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Synthesis, characterizations, biological activities and docking studies of novel dihydroxy derivatives of natural phenolic monoterpenoids containing azomethine linkage

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Abstract In the present work, we report the synthesis of six new azomethine linkage containing dihydroxy derivatives of carvacrol, thymol, and eugenol. All the synthesized derivatives have been characterized by spectroscopic techniques and their structures were confirmed by X-ray single crystallography. Synthesized derivatives were screened for anti-oxidant activity using DPPH radical scavenging assay, and anticancer activity by using SRB assay against pancreatic cancer with MIAPaCa-2 and colon cancer with HCT-15 cell lines. The molecular docking studies of all the synthesized derivatives were performed on cyclooxygenases (COX-2) protein enzyme. In the anti-oxidant test, the values of EC_{50} indicated that all the compounds show excellent anti-oxidant potency, and similarly the GI_{50} values in anticancer tests indicated that most of the compounds possess good anticancer efficacy. The overall docking score suggested that all the synthesized compounds exhibit good binding affinity towards cyclooxygenases (COX-2) protein enzyme.

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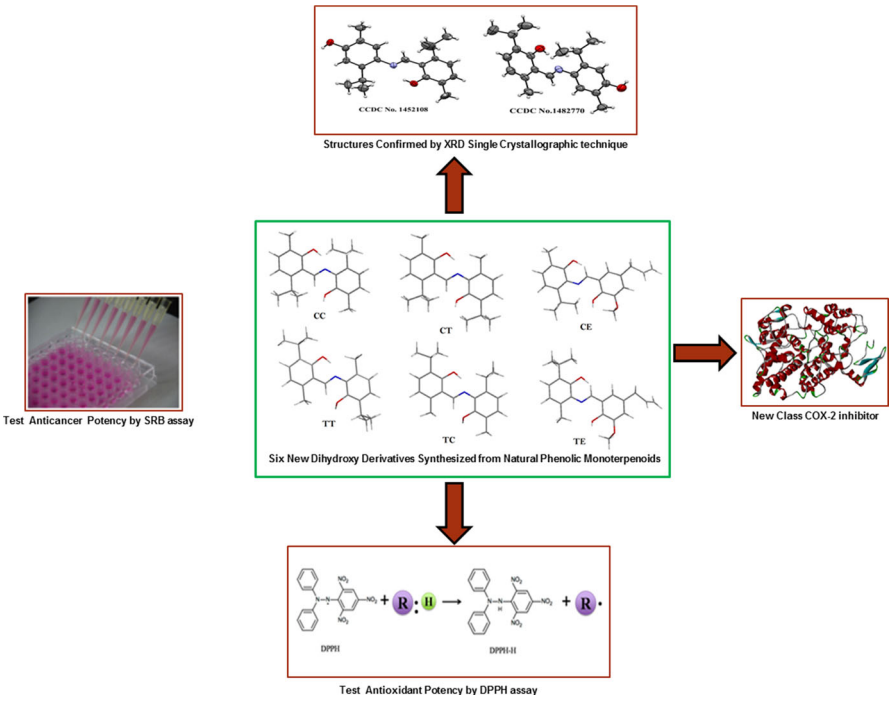
Graphical Abstract



Keywords Phenolic monoterpenoids · COX-2 · Molecular docking · Biologically active scaffolds · Anticancer · Anti-oxidant

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in a number of biological processes, such as degenerative, carcinogenesis, mutation and other diseases, inflammation, development, and aging [1–5]. Both ROS and RNS are well documented for performing a double role as both injurious and beneficial species [6, 7]. Free radicals can destroy DNA and cause mutagenicity and cytotoxicity, and thus perform a significant role in carcinogenesis. It is believed that ROS can induce mutations and inhibit the DNA repair process that results in the activation of certain tumor repressors, leading to cancer [8, 9]. To counterbalance such adverse effects, there is a demand for natural and synthetic anti-oxidants to support the endogenous defenses [10]. Anti-oxidants must react with radicals and other reactive species more quickly than the biological entity, thus protecting biological targets from oxidative injury [11, 12]. In addition, the ensuing anti-oxidant radical must possess excessive stability, that is, the anti-oxidant radical must disturb a chain reaction [13, 14]. Recently, research has shown that cancer cells

produce higher levels of ROS to help feed their abnormal growth [15]. In laboratory and animal studies, the presence of increased levels of exogenous anti-oxidants has been shown to prevent the types of free radical damage that have been associated with cancer development [16, 17].

The literature has suggested that phenolic compounds usually have probable anti-oxidant and anticancer activity due to phenolic hydroxyl groups which act as a proton donor and are responsible for a redox-type mechanism [18, 19]. Mastelic et al. reported anti-oxidant activity of four dihydroxy derivatives of carvacrol, thymol and eugenol for the first time [**1–4**], concluding that the results of DPPH and Rancimat assay demonstrated that all synthesized derivatives possess remarkable anti-oxidative properties as well as antiproliferative effects against human uterine carcinoma cells (HeLa) [20].

Carvacrol, thymol and eugenol are present in the essential oils of several plants [21, 22]. These naturally occurring phenolic monoterpenoids are outstanding creative molecules incorporated as useful constituents in various products and have found wide applications in pharmaceutical, agricultural, fragrance, flavor, cosmetic and various other industries [23, 24]. Their vast range of pharmacological actions including antimicrobial, anti-inflammatory, analgesic, anti-oxidant and anticancer activities have been well researched [25, 26] (Fig. 1).

In the present study, we report six new dihydroxy derivatives of carvacrol, thymol, and eugenol containing azomethine functionality which possess excellent anti-oxidant and good anticancer activities. Docking simulations of all the derivatives was performed on the COX-2 protein enzyme which is responsible for undeclared changes in biochemical processes.

Experimental

All the chemicals and reagents necessary for the reactions were procured from Sigma-Aldrich and Fisher scientific chemicals with purity 98% and used without further purification. The products were characterized using ^{1}H NMR, ^{13}C NMR, IR, LC–MS spectra and X-ray single crystallography. NMR spectra of the products were obtained using Bruker AC-400 MHz spectrometers with TMS as the internal standard. Mass spectra of the products were obtained using Waters 2795 micromass Q-TOF micro LC–MS.

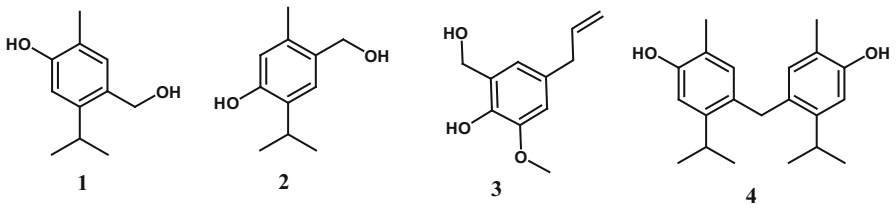


Fig. 1 Reported dihydroxy derivatives of carvacrol, thymol and eugenol

X-ray single crystallographic analysis

Appropriate crystals were selected and mounted on a Bruker APEX-II CCD diffractometer. During data collection, the crystal was kept at 296.15 K. The structure was solved using Olex2 [32] with the ShelXS [33] structure solution program by Direct Methods and refined with the ShelXL [34] refinement package using Least Squares minimization. The crystal structures of **IIA** and **IIB** with atomic numbering are presented in Fig. 2. Both the crystal structures show monoclinic crystal system. The 3D packing diagram of anhydrous carvacryl thymol (**IIA**) and carvacryl carvacrol (**IIB**) are shown in Fig. 3. Hydrogen bonding present in all the derivatives are observable in NMR which has been confirmed by X-ray single crystallography. Figure 4 showing the intermolecular and intramolecular hydrogen bonding in **IIA** and **IIB**. The presented structures illustrate the formation of carvacryl thymol (**IIA**) and carvacryl carvacrol (**IIB**) upon the condensation of respective ortho formyl phenolic monoterpenoids with para amino phenolic monoterpenoids. The crystal structures indicated that the two-fold dimeric structures of azomethine scaffolds of phenolic monoterpenoids are connected through the nitrogens of para amino phenolic monoterpenoids and formyl functionality of phenolic monoterpenoids. The CCDC No. for carvacryl carvacrol (CC) is 1452108 and for carvacryl thymol (CT) is 1482770.

Synthesis of ortho formylation on phenolic monoterpenes [35]

NaOH (6.66 g, 0.16 M) in (7 ml) distilled water was placed in a round-bottom flask fitted with a water reflux condenser and a thermometer, to which phenolic monoterpene (0.02 mol) was added. The temperature inside the flask was adjusted to 60–65 °C. Chloroform (0.04 mol) was introduced in small portions with stirring by maintaining the temperature at 60–65 °C during addition. The mixture was stirred for 2 h. Excess of chloroform was removed by steam distillation from the alkaline solution. The solution was allowed to cool and was then acidified with dilute HCl (1%). The acidified solution was steam-distilled until no more oily drops

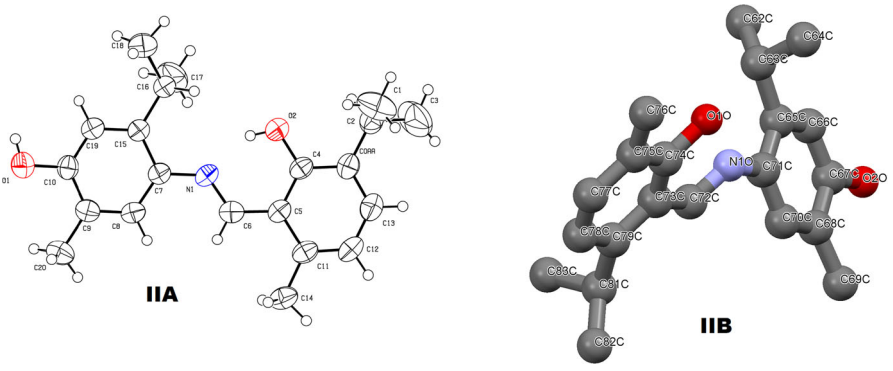


Fig. 2 ORTEPs representing 50% probability ellipsoids of **a** CT (**IIA**) and **b** CC (**IIB**)

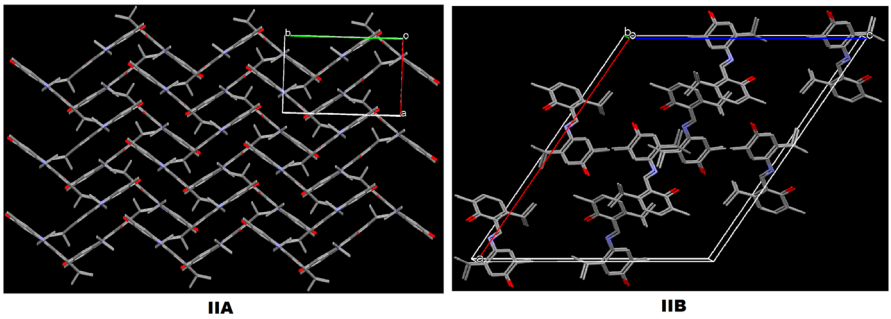


Fig. 3 Packing diagram of **a** form **IIA**, space group P21/n, and **b** form **IIB**, space group Cc

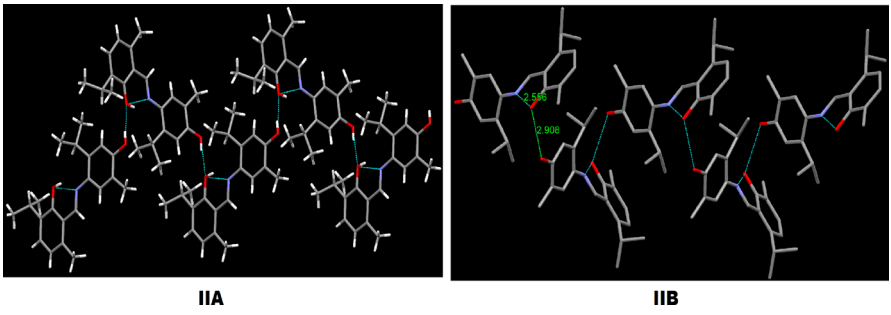


Fig. 4 Existence of intermolecular and intramolecular hydrogen bonding in **IIA** and **IIB**

were collected. The distillate was extracted with ether (3 × 30 ml). Most of the ether was removed by distillation. The residue, which contains unreacted phenolic monoterpene and ortho formylated phenolic monoterpene, was transferred to a small glass-stoppered flask to which about twice the volume of saturated sodium metabisulfite solution was added. The solution was stirred vigoursly for 30 min and allowed to stand for 1 h. The paste of the bisulfite compound was filtered and washed with a little ethanol and finally with a little ether. The bisulfite compound was decomposed by warming in a round-bottom flask on a water bath with dilute H_2SO_4. The mixture was allowed to cool and then the ortho formylated phenolic monoterpene was extracted with ether (3 × 30 ml). Removal of the ether gave pure ortho formylated phenolic monoterpene. Finally, all three ortho formyl phenolic monoterpenes were characterized by spectroscopic methods with yields of carvacrol aldehyde 70%, thymol aldehyde 70% and eugenol aldehyde 75%.

Synthesis of 4-nitroso thymol and 4-nitroso carvacrol [36]

To a solution of carvacrol or thymol (3.00 g, 0.02 mol) in 95% ethyl alcohol (20 ml), concentrated hydrochloric acid (20 ml) was added. The mixture was cooled to 0–5 °C and sodium nitrite (1.08 g, 0.015 mol) was continuously added slowly in small lots in 1 h. The mixture was stirred well after each addition. It became brown in colour and a green precipitate soon began to form. After 0.5 g of nitrite had been

added, the mixture became pasty, so the intervals between the additions were lengthened and the stirring was made more vigorous. When all the nitrite had been added, the bulk of the product was transferred to a 250-ml beaker containing 120 ml of cold water and the remainder was washed with water. After agitation with water, the product became a light yellow, fluffy solid. It was filtered off by suction, washed well with water and crystallized from ethanol with yields for both 4-nitroso carvacrol and thymol of 75%.

Synthesis of 4-amino thymol and 4-amino carvacrol [37]

The crude wet 4-nitrosocarvacrol (4-nitrosothymol) (3.1 g, 0.0174 mol) from the second step was dissolved in a mixture of 28% aqueous ammonia (30 ml) and water (50 ml). The brown solution thus obtained was filtered to remove resinous matter and hydrogen sulfide was passed into it. The brown colour disappeared and a white precipitate of 4-aminocarvacrol (4-aminothymol) was obtained, with a yield of 82%. Due to less stability, the product was used for the next step without purification.

Synthesis of Azomethine Scaffolds of thymol, carvacrol and eugenol [38]

The Schiff bases were prepared by the reaction of equimolar quantities of 4-amino thymol (or 4-amino carvacrol) and ortho formyl thymol (or ortho formyl carvacrol or ortho formyl eugenol). Each reactant was dissolved in a minimum amount of ethanol, and they were mixed together. The reaction mixture was refluxed for 4 h, then cooled to room temperature and poured into ice-cold water. The solid product obtained was collected by filtration and dried in an oven at 70 °C. The product was recrystallized from ethanol and dried to obtain pure product.

Spectroscopic characterizations and physical properties of synthesized derivatives

(*E*)-4-(2-hydroxy-6-isopropyl-3-methylbenzylideneamino)-5-isopropyl-2-methylphenol (CC) Color light red mp-118 °C, Yield: 68%, ^{1}H NMR (DMSO) δ 1.21 (d, $J = 4.0$ Hz, 6H), δ 1.27 (d, $J = 4.0$ Hz, 6H), 2.15–2.18 (s, 6H), 3.33–3.34 (m, $J = 4.0$ Hz, 1H), 3.58–3.62 (m, $J = 4.0$ Hz, 1H), 6.71–6.73 (d, $J = 8.0$ Hz, 1H), 6.79 (s, 1H), 7.04 (s, 1H), 7.15–7.17 (d, $J = 8.0$ Hz, 1H), 9.03 (s, 1H for OH), 9.26 (s, 1H for imine), 14.98 (s, 1H for OH hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 15.30, 15.57, 23.01,23.90, 27.12, 27.50, 78.4, 78.76, 79.43, 111.51, 114.30, 114.83, 120.09, 122.34, 122.53, 133.47, 137.13, 140.47, 147.28, 154.98, 157.13, 159.84. LC–MS (methanol), *m/z*: $[M + 1]^+$ 326.57, $[M + 2]^+$ 327.37.

(*E*)-4-(2-hydroxy-3-isopropyl-6-methylbenzylideneamino)-5-isopropyl-2-methylphenol (CT) Color light red, mp-112 °C, Yield: 66%, ^{1}H NMR (DMSO) δ 1.21 (d, $J = 4.0$ Hz, 6H), δ 1.24 (d, $J = 4.0$ Hz, 6H), 2.16–2.26 (s, 6H), 3.61–3.62 (m, $J = 4.0$ Hz, 1H), 3.65–3.67 (m, $J = 4.0$ Hz, 1H), 6.71–6.74 (d, Hz, 2H), 7.17–7.19 (d, Hz, 2H), 9.07 (s, 1H for OH), 9.43 (s, 1H for imine), 15.22 (s, 1H for OH

hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 15.33, 17.62, 22.36, 23.88, 26.73, 27.03, 27.50, 114.36, 114.85, 115.60, 116.76, 122.59, 130.16, 133.04, 138.00, 147.42, 153.77, 153.77, 156.68, 160.09. LC–MS (methanol), *m/z*: $[M + 1]^+$ 326.57, $[M + 2]^+$ 327.37.

(*E*)-4-(5-allyl-2-hydroxy-3-methoxybenzylideneamino)-5-isopropyl-2-methylphenol (CE) Color orange, mp-118 °C, Yield: 70%, ^{1}H NMR (DMSO) δ 1.21 (d, $J = 4.0$ Hz, 6H), 2.27 (s, 3H), 3.39-3.40 (m, $J = 4.0$ Hz, 1H), 6.71–6.73 (d, $J = 8.0$ Hz, 1H), 3.33–3.35 (d, 2H), 3.84 (s, 3H), 5.05 (dd 2H), 5.92–5.98 (m 1H), 6.71 (s, 1H), 7.84 (s, 1H), 6.98 (s, 1H), 7.17 (s, 1H), 8.77 (s, 1H for OH), 9.26 (s, 1H for imine), 14.04 (s, 1H for OH hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 17.49, 22.10, 26.38, 55.67, 78.35, 78.68, 76.01, 114.70, 114.95, 152.52, 116.66, 118.91, 122.79, 129.29, 130.35, 133.09, 137.31, 137.39, 153.78, 158.65. LC–MS (methanol), *m/z*: $[M + 1]^+$ 340.55, $[M + 2]^+$ 341.58.

(*E*)-4-(2-hydroxy-3-isopropyl-6-methylbenzylideneamino)-2-isopropyl-5-methylphenol (TT) Color red, mp-116 °C, Yield: 70%, ^{1}H NMR (DMSO) δ 1.18 (d, $J = 4.0$ Hz, 6H), δ 1.22 (d, $J = 4.0$ Hz, 6H), 2.27 (s, 3H), 2.51 (s, 3H), 3.20–3.22 (m, $J = 4.0$ Hz, 1H), 3.31–3.32 (m, $J = 4.0$ Hz, 1H), 6.63–6.65 (d, $J = 8.0$ Hz, 1H), 6.72 (s, 1H), 7.11 (s, 1H), 7.12 (d, $J = 8.0$ Hz, 1H), 8.92 (s, 1H for OH), 9.30 (s, 1H for imine), 14.98 (s, 1H for OH hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 17.15, 18.50, 22.28, 22.36, 25.67, 26.64, 78.49, 78.82, 79.15, 115.42, 116.42, 116.70, 120.01, 128.75, 130.04, 133.01, 133.46, 136.54, 138.11, 153.68, 157.14, 159.14. LC–MS (methanol), *m/z*: $[M + 1]^+$ 326.57, $[M + 2]^+$ 327.37.

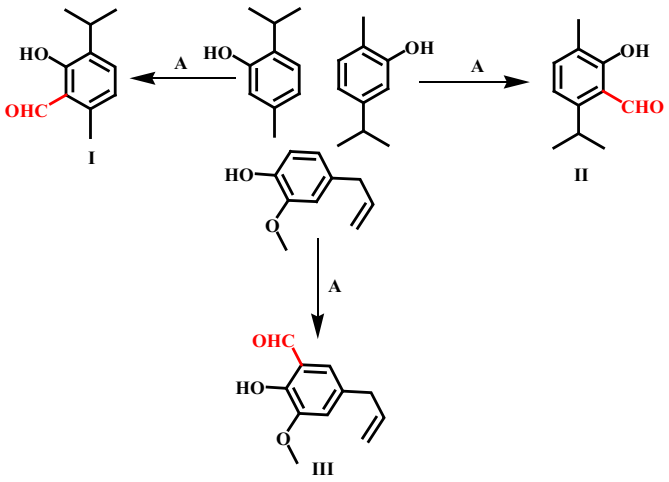
(*E*)-4-(2-hydroxy-6-isopropyl-3-methylbenzylideneamino)-2-isopropyl-5-methylphenol (TC) Color red, mp-112 °C, Yield: 68%, ^{1}H NMR (DMSO) δ 1.21 (d, $J = 4.0$ Hz, 6H), δ 1.24 (d, $J = 4.0$ Hz, 6H), 2.16–2.26 (s, 6H), 3.61–3.62 (m, $J = 4.0$ Hz, 1H), 3.65–3.67 (m, $J = 4.0$ Hz, 1H), 6.71–6.74 (d,8 Hz, 2H), 7.17–7.19 (d, 8 Hz, 2H), 9.07 (s, 1H for OH), 9.43 (s, 1H for imine), 15.22 (s, 1H for OH hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 15.33, 17.62, 22.36, 23.88, 26.73, 27.03, 27.50, 114.36, 114.85, 115.60, 116.76, 122.59, 130.16, 133.04, 138, 147.42, 153.77, 153.77, 156.68, 160.09. LC–MS (methanol), *m/z*: $[M + 1]^+$ 326.57, $[M + 2]^+$ 327.37.

(*E*)-4-(5-allyl-2-hydroxy-3-methoxybenzylideneamino)-2-isopropyl-5-methylphenol (TE) Color light orange, mp-120 °C, Yield: 70%, ^{1}H NMR (DMSO) δ 1.21 (d, $J = 4.0$ Hz, 6H), 2.27 (s, 3H), 3.39–3.40 (m, $J = 4.0$ Hz, 1H), 3.33 (d, 2H), 3.84 (s, 3H), 5.05 (dd 2H), 5.92–5.98 (m 1H), 6.71 (s, 1H), 7.84 (s, 1H), 6.98 (s, 1H), 7.17 (s, 1H), 8.77 (s, 1H for OH), 9.26 (s, 1H for imine), 14.04 (s, 1H for OH hydrogen bonded with nitrogen of imine), ^{13}C NMR (DMSO) δ 17.49, 22.10, 26.38, 55.67, 78.35, 78.68, 76.01, 114.70, 114.95, 152.52, 116.66, 118.91, 122.79, 129.29, 130.35, 133.09, 137.31, 137.39, 153.78, 158.65. LC–MS (methanol), *m/z*: $[M + 1]^+$ 340.55, $[M + 2]^+$ 341.58.

Results and discussion

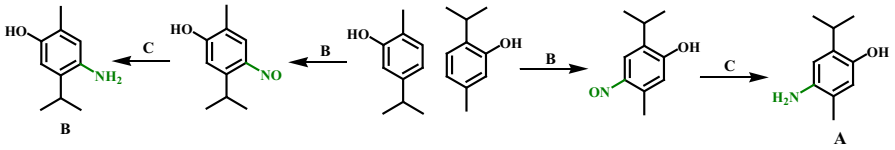
Reaction schemes

The synthesis of compounds in Schemes 1 and 2 involved a multi-step pathway leading to the formation of a variety of novel dihydroxy phenolic monoterpenoid-based azomethine scaffolds, **IA–IIIB** in excellent yields. The structures of the compounds obtained are shown in Scheme 3. Their analytical and spectroscopic data are in agreement with the predicted structures. Recently, we have reported number of derivatives of phenolic monoterpenes and their biological activities [27, 28]. Syntheses of ortho formyl derivative of (I, II and III) and 4-amino analogs (**A** and **B**) of phenolic monoterpenoids were carried out by reported methods. The reaction of ortho formyl phenolic monoterpenoids (I, II and III) and 4-amino phenolic monoterpenoids (**A** and **B**) in ethanol at 80 °C for 5 h resulted in the coupling products (**IA–IIIB**) in exceptional yields.



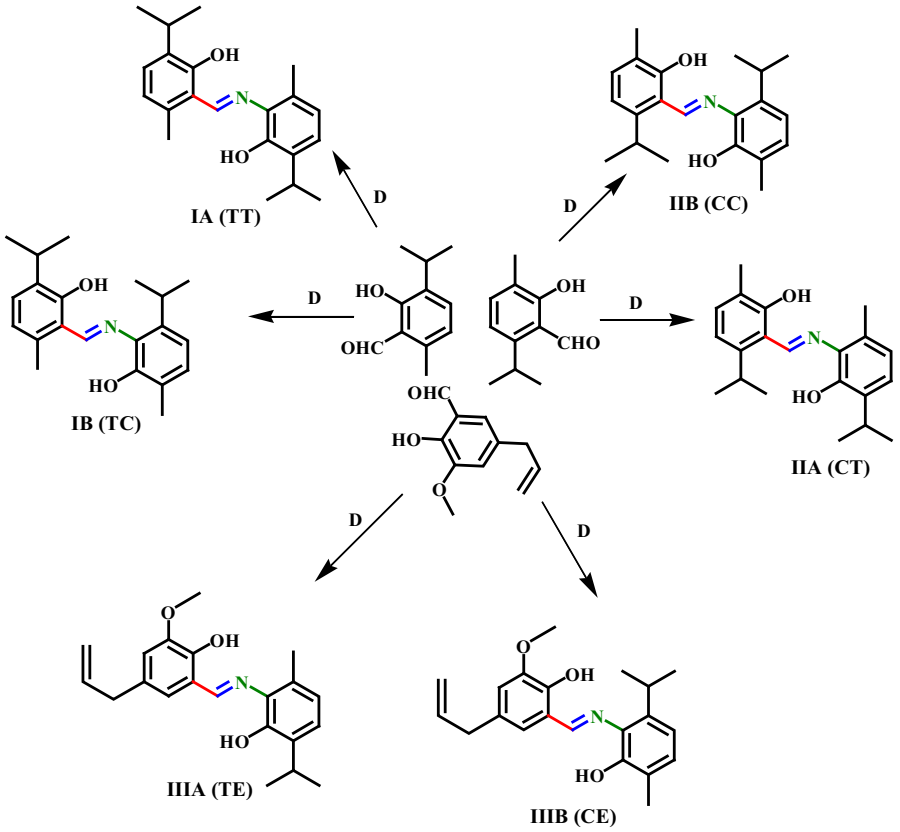
Reagents & Reaction conditions: A = $CHCl_3$, NaOH in water, reflux at 60-65 °C for 2h.

Scheme 1 Synthesis of ortho formyl phenolic monoterpenoids



Reagents & reaction conditions: B= Ethanol, $NaNO_2$ + HCl at 0-5 °C for 4h. **C=** H_2O, 28% NH_3 + H_2S for 3h.

Scheme 2 Synthesis of 4-amino phenolic monoterpenoids



Reaction Conditions: D= A or B in Ethanol, Reflux at 80 °C for 2h.

Scheme 3 Synthesis of phenolic monoterpenoids based azomethine scaffolds

Bioassay

Anticancer activity

In vitro anticancer activities of synthesized derivatives were performed using sulforhodamine B (SRB) assay on a panel of human cancer cell lines HCT-12 (colon cancer) and MIAPaCa-2 (pancreatic cancer) [29]. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For the present screening experiment, cells were inoculated into 96-well microtiter plates in 90 µl at 5000 cells per well. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of the experimental drugs, which were solubilized in the appropriate solvent to prepare a stock of 10^{-2} concentration. At the time of the experiment, four tenfold serial dilutions were made using complete medium.

Aliquots of 10 µl of these different drug dilutions were added to the appropriate micro-titer wells already containing 90 µl of medium, resulting in the required final drug concentrations. After addition of the compound, plates were incubated at standard conditions for 48 h and the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and the plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690-nm reference wavelength. The GI_{50} values, defined as the drug concentration required for inhibiting the growth of cell proliferation by 50%, were calculated from percent growth and were expressed as the ratio of average absorbance of the test well to the average absorbance of the control well $\times 100$. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration level and the data are presented in Figs. 5 and 6, and the results are expressed as GI_{50} (growth inhibitory concentration at 50%) in Table 1.

Cell growth inhibition was analyzed by SRB assay and the results show that the compounds **IA–IIIA** and **IB–IIIB** exhibit an inhibitory effect on the proliferation of HCT-15 and MIAPaCa-2 cells in a dose-dependent manner (Table 1). Compounds **IA** and **IIIA** were found to exhibit moderate cytotoxic potency (22.1 and 59.2 µg/ml) compared with that of Doxorubicin (10 µg/ml) against the HCT-15 cell. Similarly, the compounds **IA** and **IIA** displayed excellent cytotoxic potency (10.77 and 5.2 µg/ml) copmared with that of Doxorubicin (10 µg/ml) against MIAPaCa-2. Of note is that the amino carvacrol derivatives (**IB–IIIB**) presented better activity.

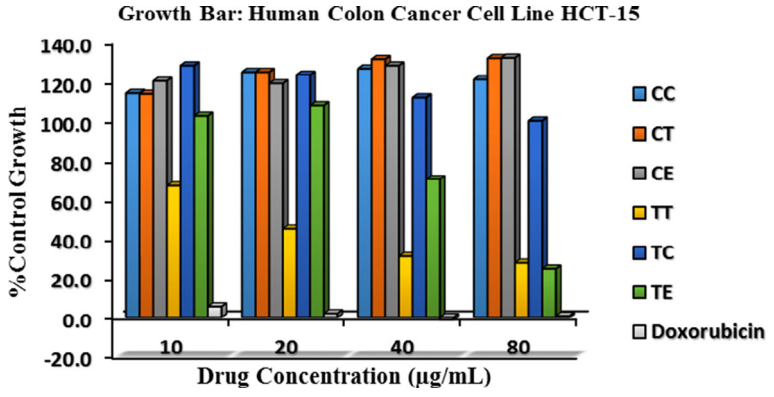


Fig. 5 Cytotoxicity effects of **IA–IIIA** and **IB–IIIB** against measured against Colon on HCT-15 cell line

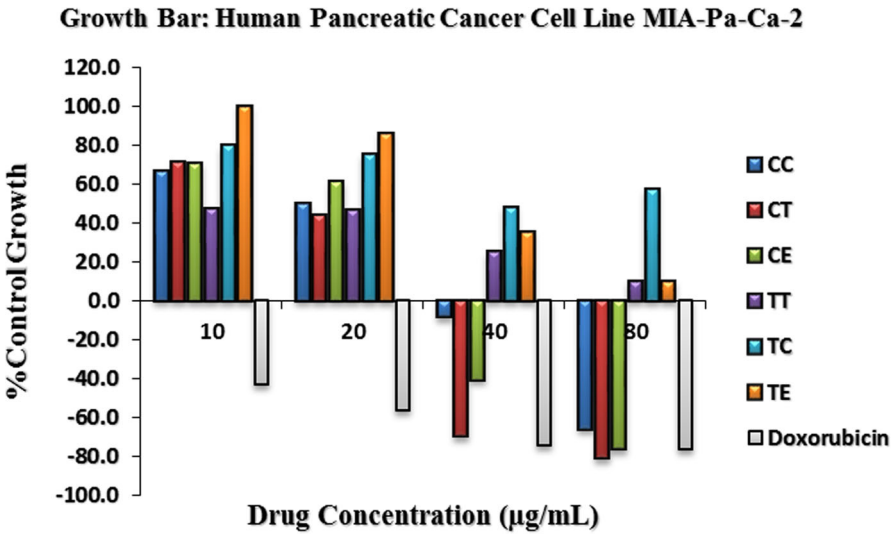


Fig. 6 Cytotoxicity effects of **IIIA** and **IB–IIIB** measured against Pancreatic on MIAPaCa-2 cell line

Table 1 EC_{50} and GI_{50} values in anti-oxidant and anticancer activities of compounds **IA–IIIA** and **IB–IIIB**

Sample no.	Name of derivatives	Anti-oxidant test EC_{50} in µg/ml	Anticancer test	
			Colon cancer GI_{50} in µg/ml	Pancreatic cancer GI_{50} in µg/ml
1.	Carvacryl carvacrol CC (**IIB**)	0.3906 ± 0.350	80	16.9
2.	Carvacryl thymol CT (**IIA**)	0.5188 ± 0.349	80	10.77
3.	Carvacryl eugenol CE (**IIIB**)	0.2316 ± 0.331	80	16.5
4.	Thymyl thymol TT (**IA**)	18.74 ± 0.214	22.1	5.8
5.	Thymyl carvacrol TC (**IB**)	0.1171 ± 0.542	80	82.5
6.	Thymyl eugenol TE (**IIIA**)	0.4136 ± 0.113	59.2	43.4
7.	STD	0.1203 ± 0.213	10	10
		Butylated hydroxy toluene (BHT)	Doxorubicin	Doxorubicin

Anti-oxidant activity

DPPH radical-scavenging activity was performed by the reported method [30]. For each determination, the stock solution (1 mg/ml) was diluted by serial dilution (25–500 µg/ml) with 60% (v/v) ethanol. An aliquot of each dilution (0.5 ml) was mixed with a methanolic solution of DPPH (5 ml, 0.06 mM). The mixtures were shaken vigorously and incubated at 37 °C in dthe ark for 30 min. At the same time,

a control containing 60% (v/v) ethanol (0.5 ml) and the methanolic solution of DPPH (5 ml, 0.06 mM) was run. The absorbance was measured at 517 nm. The percentage of DPPH scavenging versus concentration of samples was plotted. The concentration of the sample necessary to decrease the DPPH concentration by 50% was obtained by interpolation from linear regression analysis and denoted as the EC_{50} value (μg/ml). All determinations were carried out in triplicate. Butylated hydroxy toluene (BHT) was used as the reference compound. The % radical scavenging against concentration of entity has been represented in Fig. 7.

DPPH radical scavenging activity of the compounds was found to be good to outstanding as compared to the standard BHT. Derivatives **IB**, **IIIB**, **IIB**, **IIIA** and **IIA** showed outstanding EC_{50} values which are comparable with STD, while **IA** showed a decrease in % anti-oxidant activity with a higher EC_{50} value. The lowest activity was noted for IA at every concentration, perhaps due to the phenolic OH

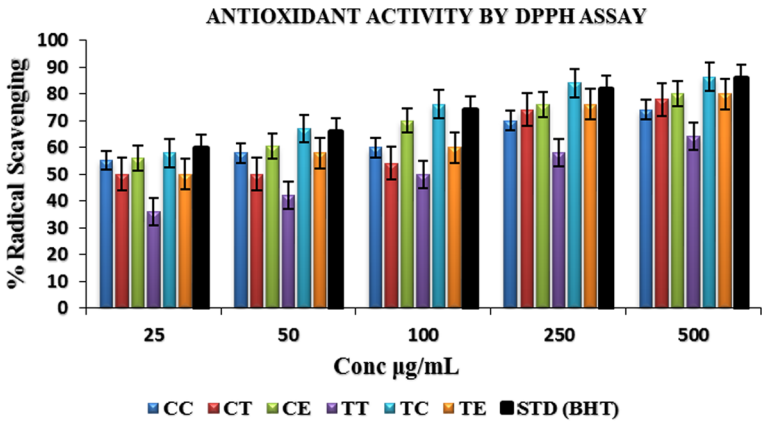


Fig. 7 Anti-oxidant activity of azomethine scaffolds (**IA–IIIB**) determined by the DPPH free radical method at various concentrations

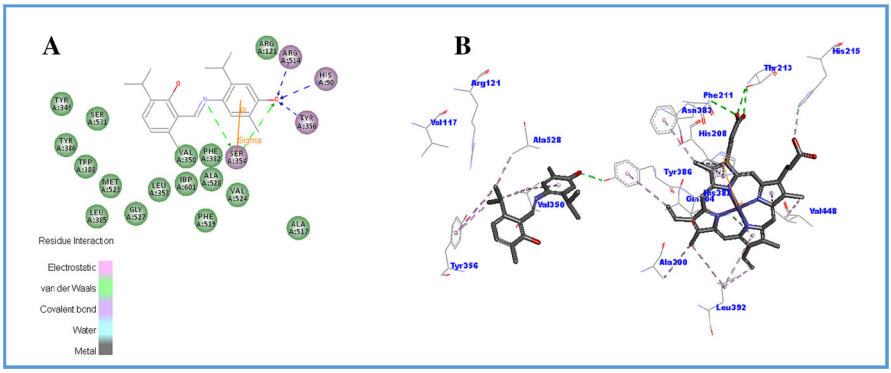


Fig. 8 Binding mode of inhibitors in active site of cyclooxygenases (4PH9). **a** 2D interaction of IB with 4PH9. **b** 3D binding of IIA with amino acids of cyclooxygenases (4PH9)

Table 2 Binding energies and entropies of protein, ligand and complex

Sample no.	Name	Binding energy	Complex energy	Protein energy	Ligand energy	Entropic energy	Complex entropy	Protein entropy	Ligand entropy
1.	TE	−29.87	−9153.19	−9222.35	39.29	19.92	−32.40	−32.40	−19.92
2.	CT	−46.80	−9116.38	−9222.35	59.17	19.68	−32.40	−32.40	−19.68
3.	CE	−8.28	−9172.86	−9222.35	41.22	19.86	−32.40	−32.40	−19.86
4.	TT	−13.53	−9182.75	−9222.35	26.07	19.73	−32.40	−32.40	−19.73
5.	CC	−23.10	−9160.73	−9222.35	38.52	19.65	−32.40	−32.40	−19.65
6.	TC	−16.46	−9170.57	−9222.35	35.32	19.69	−32.40	−32.40	−19.69
8.	Ibuprofen	−51.34	−9250.99	−9222.35	22.70	20.19	−32.41	−32.40	−20.18

Table 3 Docking score, steric score and structural parameter of synthesized derivatives

Sample no.	Name	Docking score	Steric score	Desolvation	Hydrogen bonding acceptor	Hydrogen bonding donor
1.	TE	−65.09	−66.29	8.79	−7.55	−0.03
2.	CT	−63.07	−67.19	5.66	−0.30	−1.23
3.	CE	−62.62	−69.62	8.20	−0.17	−1.03
4.	TT	−61.56	−62.90	6.50	−3.89	−1.26
5.	CC	−60.89	−62.91	6.18	−1.82	−2.34
6.	TC	−56.69	−60.05	6.30	−0.25	−2.70
7.	Ibuprofen	−81.28	−82.91	10.35	−8.72	0.00

present in the steric crowd. The anti-oxidant activity of the compounds is related to their electron or hydrogen radical releasing abilities towards DPPH, so that they become stable diamagnetic molecules. Among the series, IB showed the highest anti-oxidant activity with a remarkable EC_{50} value particularly lower in comparison with STD. All EC_{50} values are listed in Table 1.

Molecular docking studies

The docking study was performed by FRED (Open Eye.lnc.) and has been used to determine the orientation of inhibitors bound in the active site of cyclooxygenases (COX-2, PDB code: 4PH9). The PDB files were downloaded from the Protein Data Bank (www.rsc.org). The ligands were drawn in Chem Draw Ultra 12.0. The FRED docking programme was used to perform molecular docking [31]. The docking of ligand molecules with COX-2 indicated that all the inhibitor compounds exhibit bonding with more than one amino acids in the active pocket as shown in Fig. 8, and may be considered as good inhibitors of cyclooxygenases (COX-2). The study also revealed that the phenolic monoterpenoid-based scaffolds are attached to key residues, i.e. TYR386, ARG121, ARG514 and SER354 of the active site of cyclooxygenases (COX-2). Moreover, all the compounds possess the lowest binding energy and docking score as presented in Tables 2 and 3, and hence could be considered as a good inhibitors of cyclooxygenases (COX-2). The theoretical outcome highlighted that the minimum binding energy of the molecules with the targeted protein may make these newly synthesized phenolic monoterpenoid-based scaffolds good inhibitors of cyclooxygenases (COX-2). Therefore, it is striking that the docking studies have extended the scope of developing phenolic monoterpenoid derivatives as promising anti-oxidant and anticancer agents.

Conclusion

In conclusion, we have prepared novel dihydroxy analogues of phenolic monoterpenoids (**IA–IIIB**) using simple chemistry, and synthesized structures were confirmed by X-ray single crystallography. Anticancer and anti-oxidant activities

were tested in vitro. The obtained results indicated that the introduction of another hydroxy group in a given scaffold has significantly improved its anti-oxidant potency. All synthesized compounds displayed extensive in vitro anti-oxidant activities by DPPH assay. Anticancer tests were performed by using a SRB assay against pancreatic cancer with the MIAPaCa-2 cell line and colon cancer with the HCT-15 cell line. The GI_{50} value in the anticancer test of some of the compounds showed good anticancer efficacy. The molecular docking studies of all the synthesized derivatives were performed on COX-2 enzyme. Overall docking scores suggested that the synthesized derivatives will lead to new cyclooxygenases (COX-2) inhibitors.

Supplementary data (ESI)

Complete ^{1}H, ^{13}C NMR, LC–MS, IR and crystal data e included, CIF files. Also contain 3D model of molecular docking of **IA** to **IIIC**.

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References

1. R. Kohen, A. Nyska, Invited review: oxidation of biological systems: oxidative stress phenomena, anti-oxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. **30**, 620–650 (2002)
2. R.P. Patel, J. McAndrew, H. Sellak, C.R. White, H. Jo, B.A. Freeman, V.M. Darley-Usmar, Biological aspects of reactive nitrogen species. Biochim. Biophys. Acta Bioenerg **1411**, 385–400 (1999)
3. B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, anti-oxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA. **90**, 7915–7922 (1993)
4. B. Uttara, A.V. Singh, P. Zamboni, R.T. Mahajan, Oxidative stress and neurodegenerative diseases: a review of upstream and downstream anti-oxidant therapeutic options. Curr. Neuropharmacol. **7**, 65–74 (2009)
5. S. Sen, R. Chakraborty, The role of anti-oxidants in human health, in *Oxidative Stress: Diagnostics, Prevention, and Therapy* (American Chemical Society, 2011), pp. 1–37
6. A. Kumará, Sharma, reactive oxygen species: Friend or foe? RSC Adv **5**, 57267–57276 (2015)
7. A.T. Diplock, C.A. Rice-Evans, R.H. Burdon, Is there a significant role for lipid peroxidation in the causation of malignancy and for anti-oxidants in cancer prevention? Cancer Res. **54**, 1952s–1956s (1994)
8. G. Waris, H. Ahsan, Reactive oxygen species: role in the development of cancer and various chronic conditions. J. Carcinog. **5**, 14 (2006)
9. X.P. Nunes, F.S. Silva, J.R.G.D.S. Almeida, J.M. Barbosa Filhode, J.T. Lima, L.A. de Araújo Ribeiro, L.J.Q. Júnior, *Biological Oxidations and Anti-oxidant Activity of Natural Products* (INTECH Open Access Publisher, New York, 2012)
10. S.B. Nimse, D. Pal, Free radicals, natural anti-oxidants, and their reaction mechanisms. RSC Adv. **5**, 27986–28006 (2015)
11. O.I. Aruoma, A. Jenner and B. Halliwell, Application of Oxidative DNA damage measurements to study anti-oxidant actions of dietary components, in Advances in DNA Damage and Repair, pp 19–26 (1999)

12. B. Halliwell, O. Auroma, *Molecular Biology of Free Radicals in Human Diseases* (OICA, London, 1999)
13. M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur, J. Telser, Free radicals and anti-oxidants in normal physiological functions and human disease. Int J Biochem Cell Biol **39**, 44–84 (2007)
14. S.D. Bagul, J.D. Rajput, P.S. Karandikar, R.S. Bendre, Synthesis, characterization and anti-oxidant activity of carvacrol containing novel thiadiazole and oxadiazole moieties. Mod Chem Appl **4**, 193 (2016). doi:10.4172/2329-6798.1000193
15. L. Poillet-Perez, G. Despouy, R. Delage-Mourroux, M. Boyer-Guittaut, Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. Redox Biol. **4**, 184–192 (2015)
16. M.N. Alam, N.J. Bristi, M. Rafiquzzaman, Review on in vivo and in vitro methods evaluation of anti-oxidant activity. Saudi Pharm J **21**, 143–152 (2013)
17. H. Boeing, A. Bechthold, A. Bub, S. Ellinger, D. Haller, A. Kroke, P. Stehle, Critical review: vegetables and fruit in the prevention of chronic diseases. Eur. J. Nutr. **51**, 637–663 (2012)
18. N.V. Yanishlieva, E.M. Marinova, M.H. Gordon, V.G. Raneva, Anti-oxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem. **64**, 59–66 (1999)
19. S. Nandi, M. Vracko, M.C. Bagchi, Anticancer activity of selected phenolic compounds: QSAR studies using ridge regression and neural networks. Chem. Biol. Drug Des. **70**, 424–436 (2007)
20. J. Mastelic, I. Jerkovic, I. Blažević, M. Poljak-Blaži, S. Borović, I. Ivančić-Baće, N. Müller, Comparative study on the anti-oxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J. Agric. Food Chem. **56**, 3989–3996 (2008)
21. B. Tepe, D. Daferera, M. Sökmen, M. Polissiou, A. Sökmen, In vitro antimicrobial and anti-oxidant activities of the essential oils and various extracts of Thymus eigii M. Zohary et PH Davis. J. Agric. Food Chem. **52**, 1132–1137 (2004)
22. R.S. Bendre, J.D. Rajput, S.D. Bagul, P.S. Karandikar, Outlooks on medicinal properties of eugenol and its synthetic derivatives. Nat Prod Chem Res **4**, 212 (2016). doi:10.4172/2329-6836.1000212
23. J.D. Rajput, S.D. Bagul, S. Tadavi, R.S. Bendre, Comparative anti-proliferative studies of natural phenolic monoterpenoids on human malignant tumour cells. Med. Aromat. Plants (Los Angel) **5**, 2167-0412 (2016)
24. S.D. Bagul, J.D. Rajput, S.K. Tadavi, R.S. Bendre, Design, synthesis and biological activities of novel 5-isopropyl-2-methylphenolhydrazide-based sulfonamide derivatives. Res. Chem. Intermed. 1–12 (2016). doi:10.1007/s11164-016-2759-5
25. D. Kumar, D.S. Rawat, Synthesis and anti-oxidant activity of thymol and carvacrol based Schiff bases. Bioorg. Med. Chem. Lett. **23**, 641–645 (2013)
26. J.D. Rajput, S.D. Bagul, R.S. Bendre, Design, synthesis, biological screenings and docking simulations of novel carvacrol and thymol derivatives containing acetohydrazone linkage. Res. Chem. Intermed. (2016). doi:10.1007/s11164-017-2919-2
27. U.D. Pete, C.M. Zade, J.D. Bhosale, S.G. Tupe, P.M. Chaudhary, A.G. Dikundwar, R.S. Bendre, Hybrid molecules of carvacrol and benzoyl urea/thiourea with potential applications in agriculture and medicine. Bioorg. Med. Chem. Lett. **22**, 5550–5554 (2012)
28. J.D. Rajput, S.D. Bagul, S.K. Tadavi, P.S. Karandikar, R.S. Bendre, Design, synthesis and biological evaluation of novel class diindolyl methanes (DIMs) derived from naturally occurring phenolic monoterpenoids. Med. Chem. (Los Angeles) **6**, 123–128 (2016). doi:10.4172/2161-0444.1000336
29. V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat. Protoc. **1**, 1112–1116 (2006)
30. J. Bao, Y. Cai, M. Sun, G. Wang, H. Corke, Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. J. Agric. Food Chem. **53**, 2327–2332 (2005)
31. B. Brus, U. Košak, S. Turk, A. Pišlar, N. Coquelle, J. Kos, S. Gobec, Discovery, biological evaluation, and crystal structure of a novel nanomolar selective butyrylcholinesterase inhibitor. J. Med. Chem. **57**, 8167–8179 (2014)
32. O.V. Dolomanov, L.J. Bourhis, R.J. Gildea, J.A. Howard, H. Puschmann, OLEX2: a complete structure solution, refinement and analysis program. J. Appl. Crystallogr. **42**, 339–341 (2009)
33. G.M. Sheldrick, SHELXT–integrated space-group and crystal-structure determination. Acta Crystallogr. Sect. A Found Adv. **71**, 3–8 (2015)
34. G.M. Sheldrick, Crystal structure refinement with SHELXL. Acta Crystallogr. Sect. C Struct. Chem. **71**, 3–8 (2015)
35. K. Tayade, S.K. Sahoo, B. Bondhopadhyay, V.K. Bhardwaj, N. Singh, A. Basu, A. Kuwar, Highly selective turn-on fluorescent sensor for nanomolar detection of biologically important Zn^{2+} based on

isonicotinohydrazide derivative: application in cellular imaging. Biosens. Bioelectron. **61**, 429–433 (2014)
36. B.S. Vashi, V.H. Shah, Synthesis and biological screening of substituted thymolylthiazolidinones and thymolylazetidinones. J. Indian Chem. Soc. **73**, 491–492 (1996)
37. B.S. Vashi, D.S. Mehta, V.H. Shah, Synthesis of 2,5-disubstituted-1,3,4-oxadiazole, 1,3,4-triazole and 1,3,4-thiadiazole derivatives as potential antimicrobial agents. Indian J. Chem. B **35**, 111–115 (1996)
38. S.K. Tadavi, J.D. Rajput, S.D. Bagul, A.A. Hosamani, J.N. Sangshetti, R.S.Bendre, Synthesis, crystal structures, biological screening and electrochemical analysis of some salen based transition metal complexes. Res. Chem. Intermed. doi:10.1007/s11164-017-2917-4