorbance was read against a blank at 517 nm. For each hydroxytoluene derivative, not less than four different concentrations were tested. In order to make the comparison of activity based on the half-maximal inhibitory concentration (IC_{50}) uniform and meaningful, the slope of its linear regression curve of concentration *vs.* absorbance was taken to construct a new linear curve with a Y-intercept (absorbance at concentration = 0) equal to 1. Activities were then expressed as the microgram concentrations giving 50% reduction in the absorbance of a DPPH solution with an initial absorbance of 1.000.

2.2.2. Calculation of heat of formation (ΔH_f) and spin densities

Molecules were constructed with Chem3D - version 8.0. [Chemoffice package, 2004 Cambridge Soft Corporation (Cambridge Scientific Computing, Inc.)]. The models were pre-optimized by minimizing the energy with the semi-empirical modelling, MOPAC/Chem3D. Pre-optimized models were then minimized using the abinitio program, Gaussian03W/Chem3D interface. Some models were constructed by modifying others that have passed through the above steps. In most cases, radicals were constructed by deleting hydrogen from a specific hydroxyl [16].

Optimization of the molecule (minimization) using Gaussian 03W, was done at the default procedures; restricted Hartree-Fock Hamiltonian with the 6-31G (d) minimal basis set, spin 1, for parent structures and unrestricted Hartree-Fock Hamiltonian with the aforementioned basis set and spin 2, for radicals [17]. All electronic energies were then corrected by the thermal contribution to the enthalpy to obtain $H°$ (298), the standard gas-phase enthalpy at 298 K. To complete the specification of the method, the electronic energy of the H-atom was set to its calculated value of -0.50000 hartree, and obtain its enthalpy $H°$ (298) = -0.50000 + 5/2RT) = -0.49764 hartree [18]. The relative change in energy (ΔHf) in kcal/mol associated with the formation of a radical from its parent structure was calculated by subtracting the sum of heat of formation of the radical and hydrogen from that of the parent structure. In cases where there are ortho OH groups in the hydroxytoluenes (as in catechols), the term ΔH_1 was used for loss of the first (most weakly bound) H-atom to form the semiquinone and ΔH_2 for loss of the second H-atom to form the quinone. ΔH_2 is calculated by subtracting the sum of heat of formation of the quinone and hydrogen from that of the semiquinone. The spin density at each atom of a radical was calculated using Gaussian 03W/Chem3D interface (unrestricted Hartree Fock/6-31G,spin 2).

2.2.3. Chemical test

Research works proposed that reaction of methyl catechols with DPPH produce methyl quinone [6, 19]. The formation of quinone can be confirmed by chemical test. The method adopted by Ikhiri *et al* [20] was employed in this study with some modifications. The reaction mixture of hydroxytoluenes (catechol forms) and DPPH was allowed to react for 24 hours. TLC was developed in hexane: ethylacetate (3:1) and sprayed with 10%NaOH solution. Production of red colored spot confirmed the presence of quinone.

2.2.4. Isolation and structure elucidation

DPPH and representative of the active hydroxyltoluene derivative were dissolved in methanol with few drops of ethyl acetate to improve solubility of DPPH. The reaction mixture was kept in the dark room for 24 hrs [21] and then concentrated in a vacuum oven followed by isolation of components by preparative TLC, in hexane: ethyl acetate (3:1) as a solvent system. Structure of the isolated compound was elucidated based on ^{1}HNMR and ^{13}CNMR data.

3. Results and discussion

3.1. Radical scavenging activity test

The radical scavenging activity test was done using DPPH assay [14, 22]. DPPH solution preparation was uniform for all samples. It was prepared by dissolving 1.046 mg of DPPH in 25 ml to get a concentration of 0.004 % (w/v). However, absorbance of the original DPPH solution slightly varies across batches and also to a less extent depending on the storage period after preparation. Also, the Y-intercept values (absorbance of DPPH solution) in the absence of any

radical scavenger were slightly different across the calculated linear regression curves of hydroxytoluenes.

Considering the aforementioned observation, the Y-intercepts of all the LRCs were made 1, in order to compare the activity of hydroxytoluenes based on IC_{50} uniform and meaningful. This was based on the assumption that the rate of the reaction is first order i.e. the rate is dependent on the concentration of hydroxytoluene as excellent linear correlation from concentration versus absorbance was obtained for all hydroxytoluenes tested. This experimental observation that demonstrated good linear relationships between concentration and activity for phenolic compounds was also supported by literature reports [16]. In general, the reaction of various phenols in different solvents, with 2,2-diphenyl-1-picrylhydrazyl (DPPH) is first-order in phenols but zero-order in DPPH [23]. Maximum effort was exerted to get a minimum absorbance value that lies below 0.5 for a reaction mixture containing the highest concentration of hydroxytoluenes.

The method of IC_{50} determination adopted in this study successfully eliminated time factor and enabled uniform comparison of activity. It also eliminated the significance of concentration of the starting DPPH solution, especially when fresh batch has to be prepared now and then for many samples. In general, DPPH assay is considered an easy way to evaluate radical scavenging activity and there is a possibility of modifying the procedure for the determination of the scavenging activity of antioxidants [24, 25]. Result of the IC_{50} determination is presented in Table 1.

Table 1
Experimentally determined IC_{50} values of hydroxytoluenes.

Compounds	IC_{50} ($\mu g\ mL^{-1}$)
3,4dihydroxytoluene	1.92 ± 0.00696
2,3dihydroxytoluene	1.62 ± 0.01014
2,5dihydroxytoluene	2.58 ± 0.00608
2,6dihydroxytoluene	11.11 ± 0.00089
2,4,6trihydroxytoluene	7.23 ± 0.00694
4-hydroxy-3-methoxytoluene	16.63 ± 0.01184
O-cresol	179.21 ± 0.00529
P-cresol	354.61 ± 0.00449
M-cresol	304.00 ± 0.16370
Rutin	5.59 ± 0.00010

3.2. Theoretical calculation

3.2.1. Heat of formation

The widely accepted notion is that the antioxidant capacity of phenolic compounds is essentially due to the ease with which a H from an aromatic hydroxyl (OH) group can be donated to a free radical, and the ability of an aromatic compound to support an unpaired electron as a result of delocalization around the π-electron system [26, 27]. Jones *et.* al., [17] provided excellent account for the use of stabilization energy (ΔH_f) for the prediction of activity in reactions involving hydrogen abstraction.

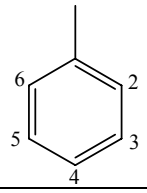
The ease of H abstraction is probably rate limiting for radical scavenging activity of flavonoids and phenols [28]. The ΔH_f is used as the primary descriptor of radical scavenging

activity as a considerable number of reports suggest that it correlates well with the observed radical scavenging activity of flavonoids [29, 30], phenols [18], and in general with reaction involving hydrogen abstraction [17, 29]. The difference between the heat of formation of the parent compound and the corresponding radical (ΔH_f), gives an insight about the stability of the radical and the ease with which it is formed. H_f associated with the formation of the corresponding phenoxy radical could be used to predict radical scavenging activity with reasonable accuracy. The enthalpy change for the process is given by [31]

$$\Delta H = \Delta U + \Delta(pV)$$
$$\Delta H = \Delta E_e^0 + \Delta(\Delta E_e)^{298} + \Delta E_v^0 + \Delta(\Delta E_v)^{298} + \Delta E_r^{298} + \Delta E_t^{298} + \Delta(pV)$$

ΔE_e^0 was obtained by taking the difference of the total energies predicted in single point energy calculations for reactants and products. Energy calculations were performed at reasonable basis set, i.e. using B3LYP/6-311G (d) //HF/6-31G. Computing frequency at B3LYP/6-311G (d) level is accurate enough for predicting the zero point and thermal energy terms [26, 27, and 32]. The theoretical determined values are very close to experimental values. All of the other ΔE and $\Delta(pV)$ terms are combined into the thermal energy correction to enthalpy predicted by frequency calculations [33]. Calculated enthalpies are presented in the Tables 2.

Table 2
Calculated differences in heat of formation (ΔH_f) associated with the production of hydroxytoluene-derived radicals.



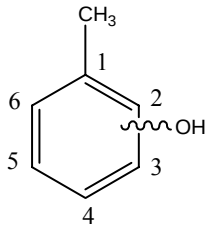
Substitute	2r	3r	4r	5r	6r
2-OH	71.35				
3-OH		73.3			
4-OH			71.41		
3,4-OH		64.77	71.50		
2,3-OH	63.33	73.72			
2,5-OH	66.05			67.44	
2,6-OH	70.35				70.35
2,4,6-OH	69.76		69.43		69.76
3-OMe-4-OH			71.23		

3.2.2. Spin density

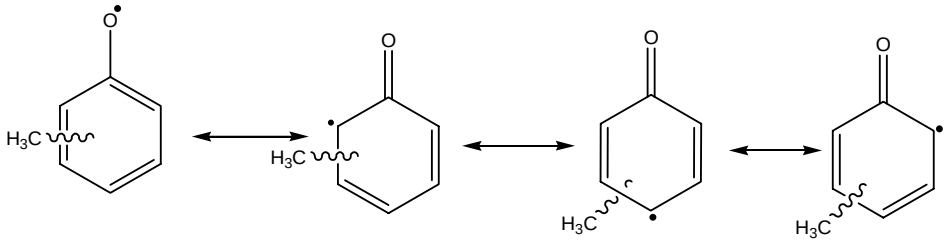
The spin density of cresols at each atom of radical was calculated using Gaussian 03W/Chem3D interface (unrestricted Hartree Fock/6-31G, spin 2) from the final optimized geometry. The result is presented in Table 3.

As clearly depicted in Table 1, unsubstituted hydroxytoluenes are poor radical scavengers. *Para* and *ortho* hydroxyltoluenes are more active than *meta* hydroxytroxytouenes. This is because these compounds have lower phenolic O−H bond dissociation enthalpy. *Ortho* and *para* alkyls (at positions 2, 4, 6) of phenols stabilize the phenoxy radical by inductive and hyperconjugative effects and, in addition, *ortho* groups provide steric hindrance to minimize undesirable wasting reactions such as pro-oxidation.

Table 3
Spin density of 2-hydroxytoluene radical form.



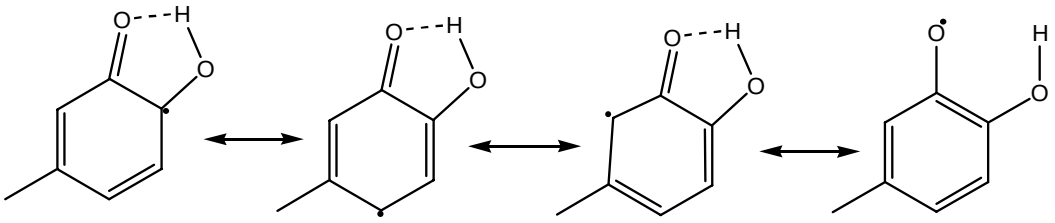
Carbon No.	Spin density of radical form of		
	o-hydroxytoluene	*m*-hydroxytoluene	p-hydroxytoluene
1	0.973672	-0.904121	1.011856
2	-0.858323	0.957272	-0.897062
3	0.963641	-0.727432	0.961299
4	-0.905837	0.941498	-0.797904
5	0.967663	-0.882725	0.948562
6	-0.914865	1.055018	-0.896718



Scheme 1. The resonance structure of hydroxytoluene.

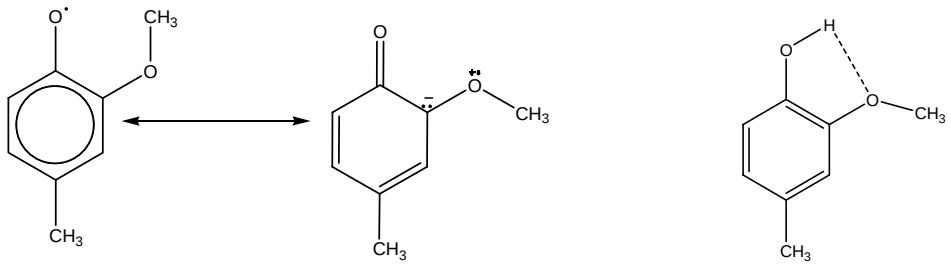
In accordance with literature reports, a glance at Table 1 would be quite enough to develop the intuition that *ortho*-dihydroxy toluenes (methyl-catechols) and 2,5 dihydroxytoluene are remarkably active radical scavengers compared to monohydroxytoluenes and 3-methoxy, 4-hydroxytoluene. Considering the similarity in basic structure of 3,4 dihydroxytoluene with 3-methoxy,4- hydroxytoluene, it is amusing that the activity of the catechol is prominent over its methoxy analogue. This may be because of the role of intramolecular hydrogen bonding.

The intramolecular hydrogen bonding increases stabilization of the semiquinone radical formed from catechol, and of the corresponding transition state, through strong hydrogen bonding in resonance structures [34], as shown in Scheme 2 and stabilized by the electron-donating effect of *ortho* OH [29]. Increased stabilization of the radical over that of the parent catechol and *3*-methoxy-4-hydroxytoluene provided by hydrogen bonding was confirmed by calculations. The parent catechol is stabilized by a moderately strong hydrogen bond while the radical has a much stronger hydrogen bond [35]. It was found that although the charge difference between hydrogen-bonded H and O played a role in determining hydrogen bond. As the oxygen-centered radical has great tendency to form a chemical bond with the H atom, hydrogen bond lengths in catecholic radicals are systematically shorter than those in catechols. Hence, the enthalpies of hydrogen bond for the former are higher than those for the latter [36]. Thus overall, *3*-methoxy-4-hydroxytoluene is somewhat deactivated as a radical scavenger by intramolecular hydrogen bonding whereas a catechol is activated [36].



Scheme 2. Resonance and hydrogen bonding in 3,4-dihydroxytoluene.

3-methoxy-4-hydroxytoluene (2-hydroxy-5-methylanisol), however, is more active than monohydroxytoluenes as an *ortho*-methoxy group could provide stabilization of the phenoxyl radical formed by resonance of the type shown in Scheme 3. The parent methoxytoluene is intramolecularly hydrogen bonded as shown in Scheme 3. This hydrogen bond opposes the electronic effect of the methoxy group to decrease its reactivity. The net result of these opposing effects, the activating effect of the *ortho*-methoxy versus the stabilizing effect of H-bonding, is a decreased reactivity of the 2-methoxy isomer [37]. Relative stability of the parent molecule and phenoxy radical plays a vital role in the radical scavenging activity of hydroxytoluenes in particular and phenolic compounds in general [36].



Scheme 3. Resonance and hydrogen bonding in 3-methoxy-4-hydroxytoluene.

There are contradicting reports on the kinetics of the reaction of hydroquinone with DPPH. Some works reported that the termination of hydroquinone is *via* the formation of 1,4 benezoquionone where two molecules of DPPH are reduced by one molecule of hydroquinone [38]. The stoichiometric factor of hydroquinones (0.6-1.1) reported by others is almost half of catechols [39]. The later report sounds evenhanded as activity is invariably dependent on the number of molecules consumed in the course of reaction [38]. The 1,4-dihydroxybenzene present an overshooting with $ABTS^{\cdot}$ concentration at about 100 sec, followed by a partial recovery of the ABTS derived radical absorbance. A plausible explanation of the observed overshoot can be given in terms of the following reaction scheme that takes into account the occurrence of reversible cross-combination reactions

$$ABTS^{\cdot} + XH\ ß \longleftrightarrow ABTS + X^{\cdot} + H^{+} \qquad (1)$$

$$ABTS^{\cdot} + X^{\cdot} \longrightarrow Adduct \qquad (Fast) \qquad (2)$$

$$Adduct \longrightarrow ABTS^{\cdot} + X^{\cdot} \qquad (Slow) \qquad (3)$$

$$X^{*} + X^{*} \longrightarrow Products \qquad (Slow) \qquad (4)$$

In this scheme, overshooting in the ABTS radical consumption is due to the occurrence of reaction (2). The following (slower) decomposition of this adducts and the self reaction of X radicals could explain the partial recovering of the ABTS derived radical concentration [40]. The

behavior of DPPH and ABTS are similar [41]. And as phenols and cresols have the same chemical properties, properties of hydroquinone are adopted to 2-methyl hydroquinone. Hence the above scheme could account why the activity and stoichiometric factors of 2-methyl hydroquinone are almost half of methyl catechols.

Comparing the activity of 2,6 dihydroxytoluene with catechols and 2,5 dihydroxytoluene, one clearly observes that 2,6 dihydroxytoluene is less potent than catechols. Though catechols, 2,5 dihydroxytoluene and 2,6 dihydroxytoluenes have the same number of hydroxyl, their heat of formation is quite different and in turn their activity is varied a lot. 2,4,6 trihydroxytoluene also has lower activity as compared to catechols and 2,5 dihydroxytoluene. The role of an additional hydroxyl group at C4 of 2,6 dihydroxytoluene has insignificant effect on its activity. Hence it seems that not only the number but also the arrangement of hydroxyl group plays vital role on the activity of hydroxytoluene derivatives. In general, methylcatechols, which have hydroxyl groups in the *ortho* position, are the most potent radical scavengers while increasing the number of O-H groups in the alternative position C(2,6) as in methyl resorcinol and C(2,4,6) as in 2,4,6 trihydroxytoluene does not show any notable activity.

Comparing the Ic_{50} of methyl-catechols with rutin (a standard compound and potent flavonoid), catechols are more active than rutin on mg basis while they have nearly the same Ic_{50} on molar basis. Hence, hydroxytoluenes can be considered as a simplified flavonoid with a considerable radical scavenging activity. Rings A and C are not essential structural requirement of flavonoids to impart radical scavenging activity.

The distribution of spin density of cresols was analyzed. Table 3 summarizes the spin density of all the possible unsubstituted hydroxytoluenes. In radicals formed by the abstraction of hydrogen atom from either 2-OH or 4-OH C1, C3 and C5 are centers of positive spin density while C2, C4 and C6 are centers of negative spin density. In the case of radicals formed from 3-OH, the reverse is the case i.e. C1, C3 and C5 have negative spin density while C2, C4 and C6 have positive spin density. This is in line with theoretical calculation performed by Seyoum *et al*. [14]. The effect of a substituent is determined by comparing the ΔH_f of the unsubstituted radical with that of the substituted radical. The data on the ΔH_f of the radicals together with the corresponding spin distributions shows some important relationships between the patterns of hydroxyl substitution and the stability levels of the aroxyl radicals.

A hydroxyl group significantly increases the stability of a radical (decreases ΔH_f) if it is substituted on a carbon with a positive spin density and has minimum effect when it is substituted on a carbon with negative spin density. Hydrogen-donating group *ortho* to a radical forming hydroxyl provides an extra radical stabilizing effect. *Ortho* and *para* methyl groups more stabilize the radical than the corresponding *meta* group. This shows that the role of methyl group in stabilizing the aryloxy radical is beyond the inductive effect.

3.3. Mechanism of reaction

The reaction mechanism of DPPH with phenols and polyphenols is a subject of controversy. The initiation reaction of free oxygen-centered and nitrogen-centered radicals (2,2-diphenyl-1-picrylhydrazyl (DPPH)) is proposed to be *via* two different mechanisms: (i) a direct abstraction of phenol H-atom by radicals (HAT reactions) and (ii) an electron-transfer process from phenol or its phenoxide anion to radical (ET reactions) [42].

According to Pannala, *et, al*., [43] mono hydroxytoluenes undergo a reaction with stable free radicals *via* HAT while the most significant reaction for dihydroxytoluenes is ET. This mechanism of reaction is, at least theoretically, accepted. However, performing theoretical calculations based on this assumption would make comparisons irregular and soundless. Hence, all theoretical calculations are performed based on the assumption that all hydroxytoluenes undergo reaction *via* HAT.

For a successful radical scavenging reaction, not only the ease of hydrogen abstraction but also the ease of the termination of the newly formed radical appears to be crucial [29]. Hence predicting both the stability of hydroxytoluene radical and a reasonable termination mechanism at the same time enable to determine which one is the most decisive parameter for radical scavenging activity. A representative active hydroxytoluene, 4-methycatechol, was taken to discern a parameter which is responsible for its activity. The concentrated reaction mixture of DPPH and 4-methyl catechol was then chromatographed on TLC and sprayed with10% NaOH. Red colored spot was observed indicating formation of a quinone as reaction product [20].

The remaining larger portion of the concentrated reaction mixture was purified by TLC using hexane: ethylacetate (3:1) as a mobile phase. The newly formed product had an R_f value of 0.6. This band was scraped, suspended in methanol and filtered. The filtrate was evaporated to dryness and the residue analyzed with ^{1}HNMR and ^{13}CNMR. The isolated compound was a brown red viscous liquid. ^{1}H NMR (400MHz, CD_3OD) gave signals at δ 2.10(3H, s); 6.5 (1H, d); 6.7(1H, s); 6.8(1H, d. The ^{13}C NMR spectrum indicated presence of seven carbons at δ 144.25, 142.07, 129.89, 120.60, 116.27, 115.36, and 19.75.

In the ^{1}H NMR the signal at δ 2.1 (singlet and integrated for three protons) indicated that these protons are allylic protons (methyl protons attached to double bond). Doublets at δ 6.5 and δ 6.75 and a singlet at δ 6.7 all integrated for one proton each represented the three vinylic protons in the compound. The vinylic protons especially that resonated at δ 6.7 and δ 6.75 are more deshielded as they are vicinal to the carbonyl group.

^{13}C NMR spectrum of the isolated compound showed well resolved peaks representing seven carbon atoms. The spectrum indicated presence of three olefinic carbons (at δ 120.60, 116.27, and 115.36), three tertiary carbons (at δ 144.25, 142.07, and 129.89) and one methyl carbon (at δ 19.75). The information from the chemical test and the clear ^{1}HNMR and ^{13}CNMR spectra showed that the isolated compound is 4-methylcyclohexa-3,5-diene-1,2-dione.

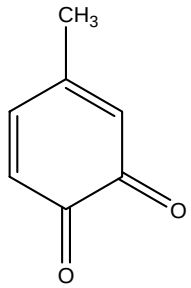
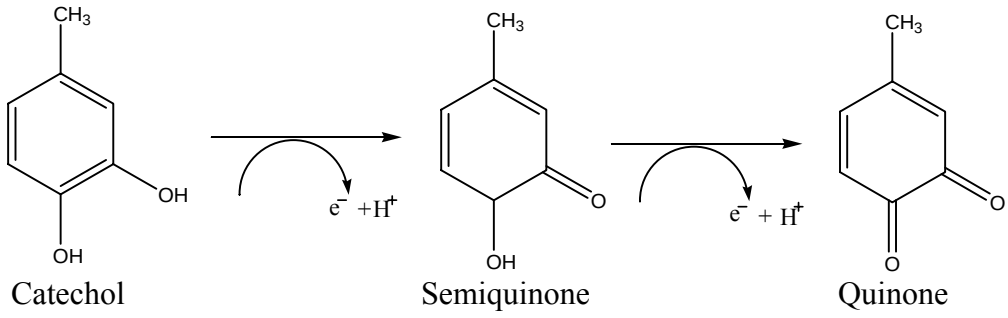


Fig. 1. Structure of the isolated compound.

All the above experimental observations lead to the conclusion that methyl catechols terminate *via* the donation of a single electron to the radical cation resulting in the formation of a semiquinone, which can further donate an electron to form the quinone.



Scheme 4. Mechanism of termination 4-methyl catechol.

For a successful radical scavenging activity, not only the ease of hydrogen abstraction but also the ease of termination of the newly formed radical appears to be crucial [29]. It would be crucial to discern which one is a decisive factor to be used as a guide to design active radical scavengers. To put this to end, the heat of formation (termination of catechols and hydroquninone) of quinones was determined and data is presented in Table 4.

Table 4
Calculated bond dissociation enthalpy (in $Kcalmol^{-1}$) of the initiation enthalpy (ΔH_1) and the termination enthalpy (ΔH_2) exchangeable (OH) hydrogen atom.

Compounds	ΔH_1	ΔH_2
3,4-dihydroxytoluene	64.77	59.46
2,3-dihydroxytoluene	63.03	60.07
2,5-dihydroxytoluene	66.05	47.36

An attempt to correlate ΔH_2 of methyl catechols and hydroquinone with radical scavenging activity showed that there is no correlation between ΔH_2 and activity. Instead a close watch of ΔH_1 shows that there is linear correlation between ΔH_1 and log IC50, with the *R* value being 0.9708 from three data points (methyl catechols and methyl hydroquinone). These three data could not be satisfactory statistically to draw a conclusion. Hence the plot of ΔH_1 and log IC50 for all hydroxytoluene under study showed a linear correlation, with the R value of 0.86 and P value of 0.00337.

The experimental observations and theoretical calculations lead to a conclusion that the initiation of reaction is the rate limiting step in the radical scavenging reaction of hydroxytoluenes with DPPH radical. The correlation between IC_{50} and calculated enthalpy showed that the primary bond dissociation energy (the enthalpy of initiation reaction) is a good predictor of radical scavenging activity with reasonable accuracy.

Since absorbance is directly proportional to the concentration of DPPH radicals, the decrease in absorbance at 517 nm was fast in the first few seconds regardless of structural difference of hydroxytoluenes. The decrease in absorbance depends on concentration of hydroxytoluene (where the concentration of DPPH is controlled). After the reaction elapsed sometime (30 minutes on average) the decrease in absorbance was minimal; however, the slopes of the LRCs of absorbance *vs* concentration of the set of readings taken at different times were similar. The reaction of hydroxytoluene with DPPH was fast before steady state was achieved. Once a steady state is achieved the concentration of hydrogen radical is constant since it will be consumed at a rate that is equal with which they are formed (Rxn.1.). That is why after 30th minute the slopes of the LRCs of absorbance *vs* concentration of the set of readings taken at different times were similar albeit the decrease in absorbance is minimal. The hydroxytoluene radical formed in (Rxn.1.) terminate *via* dimerization and formation of quinone [42].

$$ArOH \rightleftharpoons ArO^{\bullet} + H^{\bullet} \qquad \text{Rxn.1.}$$

$$H^{\cdot} + DPPH \longrightarrow DPPH_2 \qquad \text{Rxn.2.}$$

As the reaction of hydroxytoluene with DPPH undergoes *via* steady state, the reaction is first order and the initiation reaction is the rate limiting step which is in line with the kinetics of this typical reaction and the conclusion that was drawn from theoretical calculation performed in this experiment, respectively. Irrespective of the mechanism of reaction, the dissociation reaction of phenolic compounds is *via* equilibration. Considering this equilibration reaction together with the notion that the enthalpy of initiation reaction is a good predictor of radical scavenging activity, the equilibrium constant, K could be estimated mathematically.

$$ArOH \rightleftharpoons ArO^{\bullet} + H^{\bullet}$$

$$K = \frac{[ArO^{\bullet}][H^{\bullet}]}{[ArOH]} \qquad \text{Equation 1}$$

As the dissociated hydroxytoluene and hydrogen radical are equal

$$K = \frac{[H^{\bullet}]^2}{[ArOH]} \qquad \text{Equation 2}$$

The rate of equilibrium reactions do not depend on the amount of the starting martial. It only depends on the inherent property of the starting material and the state conditions. From equation 2 since the left hand side is a constant, the ratio on the right hand side should also be constant, which would mean that at equilibrium the amount of hydrogen atoms produced is directly proportional to the initial concentration of a hydroxytoluene. It is hydrogen radical that reacts with DPPH which determines radical scavenging activity of hydroxytoluene which in turn is proportional to the equilibrium constant. The last equation reveals how the enthalpy of the reaction and radical scavenging activity is explained in terms of equilibrium constant.

From energy perspective of the chemical reaction, change in free energy ($\Delta G^{\neq 0}$) in the conversion of the reactants to products is related to equilibrium constant, K, given by the relation:

$$-\Delta G^{\neq 0} = 2.303RT\log^{K} \qquad \text{Equation 3}$$

As the activation entropy $\Delta S^{\neq 0}$ for the H-atom transfer reaction can be neglected,

$$-\Delta H^{\neq 0} = 2.303RT\log^{K} \qquad \text{Equation 4}$$

Substituting the value of K in Equation 2 to Equation 4

$$-\Delta H^{\neq 0} = 2.303RT\log \frac{[H^{\bullet}]^2}{[ArOH]} \qquad \text{Equation 5}$$

Equation 3 demonstrates explicitly the linear correlation of log IC50 and ΔH. Hence it is possible to find a mathematical relationship between IC_{50} and ΔH and equilibrium constant and ΔH.

4. Conclusion

In this study methylcatechols which have hydroxyl groups in the *ortho* position were found to be the most potent radical scavengers and have a comparable IC_{50} with rutin, an active flavonoid. Hydroxytoluenes can be considered as a simplified flavonoid with a considerable radical scavenging activity. Rings A and C are not essential structural requirement of flavonoids to impart radical scavenging activity. A good linear correlation was obtained between log Ic_{50} and enthalpy, showing that the enthalpy of initiation can be used to predict radical scavenging

activity. It is also possible to explain the radical scavenging activity in terms of equilibrium constant. The information from chemical test and spectroscopic data showed that the isolated compound is 4-methylcyclohexa-3,5-diene-1,2-dione, which ultimately lead to a conclusion that methyl catechols terminate *via* the formation of quinone.

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