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REGULAR ARTICLE

Design and synthesis of sulfur-containing butylated hydroxytoluene: antioxidant potency and selective anticancer agent

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Abstract. In the present study, a series of multipotent antioxidants (MPAOs), namely sulfur-containing BHT (S-BHT), derivatives were rationally designed and synthesized, and their inhibitory activities against free radicals and human cancer cell lines, HT29 (colon cancer) and MCF7 (breast cancer) were further evaluated. The experimental results showed that the Six out-of-eight S-BHT compounds had excellent antioxidant activity against DPPH radical with major enhancement compared to BHT. Among them, compounds **2b**, **2a** and **3b** attained over 45% lower IC_{50} values than BHT. *In vitro* cytotoxicity, MTT assay was carried out using two human cancer cell lines, HT29 (colon cancer) and MCF7 (breast cancer) in addition to their non-tumorigenic counterparts to explore selectivity. In line with antioxidant activity, compounds **2a** and **2b** displayed the highest cytotoxicity effect on both cancer types. Interestingly, **2b** not only exhibited superior cancer inhibition but also scored high selectivity index (SI = 5.2, 12.5) in colon and breast tissues, respectively, exceeding that of the standard chemotherapeutic drugs used 5-Fluorouracil (5-FU) and Tamoxifen (Tmx), with lower IC_{50} values. The results indicated that the symmetric S-BHT derivatives were significantly enhanced by the antioxidant potency and their ability as useful and promising selective anticancer agents.

Keywords. Antioxidant; anticancer; sulfur-containing BHT; HT29; MCF7.

1. Introduction

Cancer is a major health problem worldwide and is also known as a silent killer. It leads the cause of death globally and is accounted for 15% of all deaths with a total of 8.2 million in 2012.[1] The number of death caused by cancer is expected to rise up to five-folds by 2025.[2] Cancer originates from the uncontrolled number of cell divisions and the mutation of normal tissues or cells when the body's normal control mechanism stops working.[3] These uncontrolled divisions are caused by the reactive oxygen species (ROS), where they extremely alter the tissue interstitium containing the protease or antiprotease enzymes and oxidize the normal cells and tissue by the degradation of essential cellular components.[4,5] ROS are the primary causes of the undesired process such as ageing, inflammatory, and other chronic diseases.[6–8] They exhibit an important physiological role as free radical but they may also engage the toxic effect for the whole human body. The increasing number of cancer incidents lead to increasing demand for cancer treatment.[9]

Chemotherapy is an effective cancer treatment procedure but it is limited to finding therapies and drugs to treat different types of cancer due to low specificity and dose-limiting toxicity.[10] Therefore, the development of new anticancer drugs has gained great attention among researchers around the world. The focus is on the design

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and a new approach by combining several entities which have antioxidant properties, into single effective and safe molecules.[11] Antioxidants play an important role in human life. Their ability as free radical scavengers help to protect the oxidant-mediated and tissue damage. Moreover, most of the antioxidant entities reported have their great effect of toxicity to a greater or lesser extent on cancer cells.[4] It has been reported that vitamin E and pyrrolidinedithiocarbamate significantly enhance the inhibition of colorectal cancer tumor growth by *in vitro* (5FU and doxorubicin) and *in vivo* (5FU) cytotoxicity approach. Hence, this research for a novel therapy with the presence of antioxidants for cancer treatment is an effort of importance.[12]

Phenolic compounds are great antioxidant agents which have the function as free radical scavengers, preventing oxidation and inhibit the formation of singlet-oxygen.[13] Butylated hydroxytoluene (BHT) is a well-known synthetic phenolic antioxidant that was established in 1947[14] and was widely used at a low concentration, which normally ranges from 0.01 to 0.1% by the Food and Drug Administration (FDA, USA) in foods and pharmaceutical industries.[15] BHT was observed to act as anticarcinogens in various animal models.[16] The two *tert*-butyl group flanking the –OH group of BHT have major potentials in anti-inflammatory activities.[17] They also provide strong steric hindrance to prevent the –OH group of BHT from undesirable reactions such as pro-oxidation.[18–21]

Over the years, sulfur-containing compounds which are among the many bioactive compounds have been identified as promising protective effect against different types of cancer.[22] Sulfur-containing compounds are reported as a radical scavenger to protecting cells from free radical.[23] They were easily reacting with reactive oxygen species due to their strong nucleophilic property to donate electron.[24] Recently, sulfur-containing compounds are widely used in anticancer research and they were showed a protective effect against several types of cancer by the electron oxidation of sulfhydryl functional groups (-SH).[23,24] The thiyl radical fragment is very important for radical scavenging and anti-proliferative effects of S-BHT. As reported, a 1,3,4-thiadiazole moiety containing compounds have exhibited potential anticancer,[25,26] antitubercular[27] and antiulcer properties.[28] The aromaticity of 1,3,4-thiadiazoles was attributed to their potential as anticancer agent.[29]

In this study, rational design and SAR approaches were utilized to merge multiple functions that include antioxidant moieties, BHT (primary Antioxidant), antiperoxide (secondary antioxidant) and anti-proliferative fragments, sulfur-containing groups into one structure in the development of new multipotent antioxidants (MPAOs) and diversified pharmacological activities with markedly enhanced radicals scavenging ability and antiproliferative activity. The DPPH and MTT assays were carried out to evaluate the structural variations of synthesized compounds for their radical scavenging effect and cytotoxicity effect against human colon adenocarcinoma cell line (HT29) and human breast adenocarcinoma cell line (MCF7), respectively.

2. Experimental

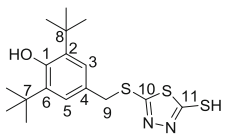
2.1 *General reagents and instruments*

Solvents and materials were purchased from Sigma-Aldrich. The ^{1}H-NMR and ^{13}C-NMR were reported in deuterated-chloroform, or deuterated-dimethylsulfoxide on Bruker AVANCE III 600 Ultrashield NMR spectrometer measured at 600 and 150 MHz for proton and carbon-13, respectively. Chemical shifts were recorded in ppm using δ scale. A hot stage Gallen Kamp melting point apparatus with microscope were used to identify the melting point. The infrared (IR) spectra were recorded on the Perkin Elmer FT-IR Spectrum 400 by attenuated total reflectane (ATR) technique. The mass spectra were identified using Agilent Technologies 6550 iFunnel Q-TOF LC/MS spectrometer 70 eV.

2.2 *Synthesis of mono-substituent of BHT-derivatives*

Benzyl alcohol (1 mmol, 0. 2364 g) and thiol (1.1 mmol, 0.1543 g) were mixed with catalyst, *p*-toluenesulfonic acid (PTSA) and were stirred for an appropriate time at room temperature. The reaction mixture was poured and diluted with ethyl acetate (5 mL) and the catalyst was allowed to settle. The ethyl acetate supernatant was poured off, rinsed with ethyl acetate (5 mL) and the mixed organic solvent was evaporated under reduced pressure to obtain crude product, which was purified using column chromatography (Silica gel: 230–400 mesh, petroleum ether (40–60 °C): ethyl acetate = 9:1) to obtain a pure product.

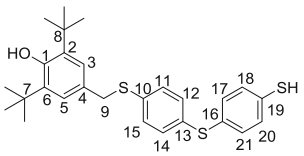
2.2a *Synthesis of 2,6-di-tert-butyl-4-(((5-mercapto-1,3,4-thiadiazole-2-yl)thio)methyl) phenol,* ***2a***:



(Yield 91%); White amorphous solid; M.p.: 178–180 °C; HREIMS *m/z*: 369.1122 $[M+H]^{+}$ (Calcd for $C_{17}H_{24}N_2OS_3$ 368.1051); IR (ATR), cm^{-1}: 3419.7 (-OH), 2963.0 (-*t*-butyl), 2855.5 (-*t*-butyl), 2527.8 (-SH); ^{1}H NMR (δ, ppm in Dmso-d_6): 7.12 (s, 2H), 4.31 (s, 2H), 1.36 (*tert*-butyl), 7.02 (-OH), 2.29 (-SH). ^{13}C NMR (δ, ppm in Dmso-d_6): 154.05 (C-1), 139.86 (C-2), 126.03 (C-3), 127.06 (C-4),

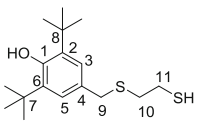
128.49 (C-5), 139.86 (C-6), 34.98 (C-7), 34.98 (C-8), 38.57 (C-9), 157.86 (C-10), 188.79 (C-11), 30.75 (*tert*-butyl).

2.2b *Synthesis of 2,6-di-tert-butyl-4-(((4-((4-mercaptophenyl)thio)phenyl)thio) methyl) phenol,* ***3a***:



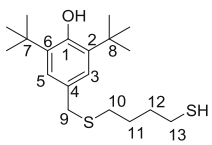
(Yield 90%); White amorphous solid; M.p.: 79–81 °C; HREIMS *m/z*: 469.1065 $[M+H]^+$ (Calcd for $C_{27}H_{32}OS_3$ 468.1615); IR (ATR), cm^{-1}: 3629.1 (-OH), 2956.2 (-*t*-butyl), 2876.7 (-*t*-butyl), 2532.3 (-SH); ^1H NMR (δ, ppm in Dmso-d_6): 6.88 (s, 1H), 7.00 (s, 1H), 4.11 (s, 2H), 7.19 (m, 4H), 7.30 (m, 4H), 1.33 (*tert*-butyl), 5.62 (-OH). ^{13}C NMR (δ, ppm in Dmso-d_6): 153.41 (C-1), 139.58 (C-2), 125.58 (C-3), 128.18 (C-4), 125.58 (C-5), 139.58 (C-6), 34.91 (C-7), 34.91 (C-8), 37.93 (C-9), 136.20 (C-10), 125.58 (C-11), 130.32 (C-12), 132.92 (C-13), 131.10 (C-14), 131.96 (C-15), 131.90 (C-16), 132.44 (C-17), 132.44 (C-18), 136.40 (C-19), 130.32 (C-20), 129.84 (C-21), 30.76 (*tert*-butyl).

2.2c *Synthesis of 2,6-di-tert-butyl-4-(((2-mercaptoethyl)thio)methyl)phenol,* ***4a***:



(Yield 48%); White amorphous solid; M.p.: 65–67 °C; HREIMS *m/z*: 313.1634 $[M+H]^+$ (Calcd for $C_{17}H_{28}OS_2$312.1582); IR (ATR), cm^{-1}: 3618.5 (-OH), 2952.2 (-*t*-butyl), 2877.0 (-*t*-butyl), 2554.6 (-SH); ^1H NMR (δ, ppm in $CDCl_3$): 7.01 (s, 2H), 4.38 (s, 2H), 3.59 (s, 2H), 2.56 (s, 2H), 1.36 (*tert*-butyl), 5.07 (-OH), 2.56 (-SH). ^{13}C NMR (δ, ppm in $CDCl_3$): 153.39 (C-1), 135.81 (C-2), 125.11 (C-3), 128.39 (C-4), 125.11 (C-5), 135.81 (C-6), 34.32 (C-7), 34.31 (C-8), 36.85 (C-9), 36.85 (C-10), 31.60 (C-11), 30.31 (*tert*-butyl).

2.2d *Synthesis of 2,6-di-tert-butyl-4-(((4-mercaptobutyl)thio)methyl)phenol,* ***5a***:

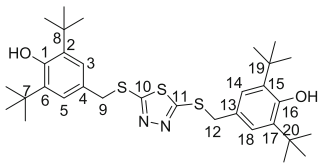


(Yield 47%); White amorphous solid; M.p.: 85–87 °C; HREIMS *m/z*: 363.1793 $[M+Na]^+$ (Calcd for $C_{19}H_{32}OS_2$ 340.1895); IR (ATR), cm^{-1}: 3564.8 (-OH), 2952.2 (-*t*-butyl), 2871.6 (-*t*-butyl), 2554.6 (-SH); ^1H NMR (δ, ppm in Dmso-d_6): 7.01 (s, 2H), 3.61 (s, 2H), 2.39 (t, 2H), 2.45 (m, 2H), 1.58 (m, 2H), 1.55 (m, 2H), 2.22 (-SH), 6.84 (-OH) 1.36 (*tert*-butyl). ^{13}C NMR (δ, ppm in Dmso-d_6): 153.08 (C-1), 139.57 (C-2), 125.37 (C-3), 129.67 (C-4), 125.37 (C-5), 139.57 (C-6), 34.92 (C-7), 34.92 (C-8), 35.98 (C-9), 30.72 (C-10), 23.81 (C-11), 27.92 (C-12), 32.99 (C-13), 30.85 (*tert*-butyl).

2.3 *Synthesis of di-substituent of BHT-derivatives*

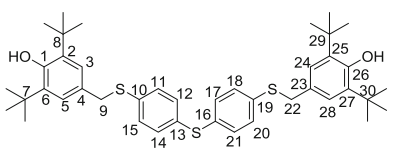
The reaction is similar to mono-substituents of BHT-derivatives but the difference is the concentration of benzyl alcohol. Benzyl alcohol (2 mmol, 0.4728 g) and thiol (1 mmol, 0.1534 g) were mixed with PTSA as catalyst and were stirred for an appropriate time at room temperature. The reaction mixture was diluted with ethyl acetate (5 mL) and the catalyst was allowed to settle. The supernatant ethyl acetate was poured off, washed with ethyl acetate (5 mL) and the mixed organic solvent was evaporated under reduced pressure to afford crude product, which is purified by column chromatography (Silica gel: 230–400 mesh, petroleum ether (40–60 °C): ethyl acetate = 9:1) to obtain pure product.

2.3a *Synthesis of 4,4′-(((1,3,4-thiadiazole-2,5-diyl)bis(sulfanediyl))bis(methylene))bis (2,6-di-tert-butylphenol),* ***2b***:



(Yield 94%); White amorphous solid; M.p.: 128–130 °C; HREIMS *m/z*: 587.2787 $[M+H]^+$ (Calcd for $C_{32}H_{46}N_2O_2S_3$ 586.2721); IR (ATR), cm^{-1}: 3566.1 (-OH), 2951.8 (-*t*-butyl), 2872.4 (-*t*-butyl); ^1H NMR (δ, ppm in $CDCl_3$): 7.15 (s, 2H), 4.28 (s, 2H), 5.40 (s, 2H), 7.42 (s, 2H), 1.45 (*tert*-butyl), 5.29 (-OH). ^{13}C NMR (δ, ppm in $CDCl_3$): 153.83 (C-1), 136.04 (C-2), 125.99 (C-3), 125.08 (C-4), 125.99 (C-5), 136.04 (C-6), 34.35 (C-7), 34.35 (C-8), 38.61 (C-9), 155.67 (C-10), 155.67 (C-11), 38.61 (C-12), 125.08 (C-13), 125.99 (C-14), 136.04 (C-15), 153.83 (C-16), 136.04 (C-17), 125.99 (C-18), 34.35 (C-19), 34.35 (C-20), 30.26 (*tert*-butyl).

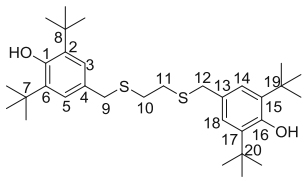
2.3b *Synthesis of 4,4′-(((thiobis(4,1-phenylene))bis(sulfanediyl))bis(methylene))bis(2,6-di-tert-butylphenol),* ***3b***:



(Yield 93%); White amorphous solid; M.p.: 66–68 °C; HREIMS *m/z*: 687.3312 $[M+H]^+$ (Calcd for $C_{42}H_{54}O_2S_3$ 686.3286); IR (ATR), cm^{-1}: 3634.4 (-OH), 2950.9 (-*t*-butyl), 2871.4 (-*t*-butyl); ^1H NMR (δ, ppm in Dmso-d_6): 7.01 (s, 2H), 4.37 (s, 2H), 7.21 (m, 4H), 7.31 (m, 4H), 4.12 (s, 2H), 7.07 (s, 2H), 1.38 (*tert*-butyl), 6.88 (-OH). ^{13}C NMR (δ, ppm in Dmso-d_6): 153.42 (C-1), 139.47

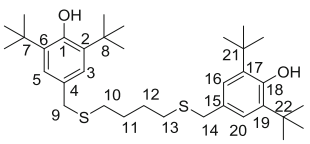
(C-2), 125.58 (C-3), 128.30 (C-4), 125.58 (C-5), 139.47 (C-6), 34.91 (C-7), 34.91 (C-8), 37.89 (C-9), 136.72(C-10), 131.57 (C-11), 130.27 (C-12), 132.56 (C-13), 130.27 (C-14), 131.57 (C-15), 132.56 (C-16), 130.27 (C-17), 131.57 (C-18), 136.72 (C-19), 131.57 (C-20), 130.27 (C-21), 37.89 (C-22), 129.93 (C-23), 124.54 (C-24), 139.58 (C-25), 153.66 (C-26), 139.58 (C-27), 124.54 (C-28), 34.91 (C-29), 34.91 (C-30), 30.77 (*tert*-butyl).

2.3c *Synthesis of 4,4′-((ethane-1,2-diylbis(sulfanediyl))-bis(methylene))bis(2,6-di-tert-butylphenol),* ***4b***:



(Yield 89%); White amorphous solid; M.p.: 121–123 °C; HREIMS *m/z*: 553.3139 $[M+Na]^+$ (Calcd for $C_{32}H_{50}O_2S_2$ 530.3252); IR (ATR), cm^{-1}: 3618.5 (-OH), 2950.9 (-*t*-butyl), 2871.4 (-*t*-butyl); ^1H NMR (δ, ppm in $CDCl_3$): 7.11 (s, 4H), 3.69 (s, 4H), 2.66 (s, 4H), 1.46 (*tert*-butyl), 5.17 (-OH). ^{13}C NMR (δ, ppm in $CDCl_3$): 152.92 (C-1), 136.01 (C-2), 125.47 (C-3), 128.38 (C-4), 125.47 (C-5), 136.01 (C-6), 34.37 (C-7), 34.37 (C-8), 36.85 (C-9), 31.60 (C-10), 30.31 (*tert*-butyl).

2.3d *Synthesis of 4,4′-((butane-1,4-diylbis(sulfanediyl))-bis(methylene))bis(2,6-di-tert-butylphenol),* ***5b***:



(Yield 87%); White amorphous solid; M.p.: 85–87 °C; HREIMS *m/z*: 559.3616 $[M+H]^+$ (Calcd for $C_{34}H_{54}O_2S_2$ 558.3565); IR (ATR), cm^{-1}: 3570.8 (-OH), 2950.9 (-*t*-butyl), 2871.4 (-*t*-butyl); ^1H NMR (δ, ppm in Dmso-d_6): 7.01 (s, 4H), 3.61 (s, 2H), 2.38 (t, 2H), 1.55 (m, 4H), 2.38 (2H, *t*, *J* = 6 Hz, H-13), 3.61 (s, 2H), 6.83 (-OH), 1.36 (*tert*-butyl). ^{13}C NMR (δ, ppm in Dmso-d_6): 153.07 (C-1), 139.58 (C-2), 125.37 (C-3), 129.68 (C-4), 125.37 (C-5), 139.58 (C-6), 34.91 (C-7), 34.91 (C-8), 35.99 (C-9), 30.84 (C-10), 28.38 (C-11), 30.84 (*tert*-butyl).

2.4 *Antioxidant activity (DPPH scavenging assay)*

DPPH assay protocol reported by Gorinstein *et al.*[30] was used to evaluate BHT-derivatives in terms of radical scavenging ability or hydrogen donating. The colour change of the reaction mixture was measured at 517 nm against the blank. Samples without treatment and BHT were used as negative controls and positive, respectively. The percentage of DPPH decolourization by the sample was calculated as per below equation:

$$\text{DPPH scavenging effect} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

$A_{control}$ is denoted as the absorbance of the control reaction and, A_{sample} is as the absorbance of the test compounds measured at 517 nm. The test was conducted in triplicate.

2.5 *Cytotoxicity assay*

In the present study, two types of human cancer cell lines were used such as HT-29 (colon adenocarcinoma) and MCF-7 (breast adenocarcinoma). Both cell types were received from the American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured under humidified 5% CO_2 incubator at 37 °C (ThermoFischer Scientific, USA) in RPMI-1640 medium (SigmaAldrich, USA), supplemented with 10% fetal bovine serum (Gibco,USA), and 1% Pen-Strip antibiotic (10,000 units penicillin-10 mg streptomycin/mL, SigmaAldrich, USA).

2.5a *MTT assay*: The MTT assay was conducted based on the previous protocol.[31] Briefly, the cells with the density of 5000 cells/well were plated into 96-well plates in the final volume of 100 µL culture medium per well. On the following day, the synthesized compounds, BHT as well as the standard cytotoxic drugs (TMX)and (5-FU) were used to treat the cells at a gradual increasing concentrations (12.5, 25, 50, 100, 200 and 400 µM) and maintained for 24 h under 5% CO_2 at 37 °C. Cells without treatment were used as a negative control. At the end of the incubation period, each well was pipetted with 10 µL of MTT reagent (5 mg/mL) and incubated at the same condition for 4 h. After that supernatant was removed, the addition of 100 µL of dimethylsulphoxide (DMSO) into each well was followed and the absorbance was determined using microplate reader (Infinite-M200Pro-TECAN) at 570 nm. The experiment was carried out in triplicates and the cellular viability was calculated according to the below equation:

$$\text{Cell viability } (\%) = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

2.5b *Selectivity Index*: Selectivity index (SI) was calculated as reported previously,[32] and according to the equation as described below:

$$SI = \frac{IC_{50} \text{ of compound on normal cell line}}{IC_{50} \text{ of compound on cancer cell}} \times 100$$

3. Results and Discussion

3.1 *Structure-activity relationship (SAR) and rational S-BHT*

The combination of versatile antioxidant moieties into one structure constitutes a fast-applying strategy in the synthesis of multipotent antioxidant.[24,33,34] This approach was carried out to maximize the radical scavenging effect and other biological properties of the well-known antioxidant (BHT), aiming to scavenge free radicals and inhibit oxidative stress processes. These functions strategies are expected to play a vital role in medical therapeutic applications to prevent various human diseases, and in repairing cellular damage as shown in Figure 1.

The well-known primary antioxidant of phenols at the 2, 4, 6-positions contain methyl and *tert*-butyl and act as great electron-donator to scavenge free radical.[4,35] The lowest bond diffusion of the phenol O-H group gives the inductive and conjugative effects to stabilize the phenoxyl radical and to easily donate electrons.[4,36] The highest steric effect at *ortho* position helps to minimize undesirable reactions due to the presence of bulky electron cloud moieties as shown in Figure 1.[18–21] Thioethers are secondary antioxidants and can be used in the combination with primary antioxidant in order to increase the antioxidant ability to trapping free radical.[19] They go through redox reactions by suppressing the formation of radicals, and protect again oxidative damage.[37] The formation of thioether bridge could provide synergistic effects resulting from the combination of primary and secondary antioxidants in MPAOs structures (Figure 1).[7] Synergistic effect is an interaction between two or more drugs that gives the total effect of the drugs to be higher than the sum of the individual effect of each drug. It means that the combination gives a greater effect compared to a single drug because the primary and secondary antioxidants are working together to inhibit free radicals. Meanwhile, thiols are reacted as good chain transfer agents because of the high reactivity of the thiyl radicals and low bond dissociation energy of S-H bond.[38–40] The conjugated system of aromatic thiols that provided strong π electron attractor and produced stable radical cations rather than aliphatic thiols (Figure 1).[41]

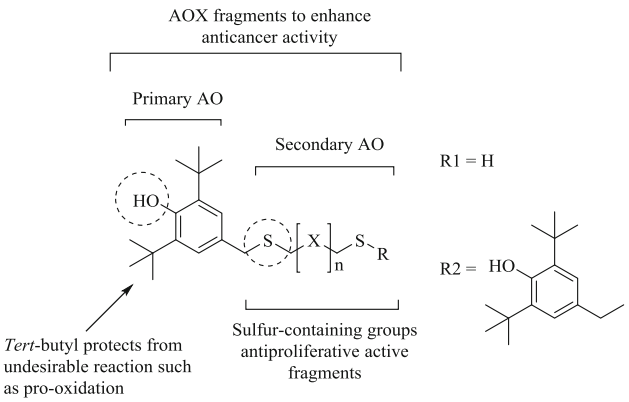
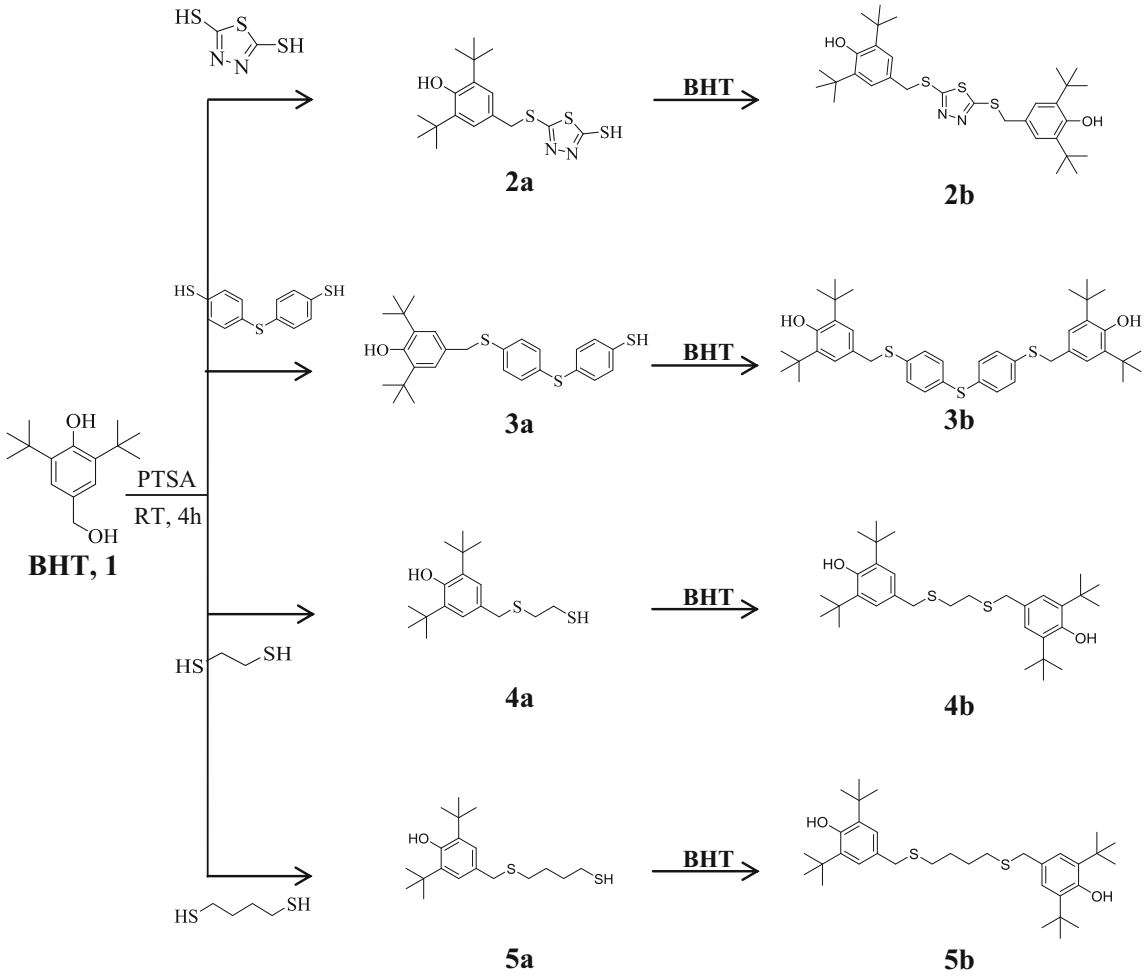


Figure 1. Rational design of sulfur-containing BHT (S-BHT).

3.2 *Chemistry*

The BHT (**1**) was modifying without altering the structure of phenolic ring to increase the antioxidant activity and cytotoxicity effect. The synthesis was very straightforward by the addition of thiols group at the para position of phenolic ring leads to the formation of high-efficiency antioxidant compounds. The presence of free *v*(Ar-O-H) was indicated by the strong absorptions in IR spectra at 3500–3600 cm^{-1} for all synthesized compounds. The absorption at 2860–2960 cm^{-1} indicated the strong stretching of C-H of phenol. The stereochemistry of some compounds to stabilize the structure makes *v*(S-H) band at 2570 cm^{-1} difficult to detect in IR spectra. NMR spectra feature in the ^{1}H-NMR spectrum for synthesized compounds can be differentiated from the appearance of peak region either on the upfield or downfield regions. Most of the aliphatic thiols appear at the upfield region due to the low frequency of proton. On the other hand, aromatic proton appears at the downfield region due to deshielding effect of high electron density on the aromatic ring. The features for ^{13}C-NMR also have similar characteristics with ^{1}H-NMR spectrum. The appearance at the downfield region is also due to the effect of the electronegative element of the adjacent atom.

In compound **2a**, the presence of hydroxyl group phenol was indicated by the intense absorption signal at 3400 cm^{-1}. Moreover, the regions 2860 to 2960 cm^{-1} indicated the stretching characteristic of alkane, C-H absorption peaks of methyl (CH_3) group. Meanwhile, the absorption peak at 1429 cm^{-1} showed the appearance of aromatic groups of compound **2a**. In ^{1}H NMR of compound **2a**, there is no peak representing thiadiazole because it has no proton in the ring structure. Most of them are electronegative elements and quaternary carbons. The formation of small singlet peak at 2.29 ppm was attributed to terminal thiol, -SH in compound **2a**. Moreover, ^{13}C NMR of compound **2a** confirmed the proposed structure due to the most deshielded carbon is C-11 of thiadiazole appeared at



Scheme 1. Synthesis of BHT-derivatives.

very downfield region 188.79 ppm. On the other hand, compound **2b** was prepared by the reaction of compound **2a** with BHT (**1**) for the formation of dimerization of compound **2a** (Scheme 1). The confirmation of structure **2b** was supported with ^{1}H NMR where the *tert*-butyl moiety appeared at 1.45 and 1.46 ppm. This indicates the presence of BHT (**1**) at both side of the compound and gives the difference resonance effect.

The compound **3a** was synthesized by the reaction of BHT (**1**) with 4,4′-thiobisbenzenethiol. The strong IR signal of aromatic appeared at 1474 cm^{-1} corresponded to bisbenzenethiol. Furthermore, the multiplet signals at 7.01, 7.31, 7.32, 7.19, 7.29, 7.20, 7.22 and 7.30 ppm indicated eight protons of bisbenzenethiol in ^{1}H NMR of compound **3a**. In ^{13}C NMR, most of the aromatic carbons appeared at region 125 to 140 ppm, due to steric hindrance of the benzene ring. The compound **3b** was prepared by the same method used to prepare compound **2b**. The appearance of hydroxyl group peaks at 6.88 ppm and 6.89 ppm showed the dimerization of compound **1** in compound **3b**. The presence of aromatic protons was indicated by the abundance peaks appeared at region 7.0 to 7.5 ppm in ^{1}H NMR due to steric hindrance of the benzene ring. This is also supported the presence of aromatic carbons by ^{13}C NMR data at region 125 to 145 ppm.

The reaction between 1,2-ethanedithiol and 1,4-butanedithiol with compound **1** yielded the compound **4a** and **5a**, respectively. The structure of these compounds was established by their spectral data. The ^{1}H-NMR spectra were revealed the presence of methylene proton of aliphatic thiol by the detection of abundant peaks resonated at region 1.5 to 2.5 ppm. The ^{13}C NMR of compounds **4a** and **5a** confirmed the suggested structure due to the appearance of abundance peaks at stable DPPH molecules 30 to 40 ppm. The dimerization of these two compounds was prepared by the reaction with compound **1** afforded compound **4b** and **5b**, respectively. Both ^{1}H NMR and ^{13}C NMR results indicated that the abundance peaks of aromatic carbons appeared at region 125 to 145 ppm due to

steric hindrance of benzene ring from both sides of the proposed structure.

3.3 *Antioxidant activity*

The radical scavenging effect of synthesized compounds was evaluated by *in vitro* DPPH assay. The protonation of a hydrogen atom to form stable DPPH molecules due to the hydrogen-donating capacity of the compound and the corresponding decrease in absorbance was measured at 517 nm. It was indicated by the colour change from purple to yellow.[42,43]

Table 1 shows the free radical scavenging activity of synthesized MPAOs with their percentage change of IC_{50} compared to standard BHT. All compounds showed more potent antioxidant activity than standard BHT with lower and decreasing percentage change of IC_{50} except for compounds **4a** and **5a** which displayed moderate activity against DPPH-radical. Compound **2b** demonstrated the good antioxidant activity with IC_{50} value of 12.1 ± 0.20 μM, decreased 64.6% of IC_{50} value compared to standard BHT, followed by compound **3b** decreased 46.1% with IC_{50} value of 18.4 ± 0.21 μM. Such major enhancement reflects the dual scavenging activity exerted by two BHT moieties attached at both sides of terminal thiols of these compounds.[44,45] Accordingly, all '**b**' compounds having two BHT moieties exerted stronger DPPH radical inhibition than their one-side '**a**' counterpart structures (Scheme 2).

Scheme 2 shows the mechanism of dimerization of BHT as antioxidant scavenging compound against free radical. The hydroxyl moiety act as proton donator to neutralize peroxyls radical to non-radical species. The presence of two *tert*-butyl moiety at *ortho* position was minimized undesirable reactions such as pro-oxidation by provided enough sterical effect to stabilized the unreactive phenoxyl radical. Then, the phenoxyl radical was stabilized by hyperconjugative and inductive effect from the delocation of electron inside the aromatic ring of BHT.

The free radical scavenging activity of the different synthesized S-BHT was in the following order: **2b** > **3b** > **2a** > **3a** > **4b** > **5b** > BHT> **4a** > **5a**. The presence of thiadiazole at compound **2b** structure as well as biaryl sulfide at compound **3b** structure exhibited the highest potential as a radical scavenger as shown in Schemes 3 and 4, respectively.

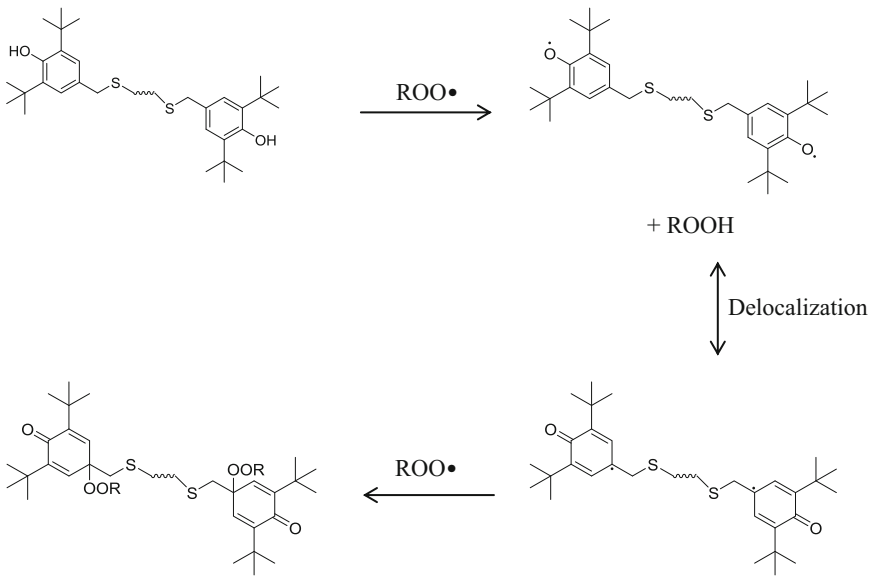
Heterocycles bearing a symmetrical thiadiazole and biaryl sulfide have a strong effect on antioxidant activity of five and six-membered heterocyclic compounds, respectively.[43,46] Scheme 3 showed the delocalization of thiadiazole in order to produce the stable ring with antioxidant ability. While Scheme 4 showed the delocatization of biaryl sulfide inside the benzene ring to stabilize its ring after inhibit free radical. Thiadiazole and biaryl sulfide derivatives have significant activity in free radical scavenging due to their character as a favorable electron donor. The antioxidant activity of thiol-containing compound was increased by the correlation between thiol and aromatic ring. The presence of aromatic ring supports the stability of self-radical of thiol by delocalization of electron inside the ring.

Secondary antioxidant does not react as radical scavengers but prevents oxidation by retarding chain initiation to form non-radical stable products.[4] Scheme 5 shows the mechanism of thioether bridge as a secondary antioxidant. Thioether prevents the radical effect by changing the hydroperoxides into alcohol and it is transformed into sulfoxides and sulfone. The combination of thioether with free radical scavenging

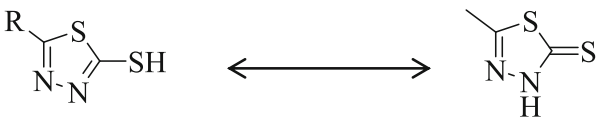
Table 1. The IC_{50} values of compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b** against DPPH radical.

Comp. no	Scavenging activity DPPH (IC_{50} μM)	% Change of IC_{50} compare to BHT
2a	18.5 ± 0.35	− 45.9%
2b	12.1 ± 0.20	− 64.6 %
3a	19.8 ± 0.26	− 42.1 %
3b	18.4 ± 0.21	− 46.1%
4a	35.1 ± 0.25	2.63 %
4b	20.1 ± 0.32	− 41.2 %
5a	42.2 ± 0.26	23.3 %
5b	21.0 ± 0.29	− 38.5 %
BHT	34.2 ± 0.31	

Each value represents the mean ± standard deviation of triplicates.



Scheme 2. The mechanism of di-substituent BHT against free radical.



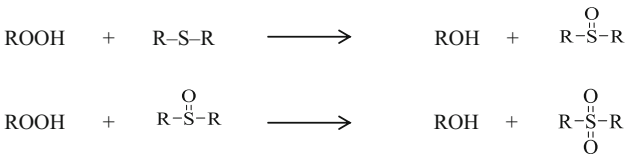
Scheme 3. Delocatization of thiadiazole.

Scheme 4. Delocatization of biaryl sulfide.

(primary antioxidant) provides the synergistic inhibition effect to improve the primary antioxidant ability.

3.4 *Cytotoxic activity*

BHT the precursor of synthesized compounds itself was proven to own cancer cytotoxic and apoptotic induction properties, as it possesses a phenoxyl radical.[47] The anticancer activity of all compounds was evaluated against breast and colon cancer, representing the two most commonly encountered cancer cases in Malaysia [National cancer registry report, Malaysia, 2007–2011]. Compounds were screened for cytotoxicity using a well-established MTT assay for 24 h at different concentrations ranging from 12.5 to 400 µM. For comparison, BHT was evaluated along with standard anticancer chemotherapeutic drugs, commonly used in breast and colon cancer treatment regimens, which were tamoxifen (TMX) and 5-fluorouracil (5-FU), respectively.



Scheme 5. The mechanism of thioether as secondary antioxidant.

All synthesized S-BHT compounds displayed cancer cytotoxic activity in a dose-dependent manner, as shown in Figures 2 and 3. However, compounds **2a** and **2b** stand out with remarkable potentiality in cancer treatment field. Table 2 lists IC_{50} values obtained in both colon and breast tissues along with the respective SI. Compound **2a** exhibited powerful cancer inhibition in both colon and breast tissues with IC50 values of 85.7 ± 7.04 and 106.2 ± 2.33 µM, respectively. Selectivity index (SI) providing a critical indicator for the anti-cancer agent efficacy, was evaluated as well in this study using normal human colon cell line (CCD-841) and non-tumorigenic human breast cells (MCF-10A). SI as displayed in Table 2, shows that compound **2a** scored 2.32 in breast tissue, passing the acceptable threshold for anti-cancer agents.[32] The selectivity achieved using **2a** couldn't be obtained using standard chemotherapy Tamoxifen (SI = 1.00).[32,48–50] This highlights the efficiency of synthesized compounds in cancer treatment.

On the other hand, compound **2b**, that is structurally similar to **2a**, displayed profound anti-cancer effect along with eminent SI scores, designating it as the best selective anti-cancer agent among all tested S-BHT synthesized in this current study. **2b** has inhibited both

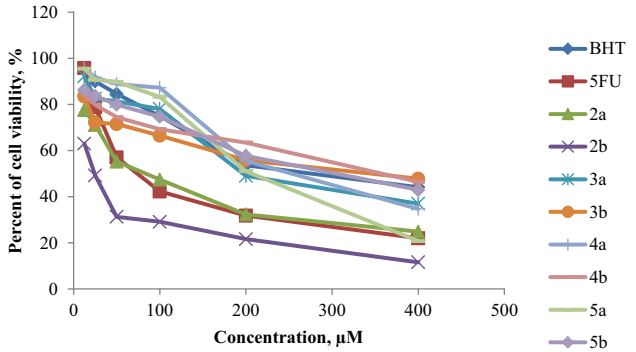


Figure 2. The percentage viability of HT29 cells treated with different concentration of compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b**.

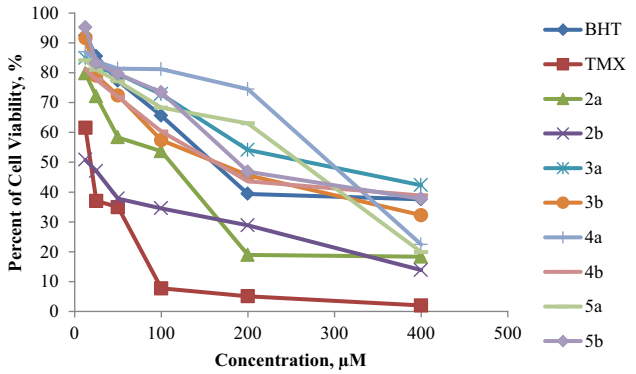


Figure 3. The percentage viability of MCF7 cells treated with different concentration of compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b**.

colon and breast cancer cells with IC50 of 33.0 ± 1.88 and 16.5 ± 1.03 µM respectively, surpassing control chemotherapy drugs commonly used in these cancer treatment regimens 5-FU[51,52] and Tmx.[32,50] Moreover, the outstanding SI of 5.2 and 12.5 in colon and breast tissues further ascertain the efficiency of this compound as a noteworthy anti-cancer agent.

The increased efficiency observed in **2b** is presumably due to the dimerization of BHT at both sides of thiols moiety as shown in Scheme 2, compared to **2a**. The structure-activity relationship showed that the aliphatic thiol gives less activity compared to aromatic thiol due to no delocalization of electron along the straight carbon chain. These results also show that the presence of thiadiazole in S-BHT is important for antioxidant and cytotoxic effects against both tested cancer cell lines compared to two standard drugs, 5-FU and TMX. Based on the results, the anticancer effect shows the correlation with antioxidant activity.

4. Conclusions

In summary, a series of sulfur-containing BHT were successfully designed and synthesized, and their antioxidant activity and cytotoxicity effect against human HT29 and MCF7 cancer cell lines were evaluated by DPPH and MTT assays, respectively. A majority of compounds in this study showed potent free radical scavenging activity, where six compounds scored lower IC_{50} than standard BHT, as measured by DPPH assay. A dose-dependent growth inhibition was obtained for all compounds on colon and breast cancer cells. Furthermore, the anti-cancer activity was demonstrated to correspond with antioxidant activity sharing similar order (**2b** > **2a**). Such trend reveals the potency of "**b**" compounds having two BHT moieties acting synergistically and producing better output than their one-side "**a**" counterparts. These interesting and promising results encourage further development of

Table 2. The IC_{50} values of compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b** against HT29 and MCF7.

	IC_{50} µM					
Comp. no	HT29	CCD	SI	MCF7	MCF10	SI
2a	85.7 ± 7.04	153.3 ± 8.34	1.78	106.2 ± 2.33	246.8 ± 7.36	2.32
2b	33.0 ± 1.88	171.7 ± 2.19	5.20	16.5 ± 1.03	212.1 ± 6.15	12.50
3a	261.0 ± 16.32	> 400	ND	350.8 ± 2.00	325.0 ± 0.83	1.00
3b	281.2 ± 18.28	338.4 ± 10.05	1.20	181.0 ± 9.27	257.0 ± 19.24	1.40
4a	338.7 ± 6.56	214.0 ± 8.65	0.78	284.4 ± 6.83	214.2 ± 9.08	0.75
4b	338.7 ± 5.91	310.8 ± 18.23	0.94	169.3 ± 7.65	276.1 ± 14.79	1.63
5a	209.6 ± 15.69	275.3 ± 9.62	1.31	241.9 ± 19.81	223.5 ± 13.75	0.92
5b	302.1 ± 16.49	312.5 ± 14.98	1.03	253.2 ± 17.84	227.9 ± 6.79	0.90
TMX	–	–	–	18.3 ± 0.61	18.3 ± 1.42	1.00
5-FU	75.2 ± 1.01	161.6 ± 4.55	2.16	–	–	–
BHT	366.6 ± 15.76	304.2 ± 12.7	0.83	146.7 ± 14.9	214.1 ± 10.6	1.46

Each value represents the mean ± standard error of triplicates.

TMX tamoxifen; *5-FU* 5-fluorouracil, *BHT* butylated hydroxytoluene, *SI* Selectivity Index, *ND* not determined.

synthesized compounds into selective *in vivo* anti-cancer drugs field, with the special attention needed for their mechanism of action studies.

Supplementary Information (SI)

The characterization of the compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b** are given in the supplementary information. Supplementary information is available at www.ias.ac.in/chemsci.

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