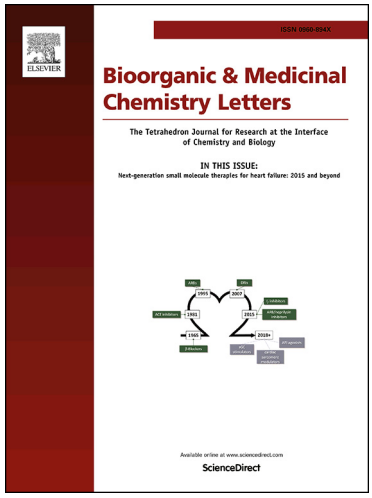
# Accepted Manuscript




Analyzing the role of cannabinoids as modulators of Wnt/β-catenin signalling pathway for their use in the management of neuropathic pain

Yedukondalu Nalli, Mohd Saleem Dar, Nasima Bano, Javeed Ur Rasool, Aminur R. Sarkar, Junaid Banday, Aadil Qadir Bhat, Basit Rafia, Ram A Vishwakarma, Mohd Jamal Dar, Asif Ali

PII: S0960-894X(19)30142-8
DOI: https://doi.org/10.1016/j.bmcl.2019.03.013
Reference: BMCL 26327

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 3 December 2018
Revised Date: 7 March 2019
Accepted Date: 10 March 2019

Please cite this article as: Nalli, Y., Dar, M.S., Bano, N., Rasool, J.U., Sarkar, A.R., Banday, J., Bhat, A.Q., Rafia, B., Vishwakarma, R.A., Dar, M.J., Ali, A., Analyzing the role of cannabinoids as modulators of Wnt/β-catenin signalling pathway for their use in the management of neuropathic pain, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.03.013

ELSEVIER

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

# Analyzing the role of cannabinoids as modulators of Wnt/β-catenin signalling pathway for their use in the management of neuropathic pain

Yedukondalu Nalli[a, 1], Mohd Saleem Dar[b, c, 1], Nasima Bano[b, c], Javeed Ur Rasool[b], Aminur R. Sarkar[a], Junaid Banday[a], Aadil Qadir Bhat[b, c], Basit Rafia[b, c], Ram A Vishwakarma[a, c], Mohd Jamal Dar[b, c, *] and Asif Ali[a, b, *]

[a] *Natural Product Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India.*
[b] *Academy of Scientific and Innovative Research, New Delhi, India*
[c] *Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India.*
[1] *These authors contributed equally to this article*

ARTICLE INFO

*Article history:*
Received
Revised
Accepted
Available online

*Keywords:*
*Cannabis sativa*
Cannabinoids
Anti-proliferative activity
Wnt/β-catenin signalling
Neuropathic pain

ABSTRACT

Neuropathic pain is a debilitating form of treatment-resistant chronic pain caused by damage to the nervous system. Cannabinoids have been known for suppressing neuropathic pain by modulating the endo cannabinoid system. Since the canonical Wnt/β-catenin signalling has recently implicated in pain sensation, we investigated the impact of major cannabinoids (**1**–**6**) from the leaves of *Cannabis sativa* and an epoxy derivative of compound **2**, here upon referred to as **2a**, on modulating Wnt/β-catenin signalling pathway. The results presented in this study show that compound **1**, **2** and **2a** exhibited potent inhibitory activity against Wnt/β-catenin pathway in a dose-dependent manner. Compound **2a** was seen to inhibit this pathway at slightly lower concentrations than its parent molecule **2**, under similar conditions. Taken together, compound **1**, **2** and **2a**, by virtue of their inhibition of Wnt/β-catenin signalling pathway, could be developed as effective neuroprotective agents for the management of neuropathic pain.



Cannabinoids, a family of terpenophenolic compounds, are comprised of naturally occurring active compounds of the *Cannabis sativa* and *Cannabis indica*, endogenous cannabinoids and synthetic cannabinoids. Cannabinoids exert most of their actions by binding to and activating specific G-protein-coupled receptors named as cannabinoid receptors (CB1 and CB2).[1] Cannabinoids are primarily used to alleviate pain, anxiety and depression. Moreover, cannabinoids are applied for reducing nausea and vomiting, inflammation, convulsion, and epilepsy.[2, 3] In addition, synthetic cannabinoids are available as prescription medicine like dronabinol and nabilone in some countries.[4] Neuroprotective properties of cannabinoids have been studied in several *in-vitro* and *in-vivo* neuro-degeneration models.[4-6] The key mechanisms involved in the neuroprotection provided by cannabinoids include cannabinoid receptor-independent effects aimed at reducing the oxidative injury, and also cannabinoid-receptor mediated effects exerted to regulate the neuronal homeostasis.[1, 7-9] However, the exact mechanism of how cannabinoids exert their neuroprotective effects is not yet fully understood.

Wnt signaling is a highly conserved pathway that plays multiple roles in regulating cell proliferation, differentiation, migration, and polarity during various stages of development, particularly, nervous system development.[10, 11] Neuropathic pain, affecting millions of people worldwide, is a neurological disorder resulting from peripheral nerve damage. Recently, Wnt/β-catenin signalling has been shown to play a key role in the pathogenesis of neuropathic pain caused upon nerve injury.[12-16] Pharmacological means used to block Wnt/β-catenin are shown to suppress neuropathic pain in rodent models which include a rat model of chronic constriction injury of the sciatic nerves and a mouse model of bone cancer pain induced by tumour cell implantation.[14] Furtheremore, a recent report showed that the anandamide, an endocannbinoid, is involved in regulating Wnt/β-catenin signalling in MDA MB 231 breast cancer cells.[17] Since cannabinoids are known to show anti-anxiety, anti-depression

* Corresponding author. Tel.: +91-191-2569222; fax: +91-191-25693331; e-mail: asifali@iiim.ac.in (A Ali); jamal@iiim.ac.in (M J Dar)

and anti-proliferative effects apart from alleviating pain.[5, 7-9] The current study was initiated to examine the cellular effects of naturally occurring cannabinoids on regulating the Wnt/*β*-catenin signaling pathway by analysing the impact of these cannabinoids on TCF-dependent *β*-catenin mediated transcription regulation.

In this study, we isolated six known compounds from *Cannabis sativa*, and an epoxy derivative **2a** was synthesized from **2**. We characterized these cannabinoids by 1D and 2D NMR spectral data. We then assessed the anti-proliferative activity of these cannabinoids on a panel of cancer cell lines. Since cannabinoids are known to alleviate pain, we examined the impact of these cannabinoids on modulating Wnt/*β*-catenin signalling pathway, one of the most critical pathways implicated in regulating neuropathic pain.

We extracted air dried and powdered leaves of *Cannabis sativa* with hexane and fractionated it in an open column using silica gel (100-200 mesh) column which resulted in 10 fractions (Fr.1-Fr.10). Fr.3 (10 g) had major cannabinoids. Upon further passing through Sephadex LH20, we collected ten fractions (Fr.3-1 to Fr.3-10). Fr.3-5 (450 mg) was filtered through a 0.45 µm filter, and the MeOH soluble fraction was analysed by semi-preparative HPLC. Its chromatogram showed six peaks in major quantity, and all the six compounds were isolated with > 95% purity: **1** (6 mg, $t_R$ 14.7 min), **2** (42 mg, $t_R$ 18.3 min), **3** (15 mg, $t_R$ 21.4 min), **4** (30 mg, $t_R$ 24.9 min), **5** (48 mg, $t_R$ 30.5 min), **6** (27 mg, $t_R$ 34.7 min). The structures of the compounds were characterized as cannabidivarin (**1**),[18] cannabidiol (**2**),[19] $\Delta^1$-tetrahydrocannabidivarol (**3**),[18] cannabinol (**4**),[19] $\Delta^9$-tetrahydrocannabinol (**5**)[19] and cannabichromene (**6**)[20] by HRESIMS, NMR (1D and 2D spectroscopy analysis, and compared with those reported in literature (Fig. 1).

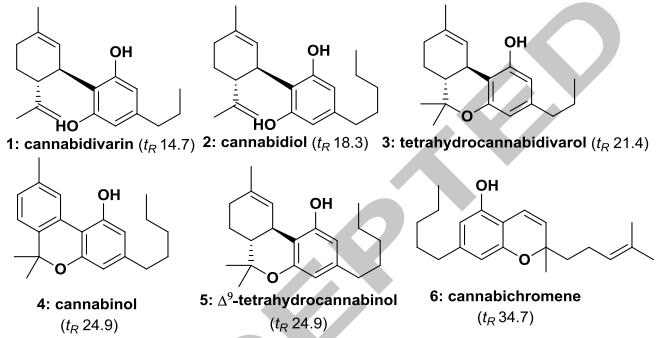
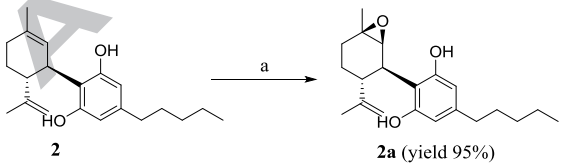



**Figure 1**. Structures of compounds isolated from *cannabis sativa*.

Further, 2, 3-epoxy derivative (**2a**) was synthesized by treatment of CBD-5 in acetone with oxone at room temperature for overnight which afforded 95% yield (Scheme 1).[21]




**Scheme 1.** Reagents and conditions: (a) Oxone (3.0 equiv.), acetone, rt, overnight

Indeed Yamamoto *et al* synthesized **2a** from **2** as colourless needles with mp 65 °C in two steps via CBD-2'-6'-diacetate and characterized it by only $^1$H NMR ($CDCl_3$).[22] However, the compound **2a** that we synthesized here is assumed to be its stereoisomer as it is a yellow colour liquid, and most of its $^1$H NMR chemical shifts mismatched with reported data.[22] Thus, we characterized the structure of **2a** by HRESIMS, and 1D- and 2D-NMR spectroscopic data analyses. The molecular formula was assigned as $C_{21}H_{30}O_3$ based on the protonated molecular ion at *m/z* 331.2272 $[M + H]^+$ (calcd for $C_{21}H_{31}O_3$, *m/z* 331.2273) in its positive-ion HR-ESI-MS, confirmed seven indices of hydrogen deficiency. Detailed analyses of 2D NMR spectroscopic data (Table S1) provided structure of **2a** as shown in Figure 2. The relative configuration of **2a** was determined by analyzing NOESY NMR correlations (Fig. 2). The NOESY correlations between $H_3$-7/H-2, H-2/H-1 and H-1/ H-8 (Fig. 2) suggested that $H_3$-7, H-2, H-1, H-8 were on same side (*α* oriented), and epoxy was *β* oriented (Fig. 2).

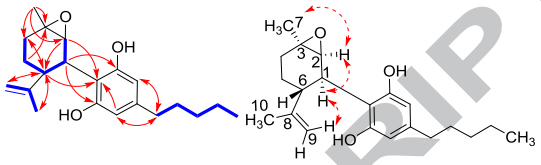


**Figure 2.** Key $^1$H–$^1$H COSY (—), HMBC (⤻) and $^1$H–$^1$H NOESY (⤻) correlations of 2, 3-epoxy derivative (**2a**).

*Determining effect of cannabinoids on cell proliferation by MTT assay*. Since the major drawback of many chemotherapeutic agents is their cellular toxicity. Thus, these six cannabinoids were evaluated for their cytotoxicity by MTT assay on a panel of cancer cell lines that include HCT-116, OVCAR, A549, MCF7, PC-3, HepG2 and SH-SY5Y cells spanning different tumour types (colon, ovary, lungs, breast, prostate, liver and neuroblastoma). The addition of CBDs to the culture medium of these cell lines for 24, 48 and 72 h resulted in a concentration-dependent inhibition of the cell growth as determined by MTT assay. The range of concentrations tested was from 1 µM to 100 µM. The results shown in Table-1 indicate that cannabinoid treatment for 72 hours caused a significant decrease in the viability of these cell lines when compared to DMSO control. These cannabinoids showed varying degrees of growth inhibition across different cell lines. Since HepG2 cells are considered a standard cell line for screening small molecule inhibitors of Wnt/*β*-catenin signalling due to the presence of an interstitial deletion at the N-terminal domain of *β*-catenin protein, we calculated $IC_{50}$ values for these compounds in HepG2 cells. Compounds **3**, **4**, **5** and **6** were relatively less effective than **1**, **2** and **2a**, and we compared the effect of **1**, **2** and **2a** in HepG2 cells with that of salinomycin, a natural product-based inhibitor of Wnt/*β*-catenin signalling pathway. Compounds **1**, **2** and **2a** are seen to be less toxic than salinomycin in these cells under similar conditions.

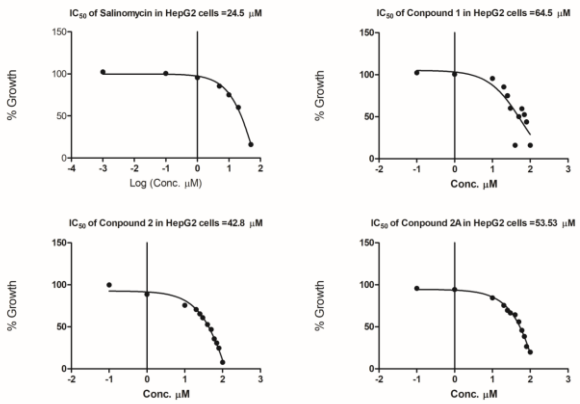



**Figure 3.** Comparison of dose-response curve of selected cannabinoids in HepG2 cells. The experiments performed in triplicate; 50% inhibition of cell growth ($IC_{50}$) used as the analysis parameter calculated by Prism GraphPad (version 5.0) software. The One-Way ANOVA analysis was used to compare the mean values ($p < 0.05$).

**Table 1.** *In-vitro* cytotoxicity (IC50) of cannabinoids on a panel of selected cncer cells post-72 hr treatment.

| Molecule/Cell Line | 1 | 2 | 2a | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| **HCT-116** | 13±3.33 | 18±1.00 | 15.3±1.30 | 83±2.98 | 80±3.23 | 42±1.97 | 60±3.22 |
| **OVCAR** | 57±3.01 | 50±3.00 | 56.8±4.28 | 75±5.32 | 77±4.01 | 63±2.75 | 65±4.44 |
| **A549** | 39±2.10 | 40±2.40 | 45.2±4.22 | 90±3.20 | 91±3.98 | 45±4.44 | 60±1.88 |
| **MCF7** | 53±2.60 | 55±2.20 | 47.2±3.50 | 80±2.97 | 81±3.44 | 57±3.86 | 40±2.32 |
| **PC-3** | 44±1.88 | 12±0.90 | 17.6±3.06 | 79±3.09 | 78±4.11 | 65±5.07 | 40±1.79 |
| **HEPG2** | 64±40 | 42.8±1.19 | 53±2.54 | 75±4.22 | 74±5.08 | 59±3.29 | 60±4.02 |
| **SH-SY5Y** | 55±5.32 | 48±5.12 | 51±2.49 | 95±4.27 | 97±5.05 | 50±2.99 | 77±5.82 |

As mentioned above, Wnt/*β*-catenin signalling has recently implicated in neuropathic pain and the fact that cannabinoids are known to alleviate pain in numerous studies. We then examined the impact of these cannabinoids on Wnt/*β*-catenin signaling pathway in HepG2 cells. We monitored TCF-dependent *β*-catenin mediated transcription activity using Top-Flash reporter assay in which co-transfection of TopFlash (TOP) or FopFlash (FOP) and Renilla plasmids into HepG2 cells was carried out under basal and serum-free conditions as described by dar et al.[23] Four hours post-transfection, cells are treated with cannabinoids in a concentration-dependent manner (well below their $IC_{50}$ values), and luminescence measured at 24h post-treatment. To perform these experiments, we used salinomycin –a natural product-based small molecule inhibitor of Wnt/*β*-catenin signalling, as a positive control.[24] Interestingly, compound **1**, **2** and **2a** were seen to significantly decrease the TopFlash activity in a dose-dependent manner when compared to solvent control. Salinomycin treatment resulted in a decrease in Top-Flash activity in a dose-dependent manner under similar conditions (**Fig. 4).**

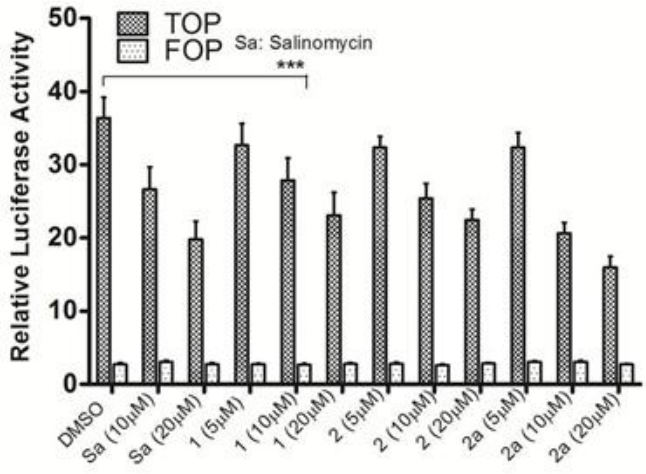



**Figure 4.** Top-Flash activity of cannabinoids in HepG2 cells: Reporter activity was determined upon co-transfection of TopFlash (TOP) or FopFlash (FOP) and Renilla plasmids into HepG2 cells under basal and serum-free conditions (materials and methods). The graph shown is representative of three independent experiments (Mean ± SD, ***P < 0.001).

We also analysed the Top-Flash activity of **3**, **4**, **5** and **6**. However, these cannabinoids failed to show any significant activity (Fig. 5). Taken together, we show that **1**, **2** and **2a** significantly decreased *β*-catenin transcriptional activity in HepG2 cells in a dose-dependent manner. Thus, we anticipate compound **1**, **2** and **2a** could be developed as effective neuroprotective agents for the management of neuropathic pain.

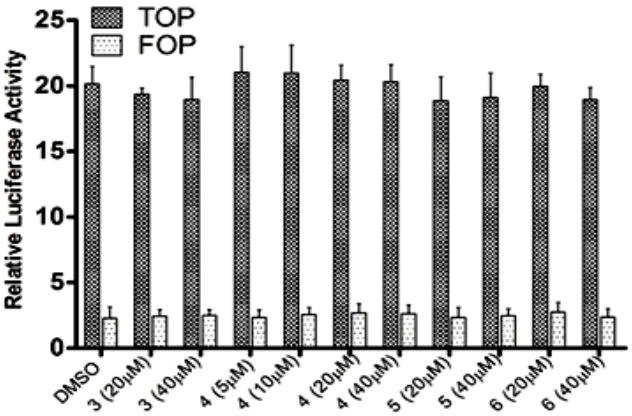



**Figure 5.** Top-Flash activity of cannabinoids in HepG2 cells. Reporter activity was determined upon co-transfection of TopFlash (TOP) or FopFlash (FOP) and Renilla plasmids into HepG2 cells under basal and serum-free conditions (materials and methods). The graph shown is representative of three independent experiments. (Mean ± SD, ***P < 0.001).

In summary, we report the isolation and characterization of six cannabinoids from *Cannabis sativa* plant and synthesis of an epoxy derivative of **2**. Next, we examined the anti-proliferative activities of these cannabinoids on a panel of cancer cell lines spanning different cancers. We then analyzed the impact of these cannabinoids on Wnt signaling by monitoring the TCF-dependent *β*-catenin Top-Flash reporter activity in HepG2 cells. Pertinently, Compound **1**, **2** and **2a** were seen to significantly decrease the Top-Flash reporter activity in a dose-dependent manner. Thus, we report that these compounds are relatively lesss toxic and could be developed as effective neuroprotective agents for the management of neuropathic pain because of their ability to modulate activity of Wnt/*β*-catenin signaling pathway.

## Acknowledgement

This work supported by a grant from the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi, under- HCP0008.

## References and notes

1. Soderstrom K, Soliman E, Van Dross R. Cannabinoids modulate neuronal activity and cancer by CB1 and CB2 receptor-independent mechanisms. *Frontiers in Pharmacology.* 2017;8: 720.
2. Perucca E. Cannabinoids in the treatment of epilepsy: hard evidence at last? *Journal of Epilepsy Research.* 2017;7(2): 61-76.
3. Kogan NM, Mechoulam R. Cannabinoids in health and disease.

*Dialogues in Clinical Neuroscience.* 2007;9(4): 413-430.

4. Krcevski-Skvarc N, Wells C, Hauser W. Availability and approval of cannabis-based medicines for chronic pain management and palliative/supportive care in Europe: a survey of the status in the chapters of the European Pain Federation. *Eur J Pain.* 2018;22(3): 440-454.
5. Meng H, Johnston B, Englesakis M, Moulin DE, Bhatia A. Selective cannabinoids for chronic neuropathic pain: a systematic review and meta-analysis. *Anesth Analg.* 2017;125(5): 1638-1652.
6. Maroon J, Bost J. Review of the neurological benefits of phytocannabinoids. *Surg Neurol Int.* 2018;9: 91.
7. Fernández-Ruiz J, Moro MA, Martínez-Orgado J. Cannabinoids in neurodegenerative disorders and stroke/brain trauma: from preclinical models to clinical applications. *Neurotherapeutics.* 2015;12(4): 793-806.
8. Sagredo O, Garcia-Arencibia M, de Lago E, Finetti S, Decio A, Fernandez-Ruiz J. Cannabinoids and neuroprotection in basal ganglia disorders. *Mol Neurobiol.* 2007;36(1): 82-91.
9. Rahn EJ, Hohmann AG. Cannabinoids as pharmacotherapies for neuropathic pain: From the bench to the bedside. *Neurotherapeutics.* 2009;6(4): 713-737.
10. Kahn M. Can we safely target the WNT pathway? *Nat Rev Drug Discov.* 2014;13(7): 513-532.
11. Rosso SB, Inestrosa NC. WNT signaling in neuronal maturation and synaptogenesis. *Front Cell Neurosci.* 2013;7: 103.
12. Harrison C. A new player in neuropathic pain pathogenesis. *Nature Reviews Drug Discovery.* 2013;12: 422.
13. Itokazu T, Hayano Y, Takahashi R, Yamashita T. Involvement of Wnt/*β*-catenin signaling in the development of neuropathic pain. *Neurosci Res.* 2014;79: 34-40.
14. Zhang YK, Huang ZJ, Liu S, Liu YP, Song AA, Song XJ. WNT signaling underlies the pathogenesis of neuropathic pain in rodents. *J Clin Invest.* 2013;123(5): 2268-2286.
15. Shi Y, Yuan S, Li B, et al. Regulation of Wnt signaling by nociceptive input in animal models. *Mol Pain.* 2012;8: 47.
16. Kurimoto S, Jung J, Tapadia M, et al. Activation of the Wnt/*β*-catenin signaling cascade after traumatic nerve injury. *Neuroscience.* 2015;294: 101-108.
17. Laezza C, D'Alessandro A, Paladino S, et al. Anandamide inhibits the Wnt/*β*-catenin signalling pathway in human breast cancer MDA MB 231 cells. *European journal of cancer (Oxford, England : 1990).* 2012;48(16): 3112-3122.
18. Dialer L, Petrovic D, Weigl U. Preparation of cannabidiol and Δ9-tetrahydrocannabinol. Noramco, Inc., USA . 2017:94.
19. Choi YH, Hazekamp A, Peltenburg-Looman AM, et al. NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of Cannabis sativa. *Phytochem Anal.* 2004;15(6): 345-354.
20. Wang G, Zhu L, Zhao Y, et al. A natural product from *Cannabis sativa* sub sp. sativa inhibits homeodomain-interacting protein kinase 2 (HIPK2), attenuating MPP+-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Bioorg Chem.* 2017;72: 64-73.
21. *2-(6-methyl-3-(prop-1-en-2-yl)-7-oxabicyclo[410]heptan-2-yl)-5-pentylbenzene-1,3-diol (**2a**). Compound **2** (50 mg, 015 mmol) dissolved in acetone and added oxone (3 eq, 047 mmol) The resulting mixture was stirred overnight at room temperature. After completion of the reaction, acetone was removed in vacuo, and the crude residue purified by silica gel column chromatography (hexanes/EtOAc = 98:5 to 9:2) to obtained compound **2a** (350 mg, 011mmol) (yield 95%) as a yellow oil;* $[\alpha]_D^{25}$ *+88 (c 01, acetonitrile); See table 1 for* $^1$*H NMR (400 MHz,* $CDCl_3$*) and* $^{13}$*C NMR (100 MHz,* $CDCl_3$*); HRESIMS m/z 3312272* $[M + H]^+$ *(calcd for* $C_{21}H_{31}O_2$*, 3312273).*
22. Yamamoto I, Gohda H, Narimatsu S, Yoshimura H. Mechanism of biological formation of cannabielsoin from cannabidiol in the guinea-pig, mouse, rat and rabbit. *Journal of Pharmacobio-Dynamics.* 1989;12(8): 488-494.
23. Dar MS, Singh P, Singh G, et al. Terminal regions of *β*-catenin are critical for regulating its adhesion and transcription functions. *Biochimica et biophysica acta.* 2016;1863(9): 2345-2357.
24. Lu D, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(32): 13253-13257.

## Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

**Analyzing the role of cannabinoids as modulators of Wnt/*β*-catenin signalling pathway for their use in the management of neuropathic pain**

Leave this area blank for abstract info.

Yedukondalu Nalli, Mohd Saleem Dar, Nasima Bano, Javeed Ur Rasool, Aminur R. Sarkar, Junaid Banday, Aadil Qadir Bhat, Basit Rafi, Ram A Vishwakarma, Mohd Jamal Dar and Asif Ali