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# ISOLATION, CHEMICAL MODIFICATION, AND ANTICANCER ACTIVITY OF MAJOR METABOLITES OF THE LICHEN *Parmotrema mesotropum*

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*Extensive chromatographic purification of the chloroform–methanol (1:1) extract of the lichen* Parmotrema mesotropum *led to the isolation of methyl hematommate (**1**), methyl-2,4-dihydroxy-3,6-dimethylbenzoate (**2**), orcinol (**3**), and atranorin (**4**). The two major metabolites (**1** and **2**) were subjected to chemical modification and a total of 15 analogues were synthesized. The synthesized analogues and their parent compounds were evaluated for their anticancer potential against a panel of five human cancer cell lines. Among the tested samples, compound **1g** showed potent activity against three cancer cell lines, namely DU145 ($IC_{50}$ 20.07 μM), MCF-7 ($IC_{50}$ 20.94 μM), and U87MG ($IC_{50}$ 25.32 μM). This compound can be considered as lead a molecule for further development.*

**Keywords**: Lichen, *Parmotrema mesotropum*, methyl hematommate, methyl 2,4-dihydroxy-3,6-dimethylbenzoate, synthetic analogues, anticancer activity.

Lichens are symbiotic organisms, consisting of a photobiont, usually a green algae or cynobacteria, and mycobionts referred to as fungi. These are distributed worldwide and can survive under various harsh environmental conditions (e.g., at high altitude and direct sunlight). So far, about 20,000 lichen species have been recorded worldwide [1]. They are resistant to UV irradiation due to the production of a large number of unique secondary metabolites such as depsides, depsidones, dibenzofuran derivatives, anthraquinones, and xanthones [2]. These metabolites can act as UV filters as well as antioxidants [3]. Lichens have been used in folk medicine for centuries by native Americans, Europeans, and Indians [4]. Some lichens were found to be good for coughs, jaundice, rabies, and for restoring lost hair.

Several herbal medicine texts have documented the medicinal properties of various lichen species including *Cladonia*, *Evernia*, *Lobaria*, *Parmelia*, *Roccella*, *Usnia*, and *Xanthoria*. But their chemistry is poorly studied. *Parmotrema mesotropum* (Mull. Arg.) Hale (Syn: *Parmelia mesotropa* Mull. Arg.; Fam: Parmeliaceae) is a foliose lichen widely distributed in low temperate zones of India especially in Himachal Pradesh, Karnataka, Madhya Pradesh, Andhra Pradesh, etc. [5]. Literature search reveals that this species is totally unexplored both chemically and biologically. With this background in view, a focussed study is now undertaken on *P. mesotropum* to isolate various chemical constituents and to carry out chemical modification on major metabolites to evaluate their biological potential, especially anticancer activity. The results of chemical and biological studies of *P. mesotropum* are presented in this communication.

The brown colored *P. mesotropum* lichen was extracted with chloroform–methanol (1:1), and the resultant extract (10%) was subjected to silica gel flash column chromatography with *n*-hexane and ethyl acetate solvents as eluents to yield four single and pure compounds **1–4**.

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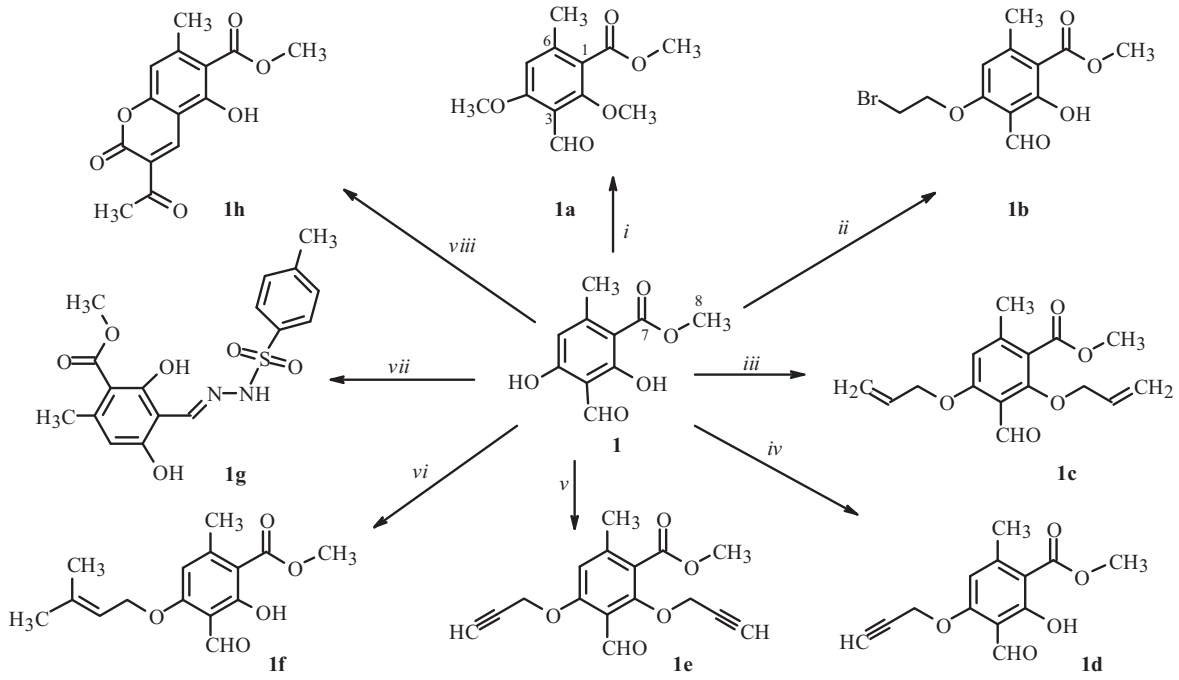


*i*. $CH_3I$, $K_2CO_3$, acetone, reflux, 75%; *ii*. 1,2-dibromoethane, $K_2CO_3$, acetone, reflux, 70%; *iii*. allyl bromide, $K_2CO_3$, acetone, reflux, 72%; *iv*. propargyl bromide, $K_2CO_3$, acetone, reflux, 55%; *v*. propargyl bromide, $K_2CO_3$, acetone, reflux, 45%; *vi*. prenyl bromide $K_2CO_3$, acetone, reflux, 64%; *vii*. *p*-toluenesulfonyl hydrazide, ethanol, reflux, 77%; *viii*. ethylacetoacetate, piperidine, toluene, r.t., 62%.

Scheme 1

Compound **1**, $C_{10}H_{10}O_5$. IR and $^1H$ and $^{13}C$ NMR spectra identifies its structure as methyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (methyl hematommate) [6].

Compound **2**, $C_{10}H_{12}O_4$. IR and $^1H$ and $^{13}C$ NMR spectra were very similar to those compound **1**. Based on its spectral data, compound **2** was identified as methyl 2,4-dihydroxy-3,6-dimethylbenzoate [7].

Compound **3**, $C_7H_9O_2$. IR and $^1H$ and $^{13}C$ NMR spectra of the compound revealed its structure as 3,5-dihydroxytoluene (orcinol) [8].

Compound **4**, $C_{19}H_{18}O_8$. IR and $^1H$ and $^{13}C$ NMR spectra of the compound revealed its identity as atranorin [9].

Among the isolated metabolites, compounds **1** and **2** were obtained in significant quantities (0.5% and 1%). In an attempt to obtain more active molecules, compounds **1** and **2** were chemically modified and synthesized to obtain a total of 15 analogues. The structures of the synthesized analogues were confirmed by their spectral data ($^1H$ and $^{13}C$ NMR, IR, HR-MS).

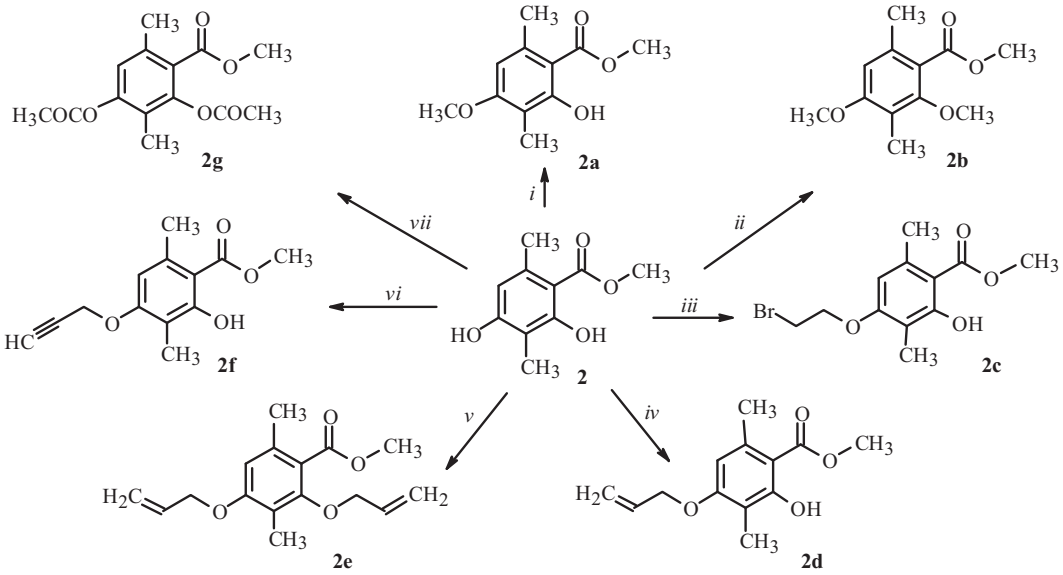
In the case of compound **1**, the chemical transformations were carried out mostly on the two phenolic hydroxyls and the aldehydic group (Scheme 1). Treatment of compound **1** with either saturated or unsaturated alkyl halides in the presence of $K_2CO_3$ in acetone yielded the corresponding mono and dialkyl ethers (**1a–1f**) in very good to excellent yields (45–75%). The formation of these ethers was easily identified by the absence of peaks corresponding to one or both phenolic hydroxyl groups of compound **1**. Further, the unsaturated ethers (**1c–1f**) showed the characteristic olefinic ($^1H$ NMR δ 5.496, 5.378, 6.051, 5.464, 5.351, 5.330; $^{13}C$ NMR δ 133.1, 118.24, 131.8, 118.1) or acetylenic ($^1H$ NMR δ 3.547; $^{13}C$ NMR δ 72.97, 77.84) peaks. Similarly, compound **1** when treated with *p*-toluenesulfonyl hydrazide in ethanol yielded the corresponding *p*-toluenesulfonyl hydrazone **1g** in 77% yield. This compound showed characteristic peaks in IR (1730, 1595, 1458, 1360 $cm^{-1}$), $^1H$ NMR δ 7.341, 8.438, $^{13}C$ NMR δ 143.03, 143, and HR-MS 379.096. Interestingly, compound **1** formed 3-acetylcoumarin in 62% yield when treated with ethylacetoacetate in the presence of piperidine in toluene. Its structure was confirmed by its spectral data IR (1702 and 1654 $cm^{-1}$), $^1H$ NMR (δ 2.645 and 8.936), $^{13}C$ NMR (δ 30.45, 121.72, 143.20, 149.25, 194.72), and HR-MS *m/z* 299.0522 $[M + H]^+$.

In the case of compound **2**, the two phenolic hydroxyls can be exploited to obtain a series of ethers and esters. Hence, the chemical modification of compound **2** resulted in the formation of six saturated and unsaturated ethers and one bis-acetate in very good to excellent yields (47–75%) (Scheme 2). The structures of the synthesized compounds were confirmed by their spectral data.

TABLE 1. Anticancer Activity of Isolated and Semisynthetic Compounds ($IC_{50}$, μM)*

| Compound | A549 | DU145 | MCF-7 | SiHa | U87MG |
|---|---|---|---|---|---|
| **2** | > 100 | 90.99 ± 4.35 | 108.29 ± 9.97 | > 100 | > 100 |
| **4** | > 100 | > 100 | 103.41 ± 6.57 | 100.70 ± 4.64 | > 100 |
| **1b** | > 100 | 85.28 ± 2.70 | 50.88 ± 6.65 | 50.13 ± 0.46 | 110.32 ± 6.25 |
| **1c** | > 100 | > 100 | 37.13 ± 2.82 | 91.14 ± 2.10 | 65.61 ± 2.43 |
| **1e** | 86.28 ± 4.37 | 46.16 ± 7.55 | 65.21 ± 5.71 | 90.78 ± 4.67 | 96.94 ± 3.86 |
| **1f** | > 100 | 43.21 ± 1.01 | > 100 | > 100 | 98.68 ± 2.29 |
| **1g** | 91.43 ± 5.83 | 20.07 ± 0.4 | 20.94 ± 4.38 | 86.97 ± 3.17 | 25.32 ± 3.00 |
| **2b** | 82.4 ± 2.06 | 36.54 ± 1.67 | 40.83 ± 1.89 | 71.49 ± 3.32 | 39.69 ± 1.74 |
| **2d** | > 100 | 65.44 ± 2.86 | > 100 | > 100 | > 100 |
| **2e** | > 100 | 40.46 ± 1.41 | 86.22 ± 1.29 | > 100 | > 100 |
| **2f** | > 100 | 29.65 ± 5.77 | 38.28 ± 2.74 | > 100 | > 100 |
| **2g** | 43.99 ± 2.26 | 40.17 ± 3.53 | 29.63 ± 2.93 | 50.37 ± 2.96 | 85.05 ± 2.52 |
| Docetaxel | 0.04 ± 0.006 | 0.64 ± 0.04 | 0.034 ± 0.005 | 0.247 ± 0.07 | 0.106 ± 0.03 |

*Compounds **1**, **3**, **1a**, **1d**, **1h**, **2a**, **2e**, and crude extract had $IC_{50}$ > 100 μM for five cancer cell lines.




*i*. $CH_3I$, $K_2CO_3$, acetone, reflux, 50%; *ii*. $CH_3I$, $K_2CO_3$, acetone, reflux, 50%; *iii*. 1,2-dibromoethane, $K_2CO_3$, acetone, reflux, 73%; *iv*. allyl bromide, $K_2CO_3$, acetone, reflux, 53%; *v*. allyl bromide, $K_2CO_3$, acetone, reflux, 47%; *vi*. propargyl bromide, $K_2CO_3$, acetone, reflux, 75%; *vii*. acetic anhydride, pyridine, r.t., 68%.

Scheme 2

Compounds (**2a–2c**) showed characteristic peaks corresponding to methoxyl ($^1$H NMR δ 3.854; $^{13}$C NMR δ 56.15) or bromoalkyl ($^1$H NMR δ 4.337, 3.670; $^{13}$C NMR δ 28.962, 67.81). In the case of unsaturated alkyl chains (**2d–2f**), they showed the characteristic peaks for allyl or propargyl functionalities ($^1$H NMR δ 4.36, 6.044, 5.238, 5.356, 4.536, 5.269, 5.394; $^{13}$C NMR δ 117.76, 75.158, 133.738, 117.21). The bis-acetate showed the characteristic acetoxy group in IR (1758, 1623, 1576, 1455, 1369 $cm^{-1}$), $^1$H NMR (δ 2.318, 2.287), and $^{13}$C NMR (δ 168.38, 20.08, 166.60, 20.403).

The four isolated compounds **1–4** and 15 synthesized analogues **1a–1h** and **2a–2g** were screened for their cytotoxic potential against five human cancer cell lines, A549 (lung cancer), DU145 (prostate cancer), MCF-7 (breast cancer), SiHa (cervical cancer), and U87MG (glioblastoma), which were studied through the MTT assay [10]. Analysis of the data (Table 1) reveals that the synthesized compounds showed greater activity than their parent compounds **1** and **2**. The biological activity was found to increased when the aldehyde group of compound **1** was converted to the corresponding imine compound (**1g**) and tested against DU145 ($IC_{50}$ 20.07 μM), MCF-7 ($IC_{50}$ 20.94 μM), and U87MG ($IC_{50}$ 25.32 μM). When different alkyl,

alkenyl, or acetylenic groups were introduced in compound **1** in the form of ethers (**1a–1f**), there was no significant enhancement in their cytotoxicity. Even the introduction of the coumarin moiety in compound **1** had no impact on its cytotoxicity. Similarly, different alkyl, alkenyl, or acetylenic groups when substituted in compound **2** in the form of ethers (**2a–2e**) gave no significant enhancement in their cytotoxicity, except for compound **2f** where activity was retained. Interestingly, introduction of the acetylenic group in compound **2** as in the case of **2f** enhanced the cytotoxicity against DU145 ($IC_{50}$ 29.65 μM). Similarly, the bis-acetate (**2g**) showed improved cytotoxic activity against the breast cancer cell line MCF-7 ($IC_{50}$ 29.63 μM).

Initially, all compounds were tested for anticancer activity in the above five human cancer cell lines at the concentration of 100 μM. The compounds that showed ≥ 50% cytotoxicity were further tested using a dose response study, and the $IC_{50}$ values of all the compounds obtained were tabulated.

## EXPERIMENTAL

**General Experimental Procedures**. The melting points were determined in open capillaries on a Buchi melting point apparatus and are uncorrected. IR spectra were recorded in KBr disks on a Nicolet 740 FTIR spectrophotometer. $^1H$ and $^{13}C$ NMR spectra were recorded on Bruker 500 MHz spectrometers, and chemical shifts were reported as parts per million (ppm) with tetramethylsilane as internal standard. Mass spectra were obtained on an Agilent ESI-QTOF instrument. Column chromatography was performed on ACME grade silica gel (60–120 mesh), and Merck precoated silica gel 60 $F_{254}$ plates were used for thin-layer chromatography.

**Collection of Lichen Material**. The lichen *P. mesotropum* was collected from the rocks (saxicolous) in Horsley Hills (Latitude 13.6507°N; Longitude 78.3970°E), Kadapa District, Andhra Pradesh, India at an altitude of 1109 m in March, 2014. This lichen was washed with fresh running tap water, shade dried, and stored in an airtight bottle at 4°C. The lichen sample was identified by taxonomists. Its identity was further confirmed by chemical examination using, at the species level, relevant keys in the spot tests.

**Spot Tests to Identify Various Secondary Metabolites of *P. mesotropum***. The brown colored upper cortex when treated with K (K = 10% aqueous solution) gave a yellow coloration, indicating the presence of atranorin and chloroatranorin.

**Extraction of *P. mesotropum***. The lichen material (200 g) was soaked in 1 L of chloroform–methanol (1:1) under maceration conditions for 72 h at room temperature. The resultant extract was filtered with Whatman No. 1 filter paper and concentrated under vacuum at 40°C using a rotary evaporator, which afforded a dark brown colored extract (20 g, 10%).

**Isolation and Identification of Lichen Metabolites**. The thin-layer chromatographic studies on the crude extract (20 g) revealed the presence of some well-resolved spots. Repetitive column chromatographic separation of the extract led to the isolation of four compounds (**1–4**), which were identified by their $^1H$ and $^{13}C$ NMR, IR, and mass spectra as methyl heamatommate (**1**, 1.0 g, 0.5%), methyl 2,4-dihydroxy-3,6-dimethylbenzoate (**2**, 2.0 g, 1%), orcinol (**3**, 0.5 g, 0.25%), and atranorin (**4**, 0.03 g, 0.015%) by column chromatography. The identity of the compounds was further established by comparing their physical and spectroscopic data with the literature values.

**Methyl 3-Formyl-2,4-dihydroxy-6-methylbenzoate (1)**. Colorless crystals, mp 142–145°C. IR (KBr, ν, $cm^{-1}$): 2962, 1644, 1577, 1439, 1325, 1199, 972, 851, 776, 619, 587, 476. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 12.875 (1H, s, OH), 12.410 (1H, s, OH), 10.340 (1H, s, CHO), 6.293 (1H, s, H-5), 3.961 (3H, s, $COOCH_3$), 2.531 (3H, s, $CH_3$-6). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 25.199, 152.303, 103.82, 166.618, 108.42, 168.26, 112.103, 171.98, 52.29, 193.879. ESI-HR-MS *m/z* 211.0608 $[M + H]^+$ (calcd for $C_{10}H_{11}O_5$, 211.0608).

**Methyl 2,4-Dihydroxy-3,6-dimethylbenzoate (2)**. Colorless crystals, mp 145–148°C. IR (KBr, ν, $cm^{-1}$): 3404, 3082, 2943, 1627, 1446, 1368, 1316, 1274, 1196, 1111, 1033, 994, 726, 624, 582, 476. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 12.045 (1H, s, OH), 6.209 (1H, s, H-5), 5.097 (1H, s, OH), 3.921 (1H, s, $COOCH_3$), 2.460 (3H, s, $CH_3$-6), 2.104 (3H, s, $CH_3$-3). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 24.061, 140.140, 110.547, 163.075, 105.176, 158.05, 108.537, 172.609, 51.809, 7.638. ESI-HR-MS *m/z* 197.0810 $[M + H]^+$ (calcd for $C_{10}H_{13}O_4$, 197.0810).

**3,5-Dihydroxytoluene (3)**. Colorless crystals, mp 82–84°C. ESI-HR-MS *m/z* 125.05 $[M + H]^+$ (calcd for $C_7H_9O_2$, 125.05) [8].

**3-Hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl 3-Formyl-2,4-dihydroxy-6-methylbenzoate (4)**. Colorless crystals, mp 196–198°C. IR (KBr, ν, $cm^{-1}$): 3433, 3078, 2943, 1632, 1437, 1303, 1206, 588. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 12.54 (1H, s, OH), 12.499 (1H, s, OH), 11.944 (1H, s, OH), 10.359 (1H, s, CHO), 6.501 (1H, s, ArH), 6.401 (1H, s, ArH), 3.986 (3H, s, $COOCH_3$), 2.689 (3H, s, $CH_3$), 2.546 (3H, s, $CH_3$), 2.092 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$,

δ, ppm): 25.556, 151.95, 102.816, 169.03, 108.52, 167.456, 112.82, 169.656, 193.77, 152.40, 110.24, 162.546, 116.75, 139.84, 115.988, 172.164, 52.31, 9.352, 23.99. ESI-HR-MS *m/z* 373.0917 $[M - H]^-$ (calcd for $C_{19}H_{17}O_8$, 373.0917).

**General Procedures for the Synthesis of Saturated and Unsaturated Alkyl Ethers (1a–1f, 2a–2f)**. Compound **1** or **2** (0.05 g, 1 eq.) was treated with the appropriate alkyl halide (0.05–0.06 g, 1.5 eq.) in the presence of $K_2CO_3$ (0.05 g, 1.5 eq.) in acetone (5.0 mL) for 2–12 h. After completion, the reaction mixture was extracted with ethyl acetate (3 × 5 mL). The combined organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a crude reaction mixture, which was chromatographed over a silica gel column to yield pure compounds (**1a–1f**, **2a–2f**).

**Methyl 3-Formyl-2,4-dimethoxy-6-methylbenzoate (1a)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 3448, 2923, 2853, 1728, 1689, 1564, 1463, 1280, 1154, 1109, 958, 836, 583, 523. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 10.384 (1H, s, CHO), 6.588 (1H, s, H-5), 3.918 (3H, s, $COOCH_3$), 3.880 (6H, s, 2 × $OCH_3$), 2.360 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.1, 64.28, 108.88, 162.53, 160.53, 160.53, 119.08, 167.56, 52.31, 188.12, 64.28, 56.18. ESI-HR-MS *m/z* 261.0729 $[M + Na]^+$ (calcd for $C_{12}H_{14}O_5Na$, 261.0729).

**Methyl 4-(2-Bromoethoxy)-3-formyl-2-hydroxy-6-methylbenzoate (1b)**. Colorless semisolid, mp 195°C. IR (KBr, ν, $cm^{-1}$): 3423, 2924, 2853, 1727, 1601, 1461, 1371, 1276, 1160, 1080, 969, 810, 705. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.517 (1H, s, OH), 10.319 (1H, s, CHO), 6.0209 (1H, s, H-5), 4.397 (2H, t, J = 2.731, $CH_2$), 4.289 (2H, t, J = 2.731, $CH_2$), 3.931 (3H, s, $COOCH_3$), 2.375 (3H, s, $CH_3$-6). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.68, 148.65, 114.037, 163.55, 115.81, 159.331, 119.078, 166.926, 52.477, 194.519, 64.516, 29.35. ESI-HR-MS *m/z* 317.0014 $[M]^+$ (calcd for $C_{12}H_{13}BrO_5$, 317.0014).

**Methyl 2,4-Bis(allyloxy)-3-formyl-6-methylbenzoate (1c)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 3447, 2923, 2853, 1731, 1691, 1597, 1461, 1358, 1283, 1158, 989, 762. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 10.427 (1H, s, CHO), 6.576 (1H, s, H-5), 6.051 (1H, m, CH), 5.496 (1H, dd, J = 2.831, CH), 5.464 (1H, m, CH), 5.378 (1H, dd, J = 2.621, CH), 5.351 (1H, dd, J = 2.631, CH), 5.330 (1H, dd, J = 2.631, CH), 4.644 (2H, d, J = 2.441, $CH_2$), 4.515 (2H, d, J = 2.541, $CH_2$), 3.887 (1H, s, $COOCH_3$), 2.344 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.6, 144.6, 110.1, 158.8, 116.9, 161.5, 122.6, 167.6, 52.3, 188.09, 77.5, 133.1, 118.24, 69.6, 131.8, 118.1. ESI-HR-MS *m/z* 313.1038 $[M + Na]^+$ (calcd for $C_{16}H_{18}O_5Na$, 313.1038).

**Methyl 3-Formyl-2-hydroxy-6-methyl-4-(prop-2-yn-1-yloxy)benzoate (1d)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 3283, 2924, 2854, 2123, 1794, 1726, 1690, 1597, 1460, 1359, 1359, 1359, 1285, 1209, 1157, 1100, 967, 772, 772, 669. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 12.504 (1H, s, OH), 10.269 (1H, s, CHO), 6.354 (1H, s, H-5), 4.807 (2H, s, $CH_2$), 3.923 (3H, s, $COOCH_3$), 3.457 (1H, s, CH), 2.392 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.679, 139.256, 104.507, 160.600, 111.88, 166.99, 114.04, 173.03, 52.282, 193.19, 56.47, 77.84, 72.97. ESI-HR-MS *m/z* 249.0757 $[M + H]^+$ (calcd for $C_{13}H_{13}O_5$, 249.0757).

**Methyl 3-Formyl-6-methyl-2,4-bis(prop-2-yn-1-yloxy)benzoate (1e)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 3448, 3281, 2924, 2853, 2121, 1713, 1600, 1458, 1358, 1278, 1190, 1150, 1094, 966, 836, 640. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 10.286 (1H, s, CHO), 6.648 (1H, s, H-5), 4.772 (2H, s, $CH_2$), 4.618 (2H, s, $CH_2$), 3.927 (3H, s, $COOCH_3$), 3.905 (1H, s, CH), 3.457 (2H, s, CH), 2.354 (3H, s, $CH_3$-6). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.681, 113.264, 114.037, 159.33, 104.507, 163.55, 139.264, 166.55, 52.477, 194.519, 64.516, 78.198, 68.146. ESI-HR-MS *m/z* 309.1046 $[M+Na]^+$ (calcd for $C_{16}H_{14}O_5Na$, 309.1046).

**Methyl 3-Formyl-2-hydroxy-6-methyl-4-((3-methylbut-2-en-1-yl)oxy)benzoate (1f)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 3448, 2923, 2853, 1733, 1692, 1595, 1458, 1373, 1282, 1157, 1093, 970, 772. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.55 (1H, s, OH), 10.25 (1H, s, CHO), 6.24 (1H, s, H-5), 5.462 (1H, t, J = 2.631, CH), 4.612 (2H, d, J = 2.441, $CH_2$), 3.914 (3H, s, $COOCH_3$), 2.371 (3H, s, $CH_3$), 1.811 (3H, s, $CH_3$), 1.755 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 22.68, 148.82, 104.39, 161.35, 108.89, 162.35, 114.044, 173.035, 52.172, 193.508, 65.662, 118.27, 139.42, 18.29, 14.1. ESI-HR-MS *m/z* 278.1434 $[M + H]^+$ (calcd for $C_{15}H_{19}O_5$, 278.1430).

**Methyl 2-Hydroxy-4-methoxy-3,6-dimethylbenzoate (2a)**. Colorless solid, mp 65–66°C. IR (KBr, ν, $cm^{-1}$): 3440, 2921, 2852, 1729, 1606, 1579, 1463, 1398, 1276, 1224, 1152, 1071, 963, 835, 761, 591. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 11.815 (1H, s, OH), 6.852 (1H, s, H-5), 3.925 (3H, s, $OCH_3$), 3.854 (3H, s, $COOCH_3$), 2.528 (3H, s, $CH_3$), 2.075 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 19.771, 134.50, 121.69, 156.72, 117.95, 157.32, 109.30, 168.80, 52.05, 8.816, 56.15. ESI-HR-MS *m/z* 211.0963 $[M + H]^+$ (calcd for $C_{11}H_{15}O_4$, 211.0963).

**Methyl 2,4-Dimethoxy-3,6-dimethylbenzoate (2b)**. Colorless liquid. IR (KBr, ν, $cm^{-1}$): 2924, 2853, 1717, 1653, 1577, 1458, 1399, 1281, 1133, 1082, 802, 745, 666, 580, 465, 417. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm): 6.459 (1H, s, ArH), 3.899 (3H, s, $COOCH_3$), 3.819 (3H, s, $OCH_3$), 3.752 (3H, s, $OCH_3$), 2.30 (3H, s, $CH_3$), 2.103 (3H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 19.51, 134.25, 121.44, 156.47, 117.70, 157.07, 109.05, 168.55, 51.80, 8.563, 61.59, 55.89. ESI-HR-MS *m/z* 247.0939 $[M + Na]^+$ (calcd for $C_{12}H_{16}O_4Na$, 247.0939).

**Methyl 4-(2-Bromoethoxy)-2-hydroxy-3,6-dimethylbenzoate (2c)**. Colorless solid, mp 145–146°C. IR (KBr, ν, $cm^{-1}$): 3420, 2924, 2859, 1644, 1557, 1450, 1361, 1278, 1135, 1037, 966, 827, 750, 674, 581, 467, 426. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 11.843 (1H, s, OH), 6.226 (1H, s, H-5), 4.337 (2H, t, J = 2.631, $CH_2$), 3.931 (3H, s, $COOCH_3$), 3.670 (2H, t, J = 2.631, $CH_2$), 2.513 (3H, s, $CH_3$), 2.108 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 24.553, 140.06, 111.60, 162.34, 106.77, 159.76, 106.01, 172.45, 51.84, 7.898, 67.81, 28.962. ESI-HR-MS *m/z* 304.2022 $[M + H]^+$ (calcd for $C_{12}H_{16}BrO_4$, 304.2022).

**Methyl 4-(Allyloxy)-2-hydroxy-3,6-dimethylbenzoate (2d)**. Liquid. IR (KBr, ν, $cm^{-1}$): 3448, 2924, 2853, 1729, 1604, 1461, 1377, 1276, 1153, 798. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 11.82 (1H, s, OH), 6.25 (1H, s, H-5), 6.05 (1H, m, CH), 5.44 (1H, dd, J = 1.526, CH), 5.29 (1H, dd, J = 1.373, CH), 4.57 (2H, d, J = 5.035, $CH_2$), 3.92 (3H, s, $COOCH_3$), 2.50 (3H, s, $CH_3$), 2.11 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 22.683, 139.975, 111.227, 160.48, 106.51, 162.227, 105.51, 172.499, 51.75, 7.916, 68.64, 132.97, 117.24. ESI-HR-MS *m/z* 237.11 $[M + H]^+$ (calcd for $C_{13}H_{17}O_4$, 237.11).

**Methyl 2,4-Bis(allyloxy)-3,6-dimethylbenzoate (2e)**. Liquid. IR (KBr, ν, $cm^{-1}$): 2924, 2853, 1729, 1640, 1461, 1377, 1119, 798. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.452 (1H, s, H-5), 6.044 (2H, m, 2 × CH), 5.394 (1H, dd, J = 1.526, CH), 5.356 (1H, dd, J = 1.526, CH), 5.269 (1H, dd, J = 1.526, CH), 5.238 (1H, dd, J = 1.526, CH), 4.536 (2H, d, J = 4.25, H-9), 4.36 (2H, d, J = 5.648, $CH_2$), 3.869 (3H, s, $COOCH_3$), 2.287 (3H, s, $CH_3$), 2.137 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 19.807, 134.56, 109.106, 155.53, 121.116, 158.268, 117.15, 168.89, 51.99, 9.046, 68.912, 133.074, 117.76, 75.158, 133.738, 117.21. ESI-HR-MS *m/z* 277.1427 $[M + H]^+$ (calcd for $C_{16}H_{21}O_4$, 277.1427).

**Methyl 2-Hydroxy-3,6-dimethyl-4-(prop-2-yn-1-yloxy)benzoate (2f)**. Colorless solid, mp 140–142°C. IR (KBr, ν, $cm^{-1}$): 3421, 3283, 2924, 2854, 2129, 1726, 1646, 1576, 1454, 1397, 1281, 1197, 1081, 969, 801, 742, 696, 584, 465. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 11.822 (1H, s, OH), 6.368 (1H, s, H-5), 4.739 (2H, s, J = 2.289, $CH_2$), 3.927 (3H, s, $COOCH_3$), 2.536 (3H, s, $CH_3$), 2.096 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 24.386, 139.64, 111.42, 159.05, 106.68, 162.06, 111.046, 172.17, 51.60, 7.702, 55.60, 78.09, 75.39. ESI-HR-MS *m/z* 235.0962 $[M + H]^+$ (calcd for $C_{13}H_{15}O_4$, 235.0962).

**Synthesis of Sulfonylhydrazine (1g)**. Compound **1** (0.05 g, 1 eq.) was refluxed with *p*-toluosulfonyl hydrazide (0.04 g, 1 eq.) in absolute ethanol for 48 h. After completion the reaction mixture was extracted with ethyl acetate (3 × 5 mL). The combined organic layer was dried over anhydrous sodium sulfate and concentrated under a rotary evaporator followed by recrystallization to afford **1g** as colorless crystals, yield 77%, mp 165–167°C.

**(*E*)-Methyl 2,4-Dihydroxy-6-methyl-3-((2-tosyl-hydrazono)methyl)benzoate (1g)**. Colorless crystals, mp 165–167°C. IR (KBr, ν, $cm^{-1}$): 3438, 2913, 2843, 1730, 1595, 1458, 1360, 1210, 111, 1083, 960, 775. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.450 (1H, s, OH), 11.4750 (1H, s, OH), 8.438 (1H, s, $CH_3$-3), 7.785 (2H, d, J = 8.253, ArH), 7.639 (2H, d, J = 7.978, ArH), 7.341 (1H, s, NH), 6.254 (1H, s, H-5), 3.915 (3H, s, $COOCH_3$), 2.454 (3H, s, $CH_3$), 2.409 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 23.55, 144.67, 103.3, 161.53, 143.03, 162.57, 111.03, 171.16, 51.185, 143.03, 143.0, 126.53, 128.82, 134.58, 20.59. ESI-HR-MS *m/z* 379.096 $[M + H]^+$ (calcd for $C_{17}H_{19}N_2O_6S$, 379.096).

**Synthesis of 3-Acetylcoumarin (1h)**. Compound **1** (0.05 g, 1 eq.) was reacted with toluene (3 mL) and ethyl acetoacetate (0.03 g, 1 eq.) in the presence of piperidine (2 drops) at room temperature for 2 h. The progress of the reaction was monitored with TLC. The reaction mixture was extracted with dichloromethane (3 × 5 mL). The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give a crude reaction mixture, which was purified by column chromatography to afford **1h** as an orange yellow amorphous powder, yield 62%, mp 144–145°C.

**Methyl 3-Acetyl-5-hydroxy-7-methyl-2-oxo-2*H*-chromene-6-carboxylate (1h)**. Colorless solid, mp 144–145°C. IR (KBr, ν, $cm^{-1}$): 3350, 2971, 2400, 1702, 1654, 1224, 962, 825, 598. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.87 (1H, s, OH), 8.936 (1H, s, CH), 6.694 (1H, s, H-5), 4.016 (3H, s, $COOCH_3$), 2.697 (3H, s, $CH_3$), 2.645 (3H, s, $CH_3$). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 25.108, 149.25, 107.17, 158.44, 110.7, 158.91, 107.9, 171.66, 52.74, 143.20, 121.72, 139.25, 194.72, 30.45. ESI-HR-MS *m/z* 299.0522 $[M + Na]^+$ (calcd for $C_{14}H_{12}O_6Na$, 299.0522).

**Synthesis of Bis-acetate (2g)**. Compound **2** (0.05 g, 1 eq.) in acetic anhydride (3 mL) was stirred for 10 min, followed by addition of 2 drops of pyridine with stirring continued for 12 h. After completion of the reaction, it was treated with dilute hydrochloric acid (3%). The resultant reaction mixture was extracted with chloroform (3 × 5 mL). The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give a crude reaction mixture, which was chromatographed over a silica gel column to afford **2g** as colorless crystals, yield 68%, mp 155–156°C.

**4-(Methoxycarbonyl)-2,5-dimethyl-1,3-phenylenediacetate (2g)**. Colorless crystals, mp 155–156°C. IR (KBr, ν, $cm^{-1}$): 2957, 2926, 2854, 1758, 1623, 1576, 1455, 1369, 1272, 1157, 1081, 1003, 974, 879, 795, 738, 679, 583, 552, 470. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm): 6.852 (1H, s, H-5), 3.875 (3H, s, $COOCH_3$), 2.361 (3H, s, $CH_3$), 2.318 (3H, s, $CH_3$),

2.287 (3H, s, $CH_3$), 1.957 (1H, s, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 20.753, 121.9, 122.002, 148.188, 117.70, 150.69, 136.14, 168.54, 52.15, 9.84, 168.38, 20.08, 166.60, 20.403. ESI-HR-MS *m/z* 303.0834 $[M + Na]^+$ (calcd for $C_{14}H_{16}O_6Na$, 303.0834).

**Anticancer Activity**. The above isolated and synthesized compounds were screened for anticancer activity against five different cell lines, A549 (lung cancer), DU145 (prostate cancer), MCF-7 (breast cancer), SiHa (cervical cancer), and U87MG (glioblastoma) [9].

Briefly, cells were plated at a density of $5 \times 10^3$ cells per well in a 96 well plate supplemented with 10% FBS. After 24 h of incubation at 37°C and 5% $CO_2$, they were treated with the respective concentration of the compounds dissolved in the culture media with vehicle controls and known standards for 48 h. Cell viability was determined by adding 100 µL of the MTT reagent 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (0.5 mg/mL) dissolved in serum-free media to each well and the whole incubated for 4 h. Then the medium was aspirated, the formazan crystals were dissolved in 200 µL of DMSO, and the absorbance was taken at 570 nm in a multimode plate reader (BioTek Instruments, Synergy 4, Winooski, VT). The percent cell inhibition in treated cells was calculated by normalizing the cells with 0% inhibition with the control group.

Then the compounds that exhibited a percentage inhibition greater than 50% at a concentration of 150 µM in the initial screening were further screened using dose response curves with a series of seven concentrations starting from the initial 150 µM. MTT was performed as previously and $IC_{50}$ values were determined from the DRC plot by the linear regression method. Graph of concentration vs. percentage inhibition were plotted. All the values were expressed as means ± SEM in three different experiments in which each treatment was performed in triplicate.

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