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Anticancer activity studies of cubebin isolated from *Piper cubeba* and its synthetic derivatives

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ABSTRACT

(−)-Cubebin, isolated from the seeds of *Piper cubeba*, and its five different types of derivatives (a total of 17), with varying functionalities, were tested for their in vitro anticancer activity against six human cancer cell lines (A549, K562, SiHa, KB, HCT116 and HT29) using MTT assay. Cubebin as well as its derivatives containing lactone and amide groups showed significant anticancer activity. In some of the tested cell lines, the amide derivatives showed higher activity. Morphological analysis indicated that these compounds act through apoptosis mediated pathway of cell death and we expect that these results will pave new paths in the development of novel anticancer agents by the derivatization of (−)-cubebin.

Cancer is one of the most fatal diseases, which claims millions of lives worldwide per annum and it stands second to cardiovascular diseases as a leading cause of death. It is the single cause of nearly 12.5% of overall mortality rates and it is expected that by 2030, the number of cases and deaths caused by cancer will rise above 21.4 million and 13.1 million, respectively.[1–4] Despite the fast growth of other drug discovery methods such as synthetic and combinatorial chemistry, plant-derived compounds and their synthetic analogs contribute their fair share in cancer chemotherapy.[5–10] Lignans, an important class of plant secondary metabolites have served as lead compounds for the development of novel anticancer agents. Success stories include that of the anticancer lignans such as etoposide and teneposide, which are the semi synthetic derivatives of the lignan lactone podophyllotoxin.[11,12] Apart from podophyllotoxin, lignans such as wikstromol, burseran, steganacin, steganangin, enterodiol, enterolactone etc. are also known to have very good in vitro anticancer activities.[13–15]

Piper species, belonging to Piperaceae family, widely distributed all over the world especially in tropical and subtropical regions, are rich source of bioactive lignans and neolignans.[16] The species comprise of a large number of medicinally and economically important plants which are extensively used in traditional systems of medicine. *Piper cubeba*, an important medicinal plant belonging to Piperaceae family, contains a large number of bioactive lignans in it.[17] *P. cubeba* extract was found to be effective against breast cancer and prostate cancer growth.[18,19] Major compound present in *Piper cubeba* seeds is the butyrolactol lignan, (−)-cubebin (**1**).[17,20] (−)-Cubebin has been reported to have trypanocidal,[21–23] anti-inflammatory,[24] analgesic,[25] vasorelaxant,[26] antimycobacterial and antiprotozoal[27] activities. (−)-Hinokinin (**4**), a butyrolactone lignan, isolated from the same plant was found to have very high trypanocidal,[21,23,28] analgesic, antiinflammatory,[29] antimutagenic,[30,31] chemopreventive,[32] modulatory effects on human monoamine, GABA transporter[33] and anticancer activities.[34,35] The compound was also found to be effective against oral pathogens including *Streptococcus mutans*.[36]

Even though (−)-cubebin is the most abundant compound in *P. cubeba*, the only studies reported on its synthetic derivatives and their bioactivity includes that of Silva et al.[37] who reported the antiinflammatory and analgesic activities. These derivatives were also evaluated for their activity against *Trypanosoma cruzi*,[38] the causative agent of Chaga's disease. There have been no further reports in the area of chemical modification of (−)-cubebin and nor has cubebin and its derivatives been explored in detail for their anticancer activity. Herein we report the isolation and anticancer

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activity studies of (−)-cubebin from *P. cubeba* seeds and its chemical diversification. Derivatives with varying functionalities were synthesised and tested for their anticancer activity against six human cancer cell lines using in vitro methods using MTT assay. Morphological analysis was also carried out to understand the pathway of cell death.

(−)-Cubebin was isolated from the defatted acetone extract of *Piper cubeba* seeds (Experimental details are given in Supplementary material). As shown in Scheme 1, lactol ring of (−)-cubebin (**1**) was converted into four different functionalities in order to get an insight into the structural requirement for anticancer activity. (−)-Dihydrocubebin (**2**) was obtained by the reduction of cubebin using sodium borohydride. Cubebin readily resulted into the cyclic ether under Wittig reaction condition using ethyl (triphenylphosphoranylidene) acetate to give 9-substituted product, which on alkaline hydrolysis using 10% aqueous sodium hydroxide gave compound **3**. Cubebin was also converted into the natural lignan lactone (−)-hinokinin (**4**) by PCC oxidation. Lactone ring of (−)-hinokinin (**4**) was then opened using a primary amine (*p*-methoxy benzylamine) to give the respective amide **5a**. Structures of all these compounds were confirmed by employing various spectroscopic techniques (Supplementary material). Synthesis of amide and cyclic ether derivates of (−)-cubebin are being reported here for the first time.

Preliminary investigation on the in vitro anticancer activity of these five compounds (**1**–**5a**) against six human cancer cell lines viz., A549 (human lung adenocarcinoma), KB (human nasopharyngeal carcinoma), K562 (human chronic myeloid leukemia), SiHa (human cervical carcinoma), HCT116 and HT29 (human colon carcinoma) was carried out using MTT assay and the results are reported in terms of IC_{50} values (the concentrations of test compounds required inhibit 50% of cell growth). Results (Table 1) indicate that the natural lignans, (−)-cubebin (**1**) and (−)-hinokinin (**4**) possess very good activity against A549, K562 and KB cell lines but was found to be less effective against other cell lines. Hinokinin showed better activity compared to cubebin in most of the tested cell lines, suggesting that compound with lactone ring is more active. The amide derivative **5a** showed better results than the parent compounds cubebin/hinokinin in most of the tested cell lines. Compounds containing dihydro (dihydrocubebin, **2**) and cyclic ether (**3**) functionalities were found to be less effective. From the results obtained, it is clear that compounds possessing lactone ring (**4**) and free amide group (**5a**) show slightly higher activity followed by the lactol compound cubebin (**1**), where as the dihydro (**2**) and cyclic ether (**3**) derivatives were not found to be very effective.

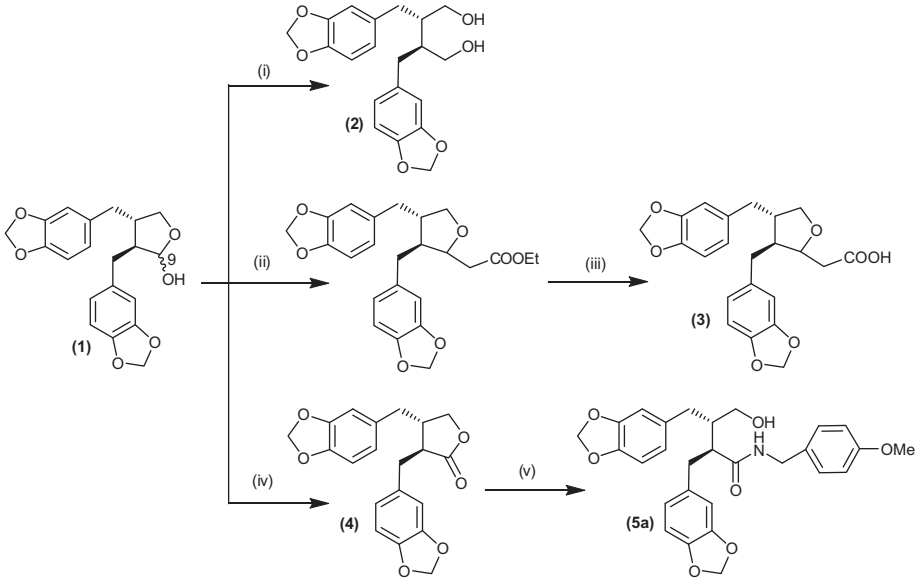
Promising results obtained in the case of amide derivative **5a**, prompted us to invest our time in synthesizing a library of amide derivatives (**5a–5i)** as shown in Table 2 and to evaluate their cytotoxic activity.

The amide derivatives were synthesised in good yield by treating (−)-hinokinin (**4**) with respective primary amines under reflux condition, without the use of any catalyst. The absence of any metal/non-metal catalyst makes the process an environmentally benign one. Structure and stereochemistry of the amide derivatives were unambiguously confirmed from spectral data (Supplementary material) and the single crystal X-ray structure obtained for **5d** (Fig. 1). All the benzyl amines gave fairly good yield, but the use of tryptamine and hydroxy substituted alkyl amine resulted in the reduction of yield. Secondary amines did not give the expected product under the above reaction conditions.

Amides **5a**–**5e** were then oxidized into corresponding succinimide derivatives (**6a**–**6e**, Scheme 2) using PCC as the oxidant, cytotoxicity of these compounds were also checked. To the best of our knowledge, this is the first report on the synthesis of succinimide derivatives of (−)-cubebin.

Amides (**5a–5i)** as well as the imides (**6a**–**6e**) derivatives were tested for their cytotoxic activity. On comparing the IC_{50} values (Table 3) it has been found that some of the amide derivatives have higher activity than the parent compounds cubebin and hinokinin.

In the case of A549 cell lines, the amide derivatives **5a**, **5d** and **5f** were more active than the natural lignans cubebin (**1**) and hinokinin (**4**). Against K562 cell lines the amide **5c** was found to be most active one and all the other benzyl amide derivatives showed comparable activity. Very interestingly, in the case of KB cell lines **5h** was found to be the most potent one, followed by **5d**. The compound **5h**, the amide derived from tryptamine, showed significant activity only against KB cell lines. All the tested compounds were found to be less effective against SiHa, HCT116 and HT29 cell lines. From these results it is clear that the ethyl phenyl substituted and



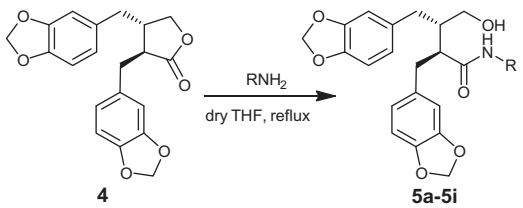
Scheme 1. Reagents and conditions: (i) $NaBH_4$, MeOH, 0 °C to rt, 24 h (ii) PPh_3=CH—COOEt, dry toluene, reflux, 24 h (iii) Aq. NaOH (10%), MeOH, reflux, 3 h (iv) PCC, dry DCM, 12 h (iv) *p*-methoxy benzylamine, dry THF, reflux, 36 h.

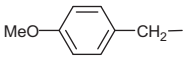
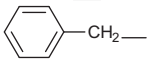
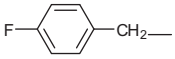
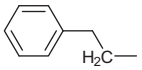
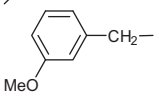
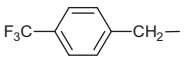
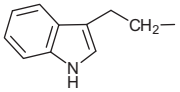
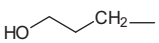
Table 1
IC_{50} value of compounds **1–5a**

Compound	[a]IC_{50} (μM)					
	A549	K562	SiHa	KB	HCT116	HT29
1	8.30 ± 0.16	8.66 ± 0.43	>100	8.16 ± 0.41	45.06 ± 3.7	45.2 ± 0.87
2	75.55 ± 1.1	30.17 ± 5.65	>100	**7.82 ± 0.38**	85.32 ± 6.4	>100
3	52.86 ± 6.9	**7.94 ± 0.45**	>100	73.88 ± 1.3	>100	>100
4	**7.86 ± 0.54**	9.07 ± 0.41	**68.4 ± 4.0**	**7.68 ± 0.53**	72.58 ± 6.2	**35.7 ± 1.23**
5a	**6.61 ± 0.42**	**8.37 ± 0.19**	**91.50 ± 0.31**	9.17 ± 0.26	46.06 ± 1.6	51.1 ± 0.90

[a] IC_{50}—data represented as mean ± S.D. from results obtained in triplicate. IC_{50} values of the compounds which showed better activity than (−)-cubebin are highlighted using bold letters.

Table 2
Synthesis of amide derivatives **5a–5i**



Entry	Compound	R	Time (h)	%Yield[a]
1	**5a**		36	73
2	**5b**		36	70
3	**5c**		36	78
4	**5d**		36	84
5	**5e**		36	71
6	**5f**		48	71
7	**5g**		48	74
8	**5h**		48	40
9	**5i**		48	54

[a] %Yield—percentage yield of isolated compound.

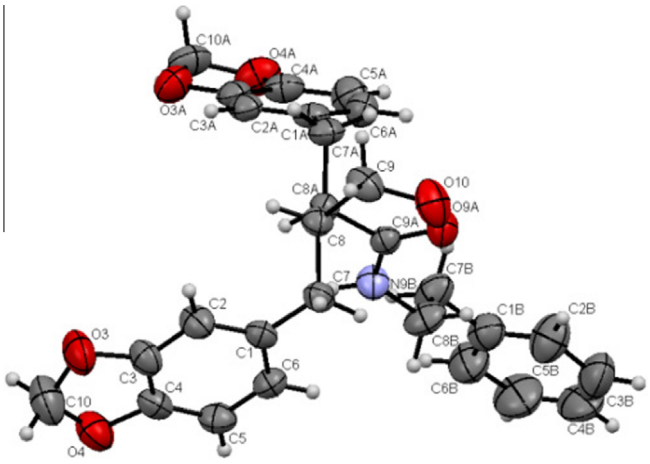
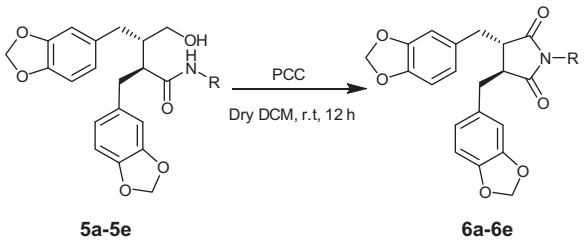


Figure 1. ORTEP diagram of **5d**.



Scheme 2. Synthesis of **6a–6e**.

benzyl substituted amides were found to be more active and among the various benzyl substituted amides one with *para* substituent is found to be more effective than unsubstituted or *meta* substituted ones. The amide formed from tryptamine was found to be effective only against KB cell lines and amide containing hydroxyl group showed only a very little effect against all the cell lines tested.

Succinimide derivatives (**6a–6e**) obtained by the oxidation of corresponding amides were also tested for their in vitro anticancer activity and were found to be less effective than the parent amide. They showed only poor anticancer activity against all the tested cell lines except in the case of K562 and KB cell lines. Comparison of the IC_{50} values of the test compounds with literature values obtained for podophyllotoxin and etoposide (currently used as chemotherapeutic agent) indicates that cubebin as well as its lactone and amide derivatives possess promising in vitro anticancer activity.

To check whether in the present study, the cell death occurred through necrosis or apoptosis, morphological analysis were carried out. For this, two cell lines A549 and KB were selected since the tested compounds showed very good activity against these cell lines. Cells were treated with (−)-hinokinin (**4**) and the respective amide which showed maximum activity, that is **5a** against A549 and **5h** against KB.

After 48 h of incubation, cell shrinkage was observed and there is loss of normal nuclear architecture. Cells were found detached and floated in the medium when analyzed using inverted microscope (Fig. S1a). In acridine orange-ethidium bromide staining assay, normal cells appeared green and the apoptotic cells showed red colored condensed nuclei when viewed under fluorescence microscope (Fig. S1b). Cell shrinkage, membrane blebbing, and chromosomal condensation were observed in cells treated with the compounds indicating that cell death occurred through apoptosis mediated path way. In Hoechst 33342 staining assay, blue fluorescence was observed for normal cells which contained regular and round shaped nuclei in them. Apoptotic cells were characterized by the condensation and the fragmentation of nuclei with increased brightness (Fig. S1c). An increased number of cells with condensed and fragmented nuclei were observed after treatment with the compounds, which is the salient morphological feature

Table 3
IC_{50} value of compounds **5a–6e**

Compound	[a]IC_{50} (μM)					
	A549	K562	SiHa	KB	HCT116	HT29
5a	**6.61 ± 0.42**	8.37 ± 0.19	91.50 ± 0.31	9.17 ± 0.26	46.06 ± 1.6	51.1 ± 0.90
5b	10.85 ± 2.1	8.60 ± 1.35	>100	9.16 ± 1.1	93.51 ± 3.1	68 ± 1.56
5c	8.94 ± 0.96	**7.76 ± 0.24**	80.0 ± 3.34	33.8 ± 0.41	34.10 ± 6.2	**24.6 ± 1.11**
5d	7.28 ± 0.35	7.91 ± 0.24	57.46 ± 9.8	7.09 ± 0.61	**7.83 ± 1.2**	58.9 ± 0.87
5e	8.75 ± 0.71	48.84 ± 4.19	95.82 ± 7.6	8.94 ± 0.42	66.83 ± 6.4	23.8 ± 0.97
5f	7.28 ± 0.39	7.98 ± 0.56	**25.32 ± 0.87**	80.5 ± 6.5	>100	25.5 ± 0.78
5g	>100	8.86 ± 0.16	73.70 ± 8.9	19.2 ± 0.51	>100	64.91 ± 0.10
5h	>100	>100	>100	**6.38 ± 0.35**	>100	52.1 ± 1.90
5i	>100	87.38 ± 11.3	85.18 ± 2.7	7.96 ± 0.45	45.19 ± 4.2	51.1 ± 2.1
6a	45.49 ± 2.9	8.69 ± 0.18	33.35 ± 2.6	9.13 ± 0.13	>100	72.57 ± 0.46
6b	58.61 ± 7.5	8.88 ± 0.18	>100	7.36 ± 0.38	65.43 ± 3.3	71.8 ± 0.13
6c	>100	>100	>100	8.95 ± 1.0	>100	72.1 ± 0.99
6d	>100	9.15 ± 0.45	96.26 ± 3.9	62.57 ± 3.4	64.04 ± 5.3	66.55 ± 0.89
6e	>100	>100	89.29 ± 3.6	7.71 ± 0.78	67.70 ± 0.97	66.21 ± 0.08
Cisplatin	–	–	–	19.2 ± 0.19	–	–
Paclitaxel	10.0 ± 0.1	–	–	–	–	–
Podophyllotoxin	13.62[39]	–	–	–	0.01[40]	2.29 ± 0.97[41]
Etoposide	14.8[42]	4.39 ± 1.2[43]	30.7[42]	3.88 ± 0.12[44]	–	1.47 ± 0.06[45]

[a] IC_{50}—data represented as mean ± S.D. from results obtained in triplicate. IC_{50} values of the amide derivatives which showed maximum activity are highlighted using bold letters.

of apoptosis. Detailed studies of the molecular target are required in order to confirm the real potency of these derivatives before entering into in vivo studies.

In conclusion the natural lignan lactol (−)-cubebin was isolated in good yield from *Piper cubeba* seeds and chemical diversification of the compound was achieved by modifying the lactol ring. Cubebin and its derivatives were screened for their anticancer activity against six human cancer cell lines (A549, K562, SiHa, KB, HCT116 and HT29) using MTT assay. From the results obtained it is clear that compounds containing amide group is the most active one followed by lactone and lactol compounds. Compounds containing dihydro, cyclic ether and imide functionalities were found to be less effective. Among the various amides, amide derived from ethyl phenyl amine and *para* substituted benzyl amides were the most active ones. From the morphological analysis it has been confirmed that these compounds act through apoptosis mediated pathway and further detailed studies are required for establishing the actual mechanism for apoptosis induction.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.041.

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