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In-vitro ANTICANCER ACTIVITY OF LICHEN *Heterodermia boryi* AND ITS SECONDARY METABOLITES

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ABSTRACT

Lichens are symbiotic organisms that have been traditionally used for treating different kinds of ailments. *Heterodermia boryi* is a common foliose lichen found growing luxuriantly in the Western Ghats. It is clear from the literature survey that there are no reports on the isolation and biological activity of lichen substances from *Heterodermia boryi,* we thought of exploring the anticancer activity of its crude extract and isolated compounds. Fractionation of crude methanolic extract of *Heterodermia boryi* by column chromatography led to the isolation of three pure compounds. The crude methanolic extract and isolated compounds were screened for *in vitro* cytotoxic activity against three different human cancer cell lines such as breast (MCF-7), lung (H1975), and colon (HCT-116). Isolated compounds with the highest activity were further evaluated for their ability to induce apoptosis by dual staining method on the MCF-7 cell line. Molecular docking studies were carried out to study the ability of isolated compounds to interact with Mitogen-activated protein kinases- 2 (Mnk2). The isolated compounds were characterized as methyl haematommate (HKB-1), atraric acid (HKB-4), and zeorin (HKB-6) by spectral methods. Among the isolated compounds, HKB-1 and HKB-6 exhibited superior IC_{50} values of 11.1 µg/ml and 17.02 µg/ml respectively. Dual staining of MCF-7 cells treated with HKB-1 and HKB-6 at 10 µg/ml displayed early signs of apoptosis. Molecular docking studies revealed excellent binding affinity and interaction of HKB-1 with the binding site residues of Mnk2 and were comparable with that of the co-crystallized ligand staurosporine indicative of their possible role as Mnk2 inhibitors.

Keywords: Anticancer Activity, Lichen, *Heterodermia boryi*, MTT Assay, Molecular Docking

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INTRODUCTION

Lichens are symbiotic organisms formed by the association of algae and a fungus. They are long-lived and slow-growing organisms that produce a wide array of unique secondary metabolites. Such slow-growing organism in low-resource environments is capable of producing self-protective chemicals.[1] So far more than 1050 diverse secondary metabolites have been reported from lichens.[2-4] In Ayurveda and Unani systems, lichens have been used for treating different ailments.[5] The lichens have been exploited by natives of America, Haiti, India, China, and Europe for centuries in their native folk medicines.[4, 6] Lichen species of the genus *Heterodermia* are commonly seen in tropical regions and they belong to the family *Physciaceae*. *Heterodermia* species are known to have ethnomedicinal and traditional uses and the crude extracts of some species have been tested for various biological activities, such as antimicrobial,

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antioxidant, anticancer, anti-inflammatory, and anthelmintic activity.[7-10] Phytochemical studies on various *Heterodermia* species have revealed the presence of lichen substances such as atranorin, zeorin, salazinic acid, and norstictic acid.[11,12] Among the various *Heterodermia* species found in the Western Ghats region, *H. boryi* (Fée) Kr. P. Singh and S.R. Singh, are the widely distributed lichens. Most of the earlier reports on the biological activity of this species have been carried out only on crude extracts, which exhibited activities such as antimicrobial, antioxidant, and anthelmintic.[13-15] Cancer remains a major feared public health issue globally, with an estimated 19.3 million new cancer cases diagnosed and nearly 10 million deaths in 2020.[16,17] Primary treatment options for cancer include surgery, radiation therapy, and chemotherapy. Patient care has improved in recent times due to the availability of anti-cancer drugs. Unfortunately, the normal cells are also adversely affected by the chemotherapy resulting in the suppression of bone marrow function and other adverse effects such as nausea, vomiting, and alopecia and concerns regarding the emergence of drug resistance.[18,19] The oncogenes are a composite, multi-step process involving multiple signaling events changing a normal cell to a cancerous one. The review of the literature suggests that biomolecules from natural sources can act against diverse signaling events in cancer cells without affecting normal cells.[20,21] Therefore in the present study, secondary metabolites from lichens are explored for their possible anticancer activity. As there are no reports on the isolation and biological activity of lichen substances from *Heterodermia boryi* we thought of exploring the anticancer activity of its crude extract and isolated compounds.

EXPERIMENTAL

General Procedure

The spectral measures (MS and NMR) were recorded by using standard techniques. Bruker 400 MHz spectrometer (Bruker, USA) was used to record ^{1}H and ^{13}C NMR spectra using DMSO-d6 (Dimethyl sulfoxide)/$CDCl_3$ (Chloroform) as solvents. Linear ion trap LC/MS in APCI mode was used for Mass spectrometric analysis (Thermo Fisher Scientific). The capillary melting point apparatus was used for melting point determination and are uncorrected. The TLC monitoring of crude extracts and eluates from the column chromatography was carried out using the solvent systems hexane: ethyl acetate (8:2). All the chemicals and media required for the MTT assay were purchased from Sigma Laboratories, USA, and Hi-media Labs, India respectively. MCF-7 (human breast cancer cell line), H1975 (non-small cell lung cancer cell line), and HCT-116 (colorectal cancer cell line) were obtained from American Type Culture Collection (ATCC), VA, USA. The cells were maintained at 37ºC in a humidified atmosphere with 5 percent CO_2 and were subcultured twice a week.

Lichen Material

Heterodermia boryi is a foliose lichen belonging to the family *Physciaceae*. It was collected from various places in Nilgiris district, Tamil Nadu, India which was authenticated by CSIR – National Botanical Research Institute, Lucknow, India. A voucher specimen was deposited with accession No 47518 at CSIR-NBRI herbarium, Lucknow, India.

Extraction and Isolation

The dried lichen thallus 135.79g of *H. boryi* obtained after cleaning debris, was powdered using a mixer grinder and was extracted with methanol by Soxhlet extraction. The extract after filtration was concentrated in a vacuum evaporator which yielded 25.95g (percentage yield-19.11) of dry extract. Fractionation of crude extract through a silica gel column (100-200 mesh) with 100% hexane yielded compounds HKB-1 and HKB-4 successively. While eluting with 5 % ethyl acetate in hexane afforded another compound HKB-6. The purity of isolated compounds was confirmed by observing single spots on TLC analysis.[22] The structure of isolated compounds was further characterized by spectral and physicochemical analysis.

Cytotoxicity Assay

The cytotoxicity assay was carried out in triplicates using a 96-well microtiter plate. Each well of the plate was seeded with 100 µl of cell suspension containing 50,000 cells. After 24 h of seeding the cells, the fresh medium having different concentrations of the extract and isolated compounds were added to the wells. Control cells were also incubated without test solutions and with DMSO solvent (maximal 0.2%) which is shown to not affect the assay.

The microtiter plate was further incubated at 37ºC for 24 h. The viability of cells was measured by adding 100 µl of 3-(4, 5 dimethyl thiazole-2yl) -25-Diphenyltetrazolium bromide (MTT) reagent (5 mg/10 ml) to the tested cell cultures and incubated at 37ºC for 4h. 100 µl of DMSO was added to solubilize the formazan complex formed by viable cells and absorbance was recorded on a plate reader at 590 nm and percentage cell growth inhibition against concentration and IC_{50} (Concentration required to inhibit cell growth by 50%)value for MCF-7,H1975 and HCT-116 cell line was calculated.[23, 24]

$$\% \text{ Inhibition} = ((\text{OD of Control} - \text{OD of Sample})/\text{OD of Control}) \times 100$$

Acridine Orange / Ethidium Bromide (AO/ EB) Staining Assay

This method is used to evaluate the ability of dual AO/ EB staining to detect tumor cell apoptosis. MCF-7 cells were grown in 12 well plates (50,000 cells/ well) and treated with isolated compounds at two different concentrations of 10 and 20 µg/ ml for 24 h. After the treatment cells were washed with phosphate buffer saline (PBS) and stained with 100 µl acridine orange and ethidium bromide mixture (1:1, 100 µg/ml). A wet mount of stained cell suspension (10 µl) was prepared and examined under a fluorescence microscope. The cells with morphological changes such as nuclear condensation and fragmentation were counted as apoptotic cells.[25]

Molecular Docking Study

To understand how strongly the isolated compounds bind to the active site of Mitogen-activated protein kinases-interacting kinase 2 (Mnk2) docking studies were carried out using the Glide module of Schrodinger molecular modeling software 2021-1.[26] Mnk2 is an enzyme responsible for phosphorylation and activation of oncogene eukaryotic initiation factor 4E (eIF4E). An increase in the level of phosphorylated eIF4E has been observed in various types of cancer. Inhibition of Mnk2 prevents the phosphorylation of eIF4E, making Mnk2 an attractive therapeutic target for cancer therapy.[27] Earlier reports on the anticancer activity of one of our isolated compounds (HKB-1) suggest its possible role in the inhibition of the Mitogen-activated protein kinase (MAPK) pathway prompted us to undertake this molecular docking study.[28] The 3D structure of Mnk2-D228G in complex with Staurosporine (PDB ID 2HW7) was used for docking. For preparing the protein, a protein preparation wizard was used and a receptor grid was generated around the active site. The low-energy conformers of HKB-1 and HKB-4 were generated. The lowest energy 3D conformers were docked to the active site of the receptor.[29,30] Staurosporine was used as the reference for comparing the binding mode and binding interactions.[31] The RMSD score for redocking the co-crystallized ligand at the same binding site from where it was removed was found to be 0.4558Å, which is well within the acceptable limit of 2 Å, validated the procedure.

RESULTS AND DISCUSSION

Isolation and Characterization of HKB-1 (Methyl Haematommate)

The crude methanolic extract of *Heterodermia boryi* was further fractionated on a silica gel (100-200 mesh) column. Elution of the extract with hexane afforded fractions exhibiting UV active single spot in TLC. These fractions were pooled together and evaporated to obtain a fluffy white powder (HKB-1, 0.545g, percentage yield, 1.5). Through spectral and physicochemical characterization, compound HKB-1 was identified as methyl haematommate (Fig.-1). The NMR spectral characterization data of HKB-1 (methyl haematommate) is presented in Table-1. The melting point and Rf value of HKB-1 (Hexane-ethyl acetate, 4:1) were found to be 144-146 ºC and 0.66 respectively. The ^{1}H NMR spectrum of HKB-1 exhibited a singlet at δ 6.347 (1H, s) for the aromatic proton at the 5th position of the phenyl ring. The ^{1}H NMR peaks for methyl protons of the ester side chain and 12th position aryl methyl protons were seen as singlets at δ 3.809 (3H, s) and 2.270 (3H, s) respectively.

Typical aldehyde proton was appearing as a single at δ 10.1992 (1H, s). The ^{13}C NMR spectra of HKB-1 exhibited peaks for ten carbons of methyl haematommate. Characteristic ^{13}C NMR signals for carbonyl carbon and aldehyde carbon was appearing at δ 168.01 and δ 194.11 respectively. The molecular ion peak of HKB-1in the mass spectrum was seen as $[M+1]^+$ peak at 210.92 (APCI Positive Mode). Moreover, the melting point and the spectral data for methyl haematommate (HKB-1) were in agreement with the previous reports.[32]

Isolation and Characterization of HKB-4 (Atraric Acid)

Successive elution of the column with hexane afforded fractions exhibiting a single UV active spot in TLC. These fractions were pooled together and evaporated to obtain an off-white powder (HKB-4, 0.783g, percentage yield: 2.14).

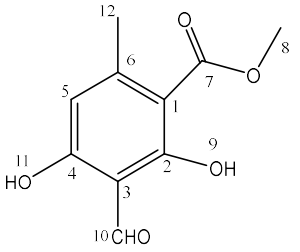

Fig.-1: Methyl Haematommate (HKB-1)

Table -1: NMR (DMSO-d_6) Data of Compound I (HKB-1)

Carbon/Proton Number	δ ^{13}C	δ ^{1}H
1	108.31	-
2	162.05	-
3	109.45	-
4	163.56	-
5	111.5	6.347 (1H, s)
6	149.32	-
7	168.01	-
8	52.53	3.809 (3H, s)
9	-	11.583 (1H, s)
10	194.11	10.192 (1H, s)
11	-	12.209 (1H, s)
12	21.85	2.270 (3H, s)

Through spectral and physicochemical characterization, compound HKB-4 was identified as atraric acid (Fig.-2). The NMR spectral characterization data of HKB-4 (atraric acid) is presented in Table-2. The melting point and Rf value of HKB-4 (hexane-ethyl acetate, 4:1) were found to be 140-144ºC and 0.43 respectively. The ^{1}H NMR spectrum of HKB-4 exhibited a singlet at δ 6.285 (1H, s) for the aromatic proton at the 5th position of the phenyl ring. The ^{1}H NMR peaks for 10th and 12th position aryl methyl protons were seen as singlets at δ 1.942 (3H, s) and δ 2.366 (3H, s) respectively. The ^{13}C NMR spectra of HKB-4 displayed peaks for ten carbons of atraric acid. The ^{13}C NMR peak for carbonyl carbon for the ester side chain was seen at δ 172.32. The molecular ion peak of HKB-4 in the mass spectrum was seen as $[M+1]^+$ peak at 196.97 (APCI Positive Mode). Moreover, the melting point and the spectra data for atraric acid (HKB-4) were in agreement with the previous reports.[33]

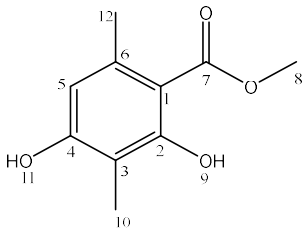

Fig.-2: Atraric acid (HKB-4)

Table-2: NMR (DMSO-d_6) Data of Compound II (HKB-4)

Carbon/Proton Number	δ ^{13}C	δ ^{1}H
1	110.97	-
2	162.18	-
3	104.44	-
4	160.48	-

5	108.6	6.285 (1H, s)
6	139.22	-
7	172.32	-
8	52.38	3.852 (3H, s)
9	-	11.641 (1H, s)
10	8.44	1.942 (3H, s)
11	-	10.140 (1H, s)
12	23.94	2.366 (3H, s)

Isolation and Characterization of HKB-6 (Zeorin)

Elution of the column with 5% ethyl acetate in hexane afforded fractions exhibiting a single non-UV active TLC spot, detected by spraying with anisaldehyde reagent. These fractions were pooled together and evaporated to obtain a white powder (HKB-6, 0.135g, percentage yield: 0.36). Through spectral and physicochemical characterization, compound HKB-6 was identified as zeorin (Fig.-3). The melting point and Rf value of HKB-6 (hexane-ethyl acetate, 4:1) were found to be 234-238 ºC and 0.21 respectively. The ^{1}H NMR spectrum of HKB-6 exhibited singlets at δ 0.68, 0.79, 0.91, 0.94, 0.97, and 1.11 for eight methyl groups integrating for twenty-four protons. The ^{1}H NMR peaks for methine (-CH-) proton with hydroxyl group attachment appeared as a multiplet at δ 3.86. The ^{13}C NMR spectra of HKB-6 exhibited peaks for thirty carbons of zeorin and a characteristic ^{13}C NMR peak for C-22 tertiary carbon was seen at δ 72.91. The molecular ion peak of HKB-6 in the mass spectrum was seen as 427.42 $[M+1-H_2O]^+$ and at 409.43 $[M+1-2H_2O]^+$ (APCI positive Mode). Moreover, the melting point and the spectra data for zeorin (HKB-6) were in agreement with the previous reports.[34] ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.68 (s, 3H, CH_3 -28), 0.79 (s, 6H, $2CH_3$ -25, 26), 0.91 (s, 3H, CH_3 -27), 0.94 (s, 3H, CH_3 -23), 0.97 (s, 3H, CH_3 -24), 1.11 (s, 6H, $2CH_3$ -29, 30), 3.86 (m, 1H, CH, 6); 0.73-2.16 (m, 27H, CH_2 & CH protons). ^{13}C NMR (400 MHz, $CDCl_3$): δ 39.28 (C1), 17.50 (C2), 42.78 (C3), 32.62 (C4), 59.96 (C5), 68.1 (C6), 44.24 (C7), 40.19 (C8), 48.66 (C9), 38.31 (C10), 20.83 (C11), 21.05 (C12), 48.37 (C13), 41.83 (C14), 35.75 (C15), 21.64 (C16), 52.99 (C17), 42.96 (C18), 40.86 (C19), 22.89 (C20), 50.16 (C21), 72.91 C22), 25.71 (C23), 27.25 (C24), 15.01 (C25), 19.98 (C26), 16.08 (C27), 13.07 (C28), 30.01 (C29), and 30.57 (C30).

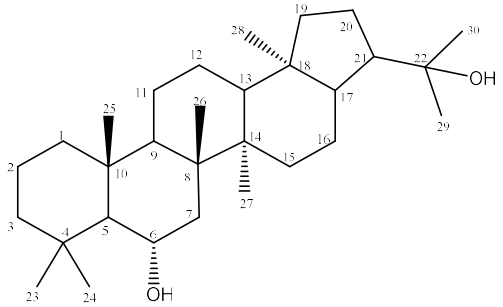

Fig.-3: Zeorin (HKB-6)

Cytotoxicity Assay

The crude methanolic extract of *Heterodermia boryi*, isolated methyl haematommate (HKB-1), atraric acid (HKB-4), and zeorin (HKB-6) were screened for *in vitro* cytotoxic activity against three different human cancer cell lines breast (MCF-7), lung (H1975) and colon (HCT-116) using doxorubicin as standard. The methanolic extract exhibited IC_{50} values of 105, 49.87, and 72.14 µg/ml against MCF-7, H1975, and HCT-116 cell lines respectively. Among tested cell lines, all isolated compounds demonstrated higher cytotoxicity against the MCF-7 cell line with IC_{50} values of (11.1 µg/ml, HKB-1), (32.2 µg/ml, HKB-4) and (17.02 µg/ml, HKB-6) as shown in the Table-3. Isolated compounds that exhibited IC_{50} value below 20 µg/ml against the MCF-7 cell line were further evaluated for their ability to induce apoptosis by dual staining method.

Acridine Orange / Ethidium Bromide (AO/ EB) Staining Assay

Acridine orange is a vital dye stain for both live and dead cells. Acridine orange stained, live (MCF-7) cells appear uniformly green. The cells which have lost the membrane integrity (late apoptotic cells) will take up the ethidium bromide stain which appears orange. MCF-7 cells when treated with isolated compounds

methyl haematommate (HKB-1) and zeorin **(**HKB-6) at 10 µg/ml displayed early signs of apoptosis such as nuclear fragmentation and chromatin condensation evidenced by the appearance of greenish-yellow bright dots in the nuclei. Whereas at 20 µg/ml, cells stained orange due to the incorporation of ethidium bromide suggestive of late apoptotic cells and necrotic cells (Fig.-4).

Table-3: Cytotoxicity Activity of Crude Methanolic Extract of *H. boryi* and Isolated Compounds by MTT Assay on MCF-7, H1975 and HCT-116 Cell Lines

Crude methanolic extract / Isolated Compound	IC_{50} ± SD (µg/ml)		
	MCF-7	H1975	HCT- 116
Crude methanolic extract	105±4.55	49.87±2.59	72.14±4.27
Methyl haematommate (HKB-1)	11.1±0.91	33.87±2.19	39.25±1.88
Atraric acid (HKB-4)	32.2±2.11	47.9±2.71	57.14±3.15
Zeorin (HKB-6)	17.02±1.29	31.58±2.17	70.55±4.11
Doxorubicin	2.38±0.17	4.79±0.21	5.81±0.37

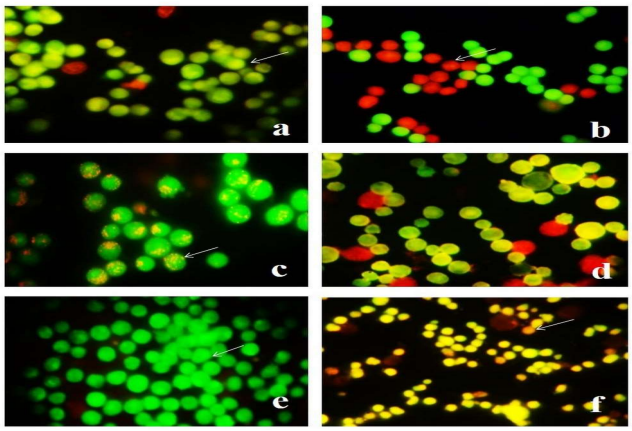

Fig.-4: Acridine Orange (AO) – Ethidium Bromide (EB) Dual Staining for Scoring Apoptosis Induced by Isolated Compounds on MCF-7 cell line; a) HKB-1 (10 µg/ml) b) HKB-1 (20 µg/ml) c) HKB-6 (10 µg/ml) d) HKB-6 (20 µg/ml) e) Untreated control f) Doxorubicin (10 µg/ml)

Molecular Docking

All the isolated compounds were studied for their binding interactions with Mitogen-activated protein kinases-interacting kinase 2 (Mnk_2). In our study, we have evaluated the possible ability of isolated compounds for the inhibition of Mnk_2 by docking them with the crystal structure of Mnk2-D228G (PDB ID 2HW7) after removing the bound ligand, Staurosporine from the 3D structure of the protein. The co-crystallized ligand, staurosporine used as the reference for comparing the binding mode and binding interaction of isolated compounds at the active site.

Docking Study of Isolated Compounds with 2HW7- Mnk2-D228G

The important binding site residues of Mnk2-D228G (PDB ID 2HW7) were GLU-160, MET-162, GLU-92, and GLU-209. The binding interactions and dock scores of isolated compounds were compared with that of reference co-crystallized ligand staurosporine. Staurosporine exhibited a docking score of -8.78 kcal/mol when docked to the ligand binding site of 2HW7. The best binding poses of staurosporine with 2HW7 exhibited a salt bridge between the protonated amino group of the pyranose ring with glutamic acid (GLU-92). This protonated amino group and NH of lactam ring form H-bonds with glutamic acid-209 (GLU-209) and glutamic acid-160 (GLU-160) respectively. While the carbonyl group of the lactam ring forms H-bond with methionine- 162 (MET-162).

Molecular docking studies of methyl haematommate (HKB-1) to the ligand-binding site of 2HW7 exhibited a docking score of -7.46 kcal/mol. The binding poses of methyl haematommate (HKB-1) with 2WH7 exhibited an H-bond interaction between the phenolic hydroxyl group and glutamic acid (GLU-160). Whereas the aryl aldehyde group forms H-bond with methionine-162 (MET-162). The binding interactions of atraric acid to the ligand binding site of 2HW7 exhibited a docking score of -7.31 kcal/mol. The binding pose of atraric acid (HKB-4) with 2HW7 exhibited H-bond interactions of the phenolic hydroxyl group

with glutamic acid -160 (GLU-160) and methionine-162 (MET-162). The binding interactions of methyl haematommate (HKB-1) and atraric acid (HKB-4) with binding site residues GLU-160 and MET-162 were similar to that of the reference compound staurosporine (Fig.-5).

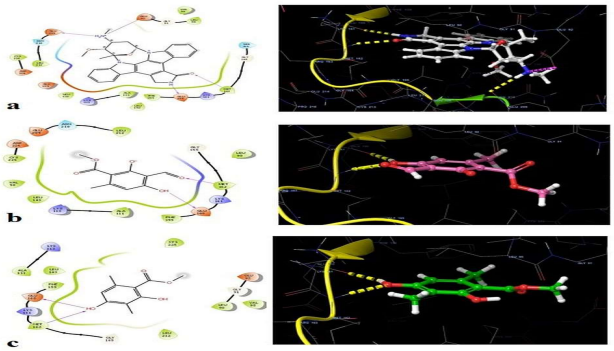

Fig.-5: 2D and 3D Binding Interactions of a) Staurosporine b) Methyl Haematommate (HKB-1) c) Atraric Acid (HKB-4) with Binding Site Residues of Mnk2-D228G

CONCLUSION

Based on the outcomes of this study, it is concluded that out of three isolated compounds from *Heterodermia boryi,* Methyl haematommate (HKB-1) and zeorin (HKB-6) exhibits superior cytotoxic activity on MCF-7 cell lines. Nuclear staining studies also revealed the ability of these compounds to induce apoptosis of MCF-7 cells. *In silico* molecular docking studies, HKB-1 exhibited excellent binding affinity and interaction with Mnk2, which was comparable with that of the co-crystallized ligand staurosporine, indicative of their possible role as Mnk2 inhibitors. Hence, we propose methyl haematommate (HKB-1) and zeorin (HKB-6) as potential lead molecules for further development as anticancer agents.

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