Design, Synthesis and Biological Evaluation of Novel Class Diindolyl Methanes (DIMs) Derived from Naturally Occurring Phenolic Monoterpenoids

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Abstract

Several Diindolyl alkanes and their derivatives have been isolated from plant and marine sources. Among the various derivatives of indoles, Diindolyl methanes have wide medicinal applications such as to induce apoptosis in human cancer cells, antibacterial, Anti-inflammatory, antiviral and hormonal control activities. Therefore, they play essential role in marine as well as terrestrial living systems. In present studies we report novel class of Diindolyl methanes prepared from natural phenolic monoterpenoids, via ortho formylation of phenolic monoterpenoids (Carvacrol, Thymol and Eugenol), followed by synthesis, characterization, anticancer, antioxidant and α-amylase inhibitory activities. All the synthesized derivatives show moderate anticancer activities against human breast cell line MCF-7, good antioxidant and α-amylase inhibitory activities using DPPH and α-amylase assay respectively.

Keywords: Carvacrol; Thymol; Eugenol; DIM; α-Amylase; DPPH; SRB

Introduction

The Diindolyl methanes (DIMs) are a class of alkaloids that includes fundamental framework of two indol-3-yl groups bridged by single methyl group and they are differentiated by the substituents attached to the bridging methyl carbon [1]. Commonly most DIMs are found in both marine and terrestrial organisms; a few of them are reported exclusively from either terrestrial or marine organisms. First DIMs derivatives were isolated from genotonic metabolite of human intestinal bacteria Streptococcus faecium, [2] (Streptindol; Figure 1). Later on number of naturally occurring analogs of DIMs were isolated and reported Some of the naturally occurring Di(Diindolyl)methane derivatives [3] are depicted in Figure 1. Moreover, there are number of reports on synthetic derivatives of DIMs [4,5]. Due to excellent bioefficacy of Diindolyl methanes, large number of reports are available in literature, [6-9] on their synthesis; most of these methods involve treatment of indoles with aldehydes in presence of homogeneous acid catalysts or lewis acids and only few reports are based on the use of heterogeneous catalysts [10-12]. Recently some organic chemists have made an effort for development of a hazard-free, waste-free and energy-efficient synthetic route and it may be of great use for economical synthesis of this class of compounds [13]. In persistency to our efforts towards development of efficient synthetic methodologies for preparation of biologically significant scaffolds; herein we have developed an efficient procedure for preparation of new class of Diindolyl methanes and performed their biological activities.

Carvacrol, Thymol, and Eugenol are found in essential oils of many plants [14,15]. These three naturally occurring phenolic monoterpenoids are outstanding resourceful molecules incorporated as useful ingredients in various products and have found applications in pharmaceutical, agricultural, fragrance, flavour, cosmetic and various other industries [16,17]. There huge range of pharmacological activities including antimicrobial, anti-inflammatory, analgesic, anti-oxidant and anticancer activities have been well-researched [18,19]. We synthesized ortho formyl phenolic monoterpenoids using Reimer-Tiemann Reaction [20] and constructed a new series of Diindolyl methanes using ortho formyl derivatives carvacrol, thymol and eugenol.

In the present work, we report a simple synthetic method for the synthesis of new class of Diindolyl methane derivatives in the presence of SnCl4, AlCl3 and citric acid as catalysts in ethanol as solvent at ambient temperature and investigated the biological activities of these synthesized derivatives.

Results and Bioassay

Reaction scheme

Keywords: Carvacrol; Thymol; Eugenol; DIM; α-Amylase; DPPH; SRB

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\[ \text{R}_1=\text{CH(CH}_3)_2, \text{R}_2=\text{H}, \text{R}_3=\text{H} \quad \text{and} \quad \text{R}_4=\text{CH}_4 \text{ then Thymyl Diindolyl methane (TDIM).} \]

\[ \text{R}_1=\text{OCH}_3, \text{R}_2=\text{H}, \text{R}_3=\text{allyl} \quad \text{and} \quad \text{R}_4=\text{H} \text{ then Eugenyl Diindolyl methane (EDIM).} \]

**Scheme 2: Reaction protocol for synthesis of Diindolyl methane derivatives.**

**Anticancer activity**

*In vitro* anticancer activities of compounds CRM, TBM and EBM were performed using Sulforhodamine B (SRB) assay on panel of human cancer cell line (MCF-7) [21]. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For 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 addition of experimental drugs. Experimental drugs were solubilized in appropriate solvent to prepare stock of 10\(^{-3}\) concentration. At the time of experiment four 10-fold 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 compound, plates were incubated at standard conditions for 48 hours and 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 minutes at 4°C. The supernatant was discarded; 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 plates were incubated for 20 minutes 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 (Table 1).

The GI\(_50\) values, defined as the drug concentration required for inhibiting 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 wells × 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 levels.

All synthesized derivatives have been found to be active against human breast cancer cell MCF, at higher concentration, with GI\(_{50}\) of Carvacryl Diindolyl methane (CDIM) 33.5 µL, Eugenyl Diindolyl methane (EDIM) as 84.8 µL and Thymyl Diindolyl methane (TDIM) as 84.4 µL, it suggests that all the synthesized derivatives possess moderate anticancer activities against human breast cancer cell MCF, (Figure 2).

**Antioxidant activity**

DPHH radical-scavenging activity was performed by the reported method [22]. For each determination, the stock solution (1 mg/ml) was diluted by serial dilution (25 µg-200 µg/ml) with 60% (v/v) ethanol. An aliquot of each dilution (0.5 ml) was mixed with methanolic solution of DPPH (5 mL, 0.06 mM). The mixtures were shaken vigorously and incubated at 37°C in the dark for 30 min. At the same time, a control containing 60% (v/v) ethanol (0.5 mL) and 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 IC\(_{50}\) value (µg/mL). All determinations were carried out in triplicate. Ascorbic acid was used as reference compound.

In anticancer activity performed by DPPH assay, all the synthesized derivatives exhibited good antioxidant activities at lower as well as higher concentrations. IC\(_{50}\) of Carvacryl Diindolyl methane (CDIM) is 53.50 ± 0.150 µg/ml, Eugenyl Diindolyl methane (EDIM) is 56.22 ± 0.142 µg/ml, Thymyl Diindolyl methane (TDIM) is 58.44 ± 0.150 µg/ml.

<table>
<thead>
<tr>
<th>Structure of DIM</th>
<th>Physical State</th>
<th>Catalyst</th>
<th>Yield</th>
<th>Reaction Condition</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDIMs</td>
<td>Solid</td>
<td>SnCl(_2) (20%)</td>
<td>82%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citric acid (20%)</td>
<td>74%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlCl(_3) (20%)</td>
<td>85%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td>TDIMs</td>
<td>Solid</td>
<td>SnCl(_2) (20%)</td>
<td>85%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citric acid (20%)</td>
<td>80%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlCl(_3) (20%)</td>
<td>90%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td>EDIMs</td>
<td>Solid</td>
<td>SnCl(_2) (20%)</td>
<td>80%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>RT=30 min Reflux=10 min</td>
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<tr>
<td></td>
<td></td>
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<td>85%</td>
<td>RT=30 min Reflux=10 min</td>
<td>Ethanol/ Methanol</td>
</tr>
</tbody>
</table>

**Table 1:** Chemical structures, physical state, catalyst, reaction condition, solvent and yields.

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ml and Ascorbic Acid (Std) is 44.33 ± 0.167 μg/ml. The results indicate that all the synthesized derivatives possess outstanding antioxidant activities (Figure 3).

**α-Amylase inhibitory activity**

This assay was performed using reported modified 4 α-amylase inhibition assay. [23] Total 250 μL of solutions of compounds having various concentrations were placed in different hard glass tubes and 250 μL of 0.02 M Sodium phosphate buffer (pH 6.9) containing α-amylase solution was added to it. All the solutions were pre-incubated at 25°C for 10 min, after which 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at time intervals and then further incubated at 25°C for 10 min. The reaction was terminated by adding 500 μL of Dinitrosalicillic acid (DNS) regent. The tubes were then incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 ml distilled water and the absorbance was measured at 540 nm using Spectrophotometer. A control was prepared using the same procedure replacing the compounds with water. Concentrations of samples resulting in 50% inhibition of enzyme activity (IC₅₀) were determined graphically.

All the synthesized derivatives show excellent α-amylase inhibitory activity at lower to higher concentration. IC₅₀ of Thymyl Diindolyl methane (TDIM) is 56.48 ± 0.123 μg/ml, Eugenyl Diindolyl methane (EDIM) is 82.70 ± 0.150 μg/ml, Carvacryl Diindolyl methane (CDIM) is 110.18 ± 0.145 μg/ml while that of Acarbose (Std) is 50.26 ± 0.114 μg/ml. Overall view of this activity suggested that Thymyl Diindolyl methane (TDIM) having 56.48 ± 0.123 μg/ml is most potent to inhibit the α-amylase (Figure 4).

**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 ¹H NMR, ¹³C NMR spectra. NMR spectra of the products were obtained using Bruker AC-400 MHz spectrometers

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**Figure 1:** Naturally occurring Diindolyl Methanes (DIMs) Derivatives.
with TMS as the internal standard. Mass spectra of the products were obtained using LC-MS.

**Procedure for synthesis of ortho formylation on phenolic monoterpenes**

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

**Figure 2:** Graph shows anticancer activity of CBIM, TBIM and EBIM.

**Figure 3:** Graph indicating Antioxidant Activity of CBIM, TBIM and EBIM.
The distillate was extracted with ether (3 × 30 mL). Most of the ether was removed by distillation. The residue, which contains unreacted phenolic monoterpenone and ortho formylated phenolic monoterpenone, was transferred to a small glass stoppered flask and about twice the volume of saturated sodium metabisulphite solution was added to it. The solution was stirred vigorously for ½ h and allowed to stand for 1 h. The paste of bisulphite compound was filtered and washed with little ethanol and finally with little ether. The bisulphite compound was decomposed by warming in round bottom flask on a water bath with dilute H₂SO₄. The mixture was allowed to cool and then the solution of bisulphite compound was extracted with ether (3 × 30 mL). Removal of ether gave pure ortho formylated phenolic monoterpenone. Finally all the three ortho formyl Phenolic monoterpenones were characterized by spectroscopic methods.

Procedure for synthesis of Diindolyl methanes (DIMs) derivatives from ortho formyl phenolic monoterpenes

A mixture of ortho formyl phenolic monoterpenes (0.0756 g, 0.5 mmol, 1 equiv.), indole (0.1171 g, 1 mmol, 2 equivalent) and catalysts (i.e., SnCl₂, AlCl₃ and citric acid) (10 mg) in ethanol (3 mL) was stirred vigorously at room temperature until the disappearance of the starting indole, checked by TLC (30 min). When the reaction was completed, the reaction mixture was filtered and washed with water. Then, the reaction mixture was concentrated under reduced pressure. The residue, which contains unreacted phenolic monoterpenone and ortho formylated phenolic monoterpenone, was removed by distillation. The residue, which contains unreacted phenolic monoterpenone and ortho formylated phenolic monoterpenone, was transferred to a small glass stoppered flask and about twice the volume of saturated sodium metabisulphite solution was added to it. The solution was stirred vigorously for ½ h and allowed to stand for 1 h. The paste of bisulphite compound was filtered and washed with little ethanol and finally with little ether. The bisulphite compound was decomposed by warming in round bottom flask on a water bath with dilute H₂SO₄. The mixture was allowed to cool and then the solution of bisulphite compound was extracted with ether (3 × 30 mL). Removal of ether gave pure ortho formylated phenolic monoterpenone. Finally all the three ortho formyl Phenolic monoterpenones were characterized by spectroscopic methods.

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Conclusion

A new class of Diindolyl methanes (DIMs) derivatives have been synthesized and characterized by various spectroscopic methods. These derivatives exhibited significant biological activities such as anticancer, antioxidant and α-amylase inhibitory activity. All the synthesized derivatives are active against human breast cancer cell line MCF, at higher concentration, similarly wonderful antioxidant and α-amylase inhibitory activities have been observed at every concentration. In
case of antioxidant activity; Carvacryl Diindolyl methane (CDIM) and Eugenyl Diindolyl methane (EDIM) showed notable IC₅₀ values while in α-amylase inhibitory activity, Thymyl Diindolyl methane (TDIM) exhibited prominent IC₅₀ values.

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References