"STUDIES ON SYNTHESIS OF DESIGNED AND ISOLATED ANTICANCER MOLECULES"

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BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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Submitted by

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2020

Dedicated
To

My Family
For Stronger, Consistent Support

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on synthesis of designed and isolated anticancer molecules"** submitted by **Mr. Balakishan Bhukya** is an original research work conducted by me and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulation-1999 as amended in 2008/2010/2013 and it is suitable for submission and evaluation for the award of the degree of Doctor of Philosophy in Applied Chemistry of the University.

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22/07/21

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DECLARATION

I, Balakishan Bhukya declare that the thesis entitled **"Studies on synthesis of designed and isolated anticancer molecules"** submitted by me for the degree of Doctor of Philosophy, is the record of work carried out by me under the supervision of **Dr. Shailesh Kumar Assistant professor at Department of Applied Chemistry, School For Physical Science, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, India and Co-supervision of Dr. Arvind Singh Negi Senior Principal Scientist at Phytochemistry Division, CSIR-Central Institute of Medicinal And Aromatic Plants, Lucknow, India** and I further confirm that said work has not been submitted anywhere else for the award of any degree, diploma, fellowship etc. either in this or any other University or other institution of higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis. I Balakishan Bhukya also declare that this thesis submitted by me is essentially free from all kinds of plagiarism (checked by URKUND, **D86832690**).

Balakishan Bhukya

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Date: 22/07/21

Place: Lucknow

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Last, but not the least, I express my sincere thanks to the authors of various research articles and books whose work has been consulted, utilized and cited in my thesis.

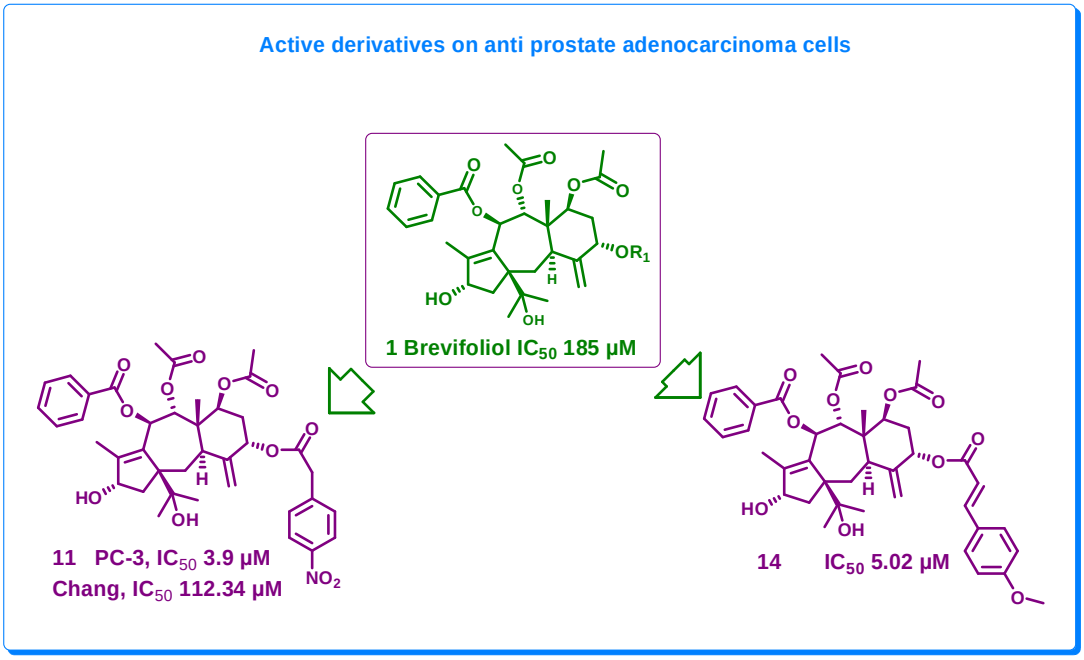
BALAKISHAN BHUKYA

(Research Scholar)

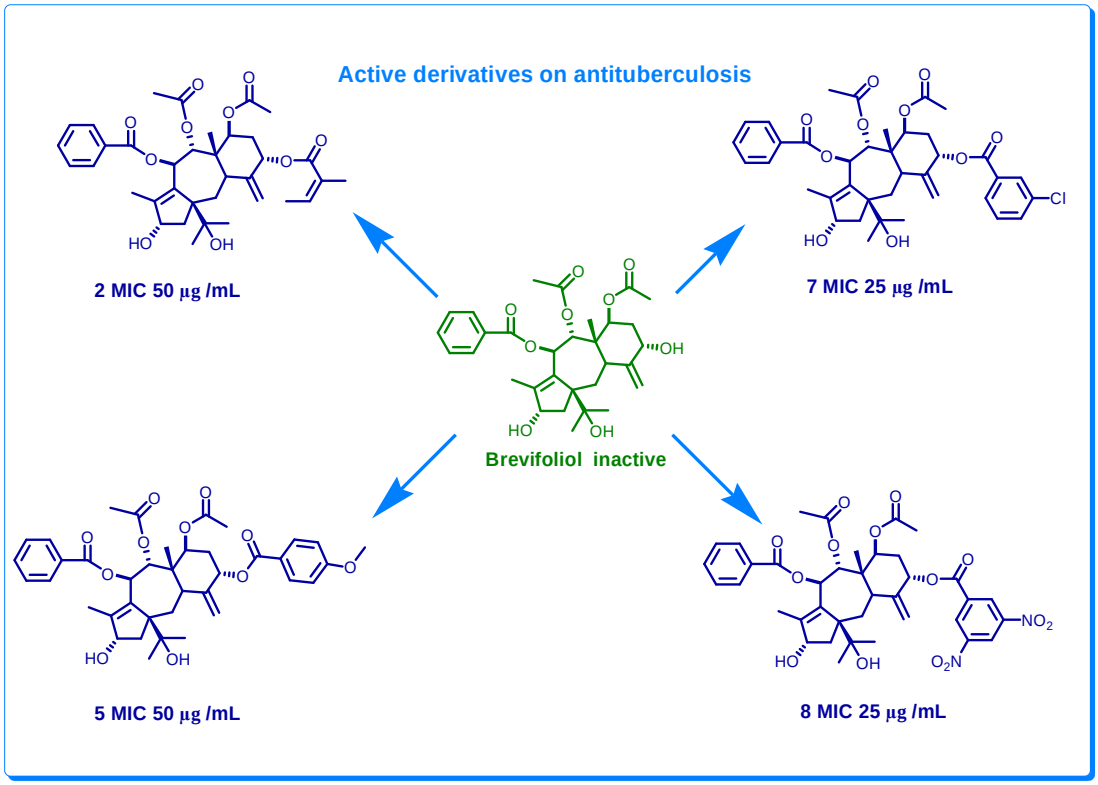
ABSTRACT

The thesis accredit, **"Studies on synthesis of designed and isolated anticancer molecules"** involves designing of novel colchicine and estradiol based 2, 3-diarylnaphthofuran and 2, 3-diarylbenzofuran core, various prototypes, their synthesis and biological evaluation. Apart from these prototypes value addition of natural product of brevifoliol and total synthesis of biologically active natural molecule rugosa flavonoid-B have been undertaken. The studies mainly involve tubulin and caspase proteins as biological targets for anticancer agents. While, polyketide synthase-13 and enoyl-ACP reductase were biological targets for antitubercular agents.

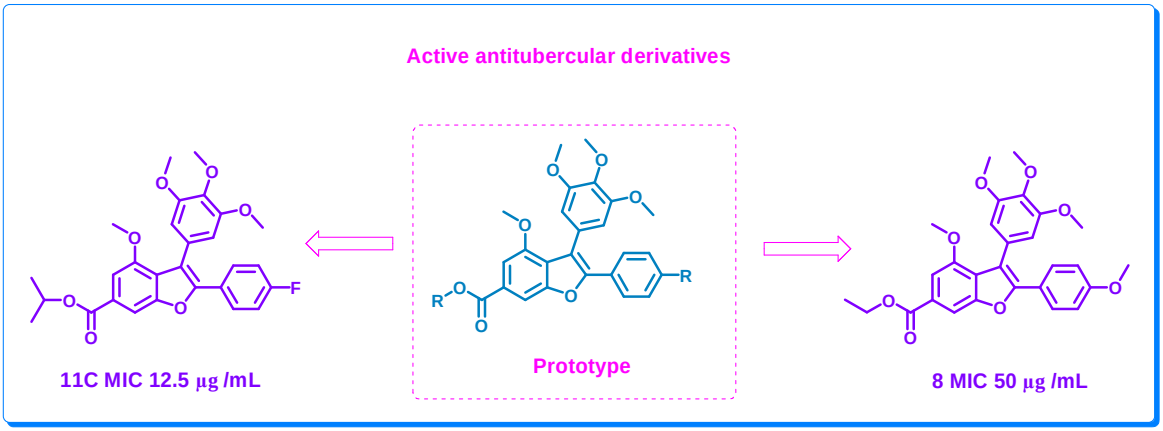
Brevifoliol, a secondary metabolite isolated from *Taxus wallichiana* needles has been derivatized as C5 esters using steglich esterification reaction. Eighteen diverse analogues were evaluated against a panel of human cancer cell lines like breast (MCF-7), colon (COLO-205), lung (A549) and prostate (PC-3) by MTT assay. Among these, two of the semi-synthetic analogues i.e. **11** and **14** (Chapter-2A) exhibited potent cytotoxicity selectively against PC-3, prostate cancer cell lines, at IC_{50} 3.89 µM and 5.02 µM respectively. In cell cycle analysis, analogue **11** induced S and G2/M phase arrest and induced apoptosis by activating caspase-3. Compound **11** showed moderate efficacy in *in-vivo* ehrlich ascites carcinoma in *Swiss albino* mice by reducing 55.85% tumour at 100 mg/kg i.p. dose. Further, compound **11** was well tolerated and found to be safe in *Swiss albino* mice up to 1000 mg/kg dose in acute oral toxicity. [B. Bhukya, *et al*, *Chem. Biol. Drug Design* **2020**; 95: 150-161]



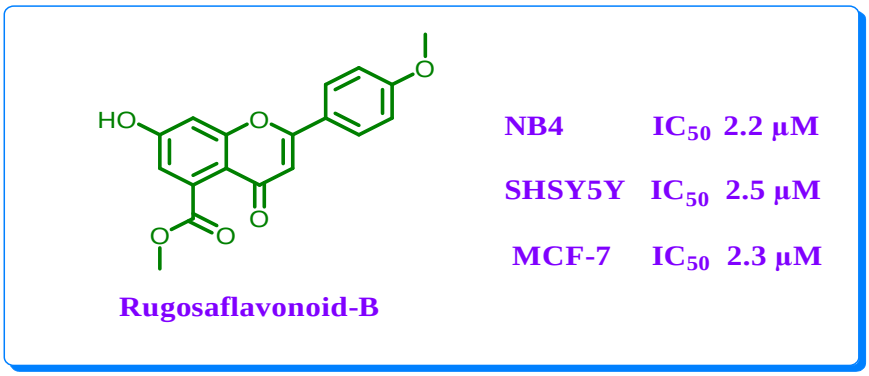
Further detailed literature search observed that many taxoids like taxol and 10-DAB derivatives were also active against the tuberculosis disease. Brevifoliol is a rearranged abeo-taxoid and its core structure is different than the taxoids class of compounds like taxol and 10-DAB. This is first report on antitubercular activity by brevifoliol. Its eighteen semi-synthetic ester derivatives of brevifoliol were screened for their anti-tubercular potential against *Mycobacterium tuberculosis* H37Ra avirulent strain. The 3-(chlorophenyl) benzoic acid and 3, 5-(dinitrophenyl) benzoic acid ester derivatives (**7**, **8**) (Chapter-2B) were most active (MIC 25 µg/ml) against the pathogen. Further, *in silico* docking studies of the active derivative **7** with mycobacterium enzyme inhA (enoyl-ACP reductase) gave a LibDock score of 152.68 and binding energy of -208.62 kcal/mol and formed three hydrogen bonds with SER94, MET98, and SER94. Similarly, when derivative **8** docked with inhA, it gave a LibDock score of 113.55 and binding energy of -175.46 kcal/mol and formed a single hydrogen bond with GLN100 and Pi-interaction with PHE97. On the other hand the known standard drug isoniazid gave a LibDock score of 61.63, binding energy of -81.25 kcal/mol and formed one hydrogen bond with ASP148. These molecular docking results and the way of binding pattern indicated that compound **7** and **8** bound well within the binding pocket of inhA and showed a higher binding affinity than the known drug isoniazid. Additionally, both the derivatives (**7** and **8**) showed no cytotoxicity towards the healthy liver cell lines CHANG. [B. Bhukya, *et al, Curr Topic Med Chem* 2020; 99:103784.]



Benzofuran is a biodynamic core, like anticancer, antitubercular, anti-inflammatory activity and many more. A total of seventeen 2, 3-diaryl benzofuran hybrids were designed, synthesized and screened for their anti-tubercular potential against *Mycobacterium tuberculosis* H37Ra avirulent strain. Out of seventeen, four derivatives showed significant activity against *M. tuberculosis* H37Ra avirulent strain (ATCC 25177) with MIC value ranging from 12.5-50 µg/mL but out of four, one derivative **11C** (Chapter-3A) was significantly active (MIC 12.5 µg/mL), which was further supported by the molecular docking score (-8.4) with respect to the first line anti-tubercular drug, isoniazid (-6.2) on the target polyketide synthase-13. All the derivatives were also evaluated for their cytotoxicity against the normal lung cell line L-132 by the MTT assay and no toxicity was observed up to 27.4 µg/mL concentration. This report on the antitubercular potential of benzofuran derivatives may be of great help in anti-tubercular drug development. [B. Bhukya, *et al*, *Bioorg Chem* 2020; 99:103784.]

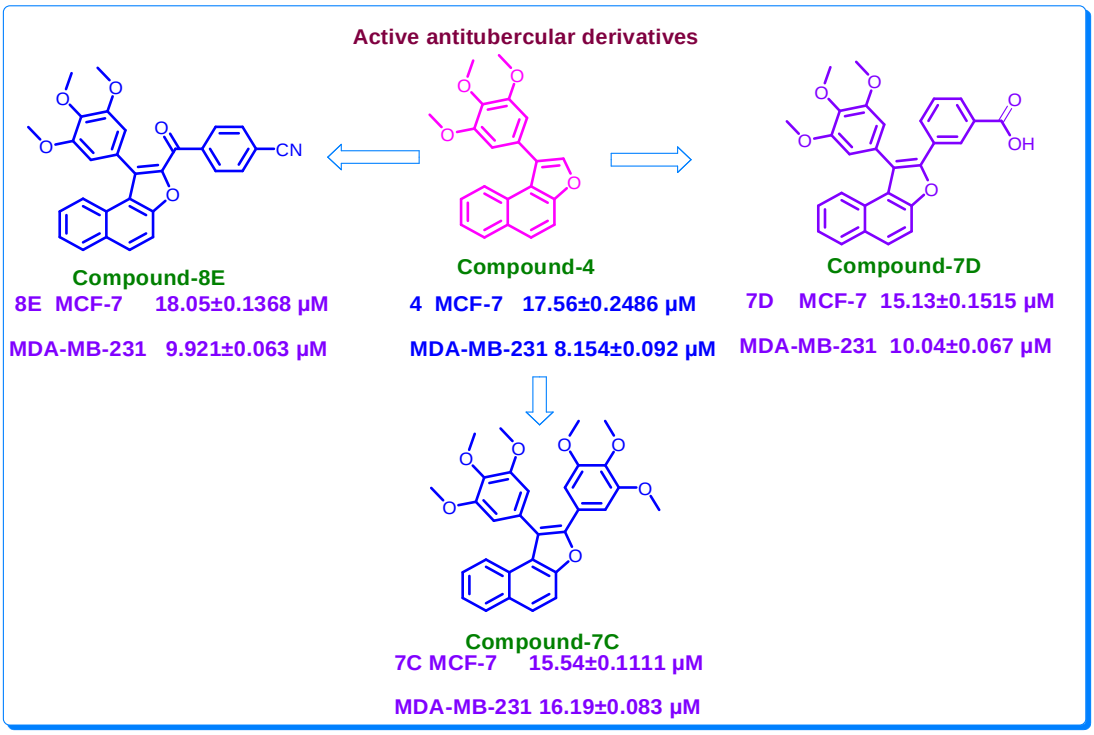


Phytomolecules have great importance in our day to day life. We are taking natural products as medicine, supplements, aroma, in the pure form as well as in the form of herbal. Natural products and their derivatives effectively served as medicine last 50 years. Almost 61% of the FDA approved and pre-NDA candidates are natural product or their derivatives. By keeping the importance of natural products we have selected the rugosaflavonoid-B for the total synthesis. It is isolated from the common ornamental plant flower *Rosa rugosa* (Rosaceae) which is distributed in the temperate regions of eastern Asia and widely cultivated in Yunnam province. *Rosa rugosa* plant petals and buds are used in the food, incense in china. Its medicinal uses are against diarrhea, stomachache and gynecological problems. Xue-Mei Gao group isolated rugosa flavonoid-B from this plant and reported its anticancer activity on three human cancer cell lines. We have selected it for total synthesis and its derivatization to generate more lead molecules to see its activity improvement. Its synthetic strategy has four steps and completed upto step-3 (Chapter-3B). Further final step and its derivatization are in progress in our lab.



Designed the pharmacophore by expecting that can exhibit dual nature drug like estradiol carrier type and tubulin binder in order to show the potent anticancer activity. Breast cancer is most common invasive cancer which is second most leading

cause of cancer deaths in women. We under took design and synthesis of diverse compounds on 2, 3-diaryl naphthofuran core. Out of sixteen new analogues four compounds exhibited significant antiproliferative activity against both hormone dependent (MCF-7) and hormone independent (MDA-MB-231) breast cancer cell lines. Among them the most active compound **7D** (Chapter-4) showed antitubulin effect. In molecular docking studies compound 7**D** occupied colchicine binding pocket with high affinity with crucial residual amino acids at β-tubulin. The compound **7D** found safe and non-toxic in *Swiss albino* mice up to 1000 mg/kg oral dose. The optimization of new lead compounds may yield some better candidates in future. [Manuscript submitted to *Bioorganic Chemistry*-2020]



LIST OF ABBREVIATIONS

Ac_2O	: Acetic anhydride
UPLC	: Ultra performance liquid chromatography
TLC	: Thin layer chromatography
TMS	: Tetramethylsilane
ESI-MS	: Electrospray ionization mass spectrometry
LC-MS	: Liquid chromatography mass spectrometry
HRMS	: High resolution mass spectrometry
Q-TOF	: Quadruple time of flight
DCM	: Dichloromethane
MeOH	: Methanol
mg	: Milligram
mp	: Melting point
g	: Gram
DMAP	: 4-(N,N-dimethylamino) pyridine
DCC	: N, N'-dicyclohexylcarbodiimide
Na_2SO_4	: Sodium sulphate
RT	: Room temperature
$CDCl_3$	: Chloroform-d
NMR	: Nuclear magnetic resonance
J	: Coupling constant
s	: Singlet
d	: Doublet
dd	: Double doublet
m	: Multiplet
brs	: Broad singlet

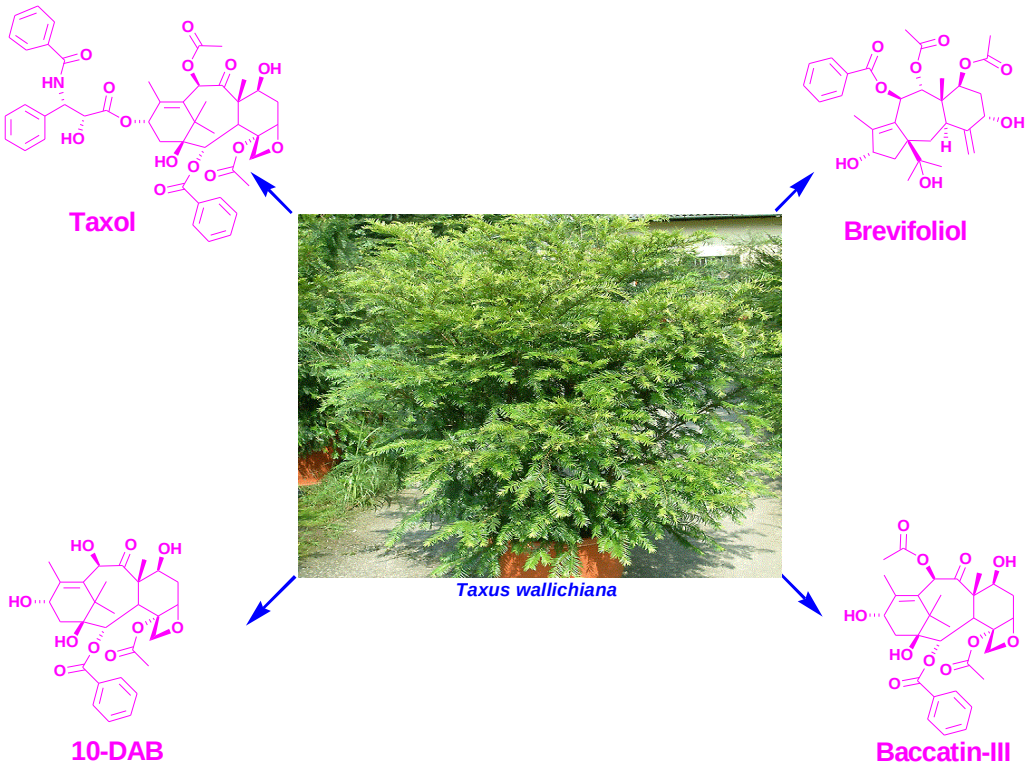
DM	: Dry Mass of plant material
EA	: Ethyl acetate fraction
t	: Triplet
µL	: Microliter
H_2SO_4	: Sulphuric acid
Conc	: Concentrated
^oC	: Degree celsius
h	: Hour(s)
NIS	: N-iodosuccinimide
K_2CO_3	: Potassium carbonate
DMF	: Dimethylformamide
$(Ph_3P)_2PdCl_2$	: Palladium bis triphenylphosphine dichloride
CuI	: Cupper iodide
$NaHCO_3$	: Sodium bicarbonate
NH_4Cl	: Ammonium chloride
KOH	: Potassium hydroxide
EtOAc	: Ethyl acetate
MHz	: Meghahertz
PPM	: Parts per million
aq.	: Aqueous
Br_2	: Bromine
DMAC	: N,N-dimethylacetamide
DMS	: Dimethyl sulphate
$POCl_3$	: Phosphoryl chloride
$Pd(OAc)_2$	: Palladium acetate
KOAc	: Potassium acetate
TFA	: Trifluoroacetic acid

MIC	: Minimum inhibitory concentration
I_2	: Iodine
mmol	: Millimole
Liq	: Liquid
m/z	: Mass per unit (+ve) charge
CS_2	: Carbon disulfide
$Na_2S_2O_3$	: Sodium thiosulfate

LIST OF TABLES

LIST OF SCHEMES

Chapter-1
Recent advances in the chemistry and pharmacology of taxol and its derivatives



Biological activity of *Taxus wallichiana*

- Anti-tuberculosis
- Stem-cell drug
- Anti-diabetic
- Antioxidant
- Antimicrobial
- Immunomodulatory
- Analgesic
- Anti-nervous
- Anti-pyretic
- Anti-asthma

- Insecticidal
- Anti-aging
- Anti-epileptic
- Anti-cancer
- Anti-bacterial
- Anti-fungal
- Anti-inflammtory
- Hepatoprotective
- Antacid
- Anti-dialysis

1.1 Introduction about *Taxus wallichiana.*

Taxus wallichiana Zuccarini is an evergreen plant of the genus taxus and family Taxaceae. *T. wallichiana* is widespread in Asia ranging from Afghanistan through the Himalayas to the Philippines. Taxaceae family is well known for the taxane class of compounds. Traditionally this *T. wallichiana* plant has been used to cure many respiratory disorders and is used as an antipyretic, anti-epileptic, anti-cancer, anti-inflammatory, anti-bacterial, analgesic, hepatoprotective, cold, cough, antioxidant, anti-fungal, antacid, antituberculosis, immunomodulatory and anti-neurological disorders. In India, its uses are described in Ayurveda and Unani medicine systems. According to traditional Chinese medicine, the China native people have been using the *T. wallichiana* roots with other medicinal plants to make decoction to treating kidney-impairing-type diabetes and moreover it does not have any other side effects like irritable bowel syndrome etc. [1]. In India, *T. wallichiana* has been used by the Uttarakhand native populations for making tea. Taxus species were having versatile activity on broad range of disease. Generally, plants synthesize the phytomolecules for their protection and these are called secondary metabolites which are most useful to the human beings. *T. wallichiana* whole plant is extensively used in traditional system, herbal formulation and also marker compounds isolation purpose.

1.1.1 Plant propagation

Due to its habitat, growth in selected areas and its long seed dormancy period, its natural regeneration from the seeds is very low. This species is currently classified as endangered by the International Union for Conservation of Nature (IUCN) France. It is one of the most exploited plants for its bark and leaves to get the taxoids. There were some surveys which have been done to know the extent of exploitation particularly the bark and leaves of this plant in one of the most interior areas of Himalaya in India and this survey reported that the 11% of the *T. wallichiana* population which amounted to 18152.86 cm^3 of bark removed [2]. Depending on its medicinal importance it has to be propagated in different ways to get its population growth [**3**]. There are different ways which have been used to propagate such as root formation in branch cutting of *T. wallichiana* and considerable success was achieved

after making use of different auxins in different concentration. This technique was the easiest method and not expensive, the developed plants can be planted anywhere in the natural habitat for the restoration of this valuable plant [4].

1.1.2 *In-vitro* Production [Ex-plant]

Isolation of taxol from the *T. wallichiana* barks and leaves, due to taxol availability in the plant, its growth can also be done by *in-vitro* method and the presence of this class of compounds was compared to the natural plant grown in the field with respective to *in-vitro* plants. The chemical analysis of ethanolic extract of *T. wallichiana* callus showed the presence of lignan, protein, carbohydrate, flavonoids and alkaloids. Then the taxol isolated from the ethanolic extract by using Prep-High Performance Liquid Chromatography. The taxol isolated about 1.22% which is more than the natural plant extracts 0.055%. If we can try this method in practical way to get the high amount of taxol, then it can be isolated from callus without putting pressure on the natural plants [5]. Further performed comparison between Cephalomannine and taxol was also done by XU group which media leaf of *T. wallichiana* and compare the above compound content with reverse-phase high performance liquid chromatography. The results were taxol and cephalomannie content very high in above plant leaves and moderate in stem. But the taxol content was also in equal amount in media leaf [6].

Isolation of taxane class of compounds is done mostly from the family of the taxaceae and only one species from the *Austrotaxus spicata* that to limited investigation. Taxol was the best taxoid from the taxanes class. Jain group applied different approach to get the taxol from *T. wallichiana*. var. mairei. They have taken the bark of the above plant then surface was sterilized, inoculated in potato dextrose agar culture medium to isolate endophytic fungi. There are 528 endophytic fungal strain were isolated from the bark of above plant and only one strain was efficiently producing the paclitaxel that strain was identified as *Phoma medicaginis* [7]

1.1.3 Seasonal effect

The seasonal variation of secondary metabolites of *Taxus wallichiana* was done by Yang *et al*.This plant material was collected in different months and the total taxoid

content was quantified by the UPLC. They have taken the paclitaxel, 10-Deacetyl baccatin, baccatin-III, cephalomannine, and 10-Deacetyltaxol. The highest content of taxoid was 1.77±0.38 mg/gram in January and the lowest was 0.61±0.08 mg/gram in September. The temperature had highest negative effect on the content of paclitaxel and positive effect on that of polysaccharides. This study suggested the suitable time of the plant material of *T. wallichiana* var.mairei to be collected as per need of research [8]

But in this review article we are giving only natural compound isolated from the *T. wallichiana* in-details, like its medicinal use, isolation of essential oil, non-taxoid isoprenoids, lignan, and isolation of Taxanes from various parts of the plant (leaves, stem, twig, barks, hardwood and roots). Different sources for Taxanes, *T. wallichiana* usage in various forms (herbal formulation, stem cell usage, marketed drugs). Some important molecules and their derivatives like (brevifoliol, 10-DAB, and taxol).

1.2 Medicinal uses of *Taxus wallichiana*

Nowadays several herbal formulations of taxus and their usage have been developed. According to recent reports the present market about herbal drugs is estimated about 40 billion dollar and which is expected to increase by 16% in future. Herbal drugs supply was less than the demand. *Taxus wallichiana* plant is most important for the treatment of various diseases and almost the entire plant is useable in different ways. Exploitation of the plant is too high, so we have to keep the plant in such a way that its population should remain unchanged. Otherwise in the Ayurveda drug supply chain will be fully adulterated and may not be traceable. Nowadays none of the pharmacopeias like USFDA, IP and others do not have suitable methods to detect like how to trace the herbal formulation, combination, and origin of the material used, exact combination, combination of the ratios thereof. [9].

T. wallichiana plant branches extract was prepared by using different solvents by increasing polarity order and tested against six bacterial and six fungal strains by using different methods. In phytotoxicity assay, all fractions showed activity at the concentration of 500 µg/ml [10, 11]. Its bark was extracted and carried out the analgesic and anti-inflammatory activity. Then to confirm the molecules responsible for the above activity from the crude, they isolated 10-deacetylbaccatin-III, 4-deacetyl

baccatin-III and tasumatrol-B from the extract. Tasumatrol-B showed significant anti-inflammatory activity and all other compounds were inactive [12]. Total flavones were extracted from the leaves and branches of *T. wallichiana* var. mairei in China. By using the extraction, they performed the cytotoxicity assay by using MTT method and found significant inhibition in sarcoma-180 cell line that to concentration-dependent relationship. [13].

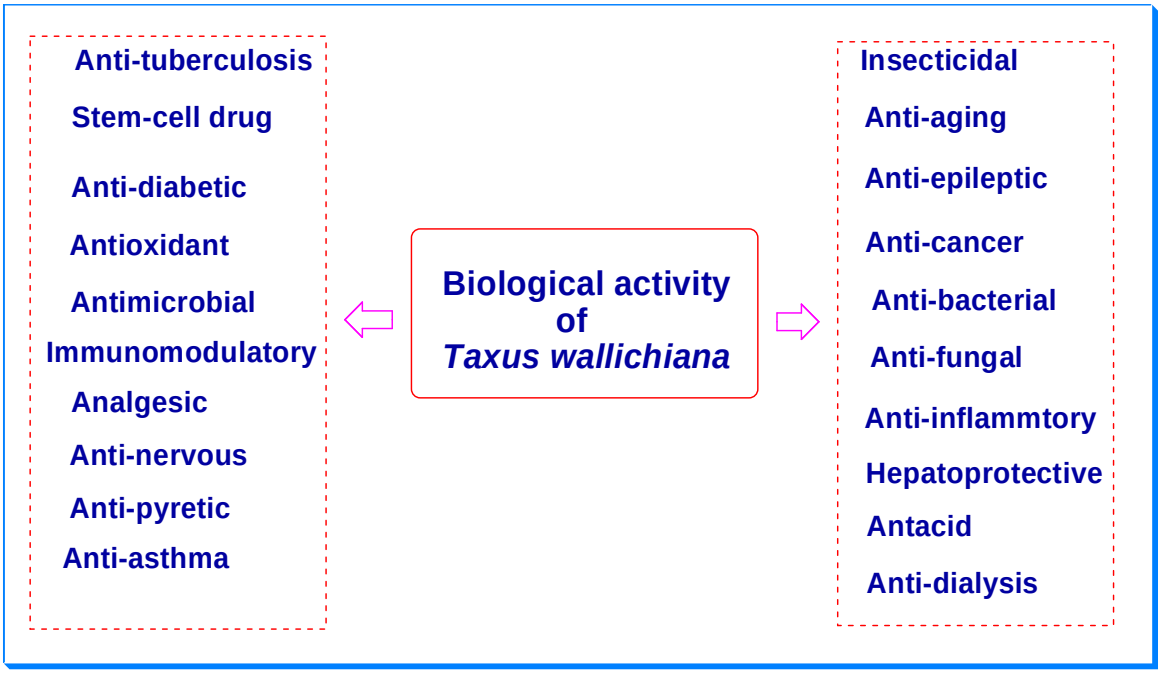


Figure (1): Various biological activities of *Taxus wallichiana*

The present review mainly focuses on isolated taxane class of compounds from the *T. wallichiana* zuccarini i.e. From the whole plant, generally taxanes classes of compounds present in only taxaceae family not any other family, except that *Austrotaxus*. *Austrotaxus* have represented by only one species *Austrotaxus spicata* was located in New Caledonia and Oceania with limited chemical investigation [14]. By the end of the 2009 totally 3500 taxanes were isolated from the various taxus species from different part of the plant such has leaves, stem hardwood, branches, twig, seeds, bark and roots.

1.3 Isolation of natural products

1.3.1 Isolation of essential oil

T. wallichiana plant contains diverse class of compounds like monoterpenes to diterpenes, triterpenes and alkaloids. The essential oil of above plant on hydro-distillation gave 0.025% oil from the leaves of Indian yew plant growing in Gulmarg, Jammu and Kashmir. Further analysis was performed by using the gas chromatography and GC-MS. The oil contains 62 constituents, representing 93.3% of the oil. The major compound for the oil was E-2-octen-1-ol [17]. Similarly, the essential oil also distilled and extracted by using dichloromethane and diethyl ether at 4^{o}C and analyzed by using GC-MS and identified the total list of compounds about 120. The major components were 3-hexen-l-ol and 2-hexanal [18].

1.3.2 Isolation of non-taxoid isoprenoids

The plant material was brought from Kathmandu Nepal. After air- drying the needle and twig, were finely powdered and extracted with the methanol by percolate procedure. The methanol extracts further fractionation was done with polarity increasing order. The hexane fraction was further subjected to VLC, then after the hexane-ethyl acetate fraction of VLC further used in column chromatography to get compounds **1**-7 [19].

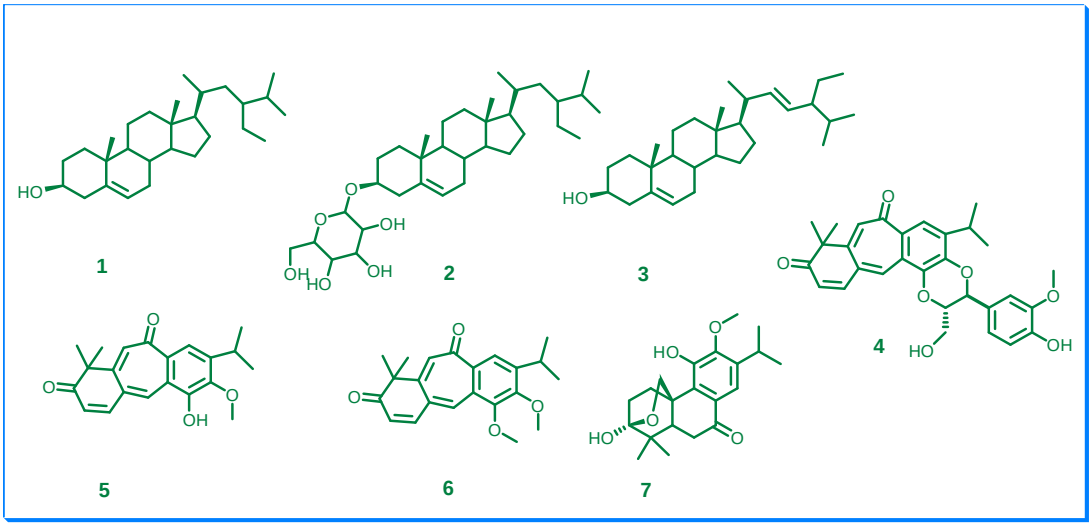


Figure (2): Non-taxoid class of compounds

1.3.3 Isolation of Lignan

Lignan was present in all the seed containing plants and have very good biological activity. Firstly the compound **8-13** were isolated from needles root and stem of the *T. wallichiana* by the miller [20]. Chattopadhyaya *et al*. 2003 added **14-18** more lignan from the heartwood crude fraction of chloroform and ethyl acetate (**19, 20** and **21**). These compounds absolute configuration also established from this group [21].

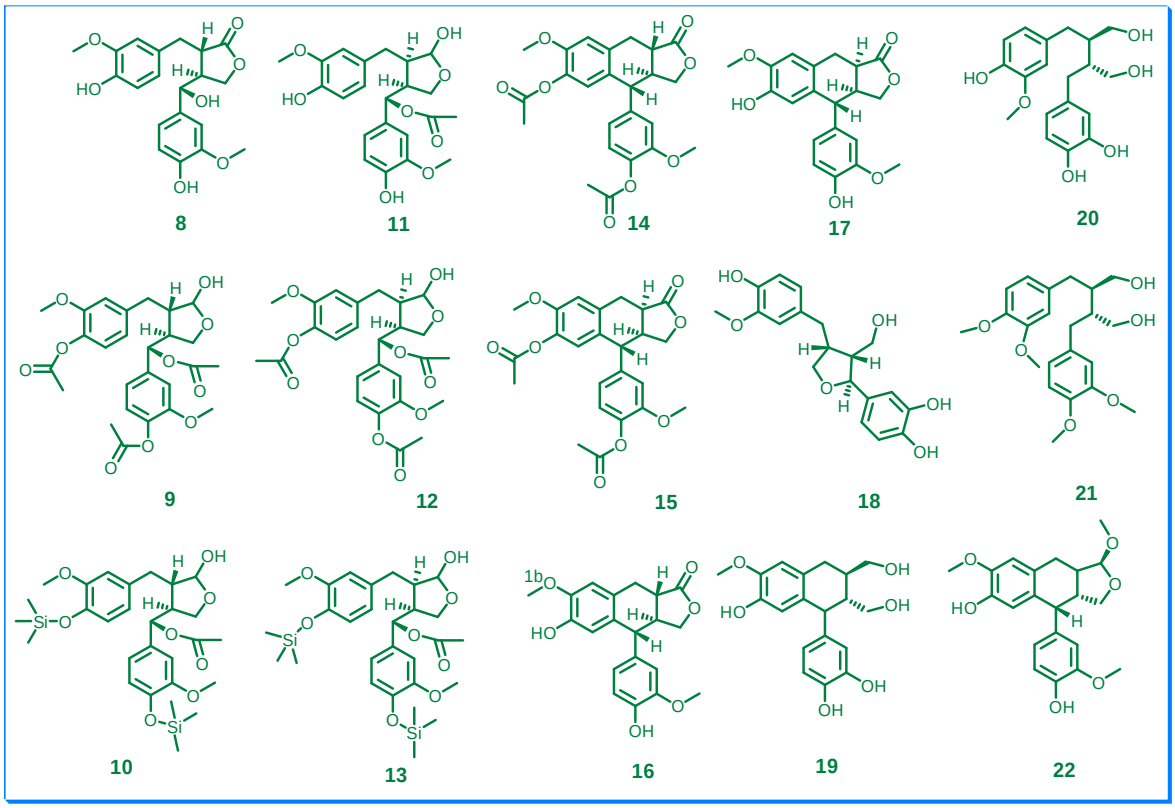


Figure (3): Lignan class of compounds

Traditionally, *T. wallichiana* has been used in various remedies in different diseases throughout the globe. Activity guided fractionation by ethyl acetate soluble extract of root yielded the compounds which was recently reported by Phu H. Dang group. This group has isolated six new lignan's **22-27** and compounds **28-38** are known. All the lignans are posses α-glucosidase inhibitory activity [22].

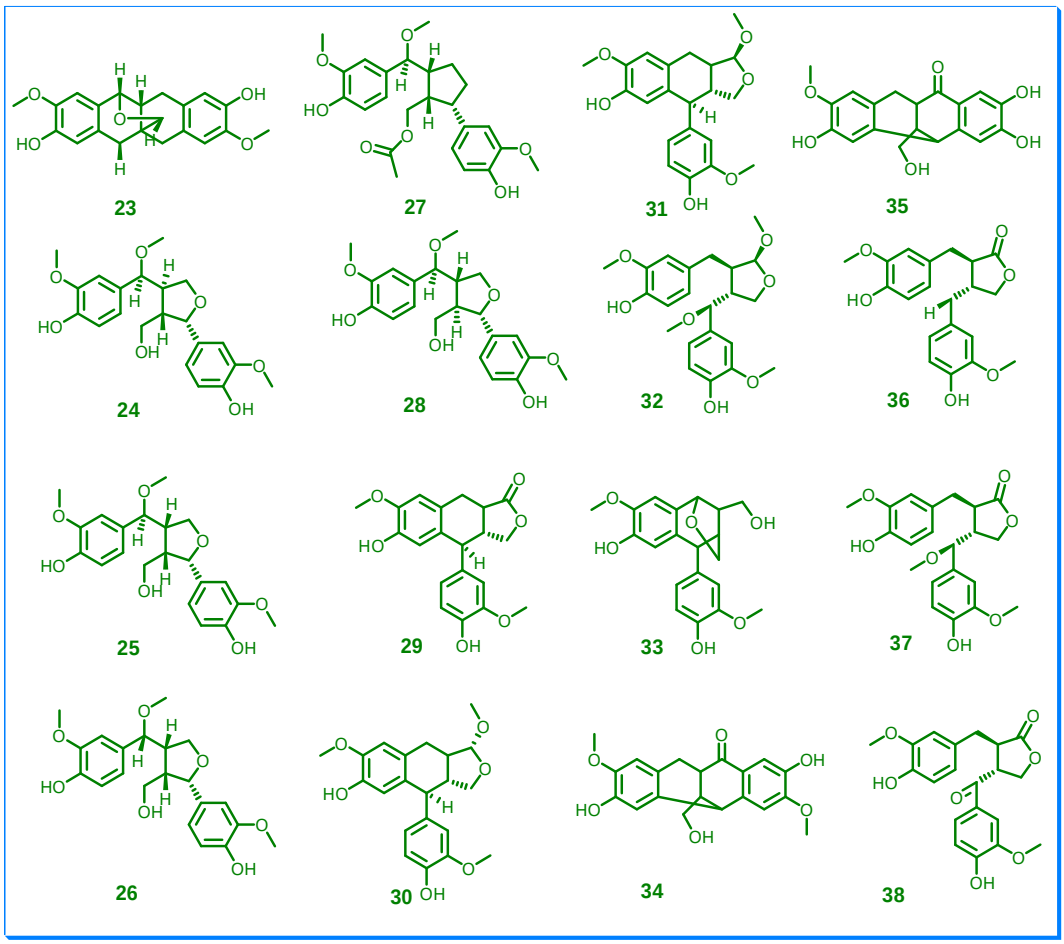


Figure (4): Lignan class of compounds

1.4. Isolation of taxanes

1.4.1 Leaves

T. wallichiana zuccarini needles were brought from plant grown in the garden of Trichandra College affiliated to university of Tribhuvan, Nepal. Finely dried powdered needles were extracted with ethyl acetate, then resulted crude mass was re-dissolved in ethyl acetate and extracted with 10% HCl. Then aqueous layer was washed with ethyl acetate and aqueous layer was neutralized with sodium carbonate and dried with sodium sulphate. This crude mass was further used for the purification by HPLC. A linear gradient of acetonitrile from 0 to 40% (0.6%/min) in 0.1% TFA; flow rate, (13 mL/ min) yielded compounds **40**, **49** and **62** at the 40%, 36% and 29% CH_3CN in the gradient. Compounds **40** and **62** showed moderate cytotoxicity against the lung cancer cell line A549 *in vitro* [23].

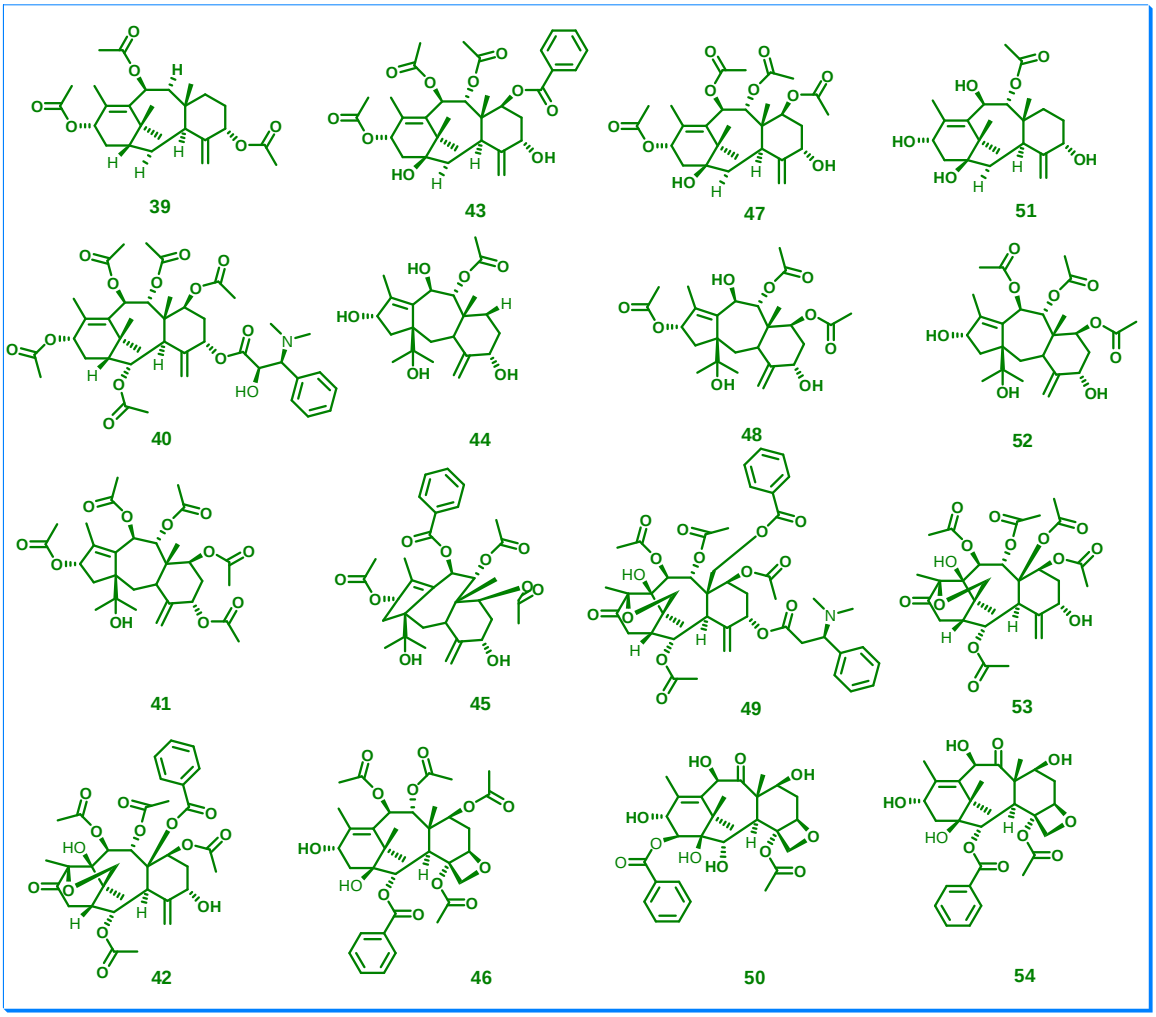


Figure (5): Taxanes isolated from leaves

Plant material was brought from Shimla nearby western Himalaya. Finley grounded needle powdered, extracted with methanol and fractionated with DCM. The dichloromethane fraction was further subjected to the column chromatography to afford compounds **42, 53** and **57** [24]. From Kashmir India needles of *T. wallichiana* were collected, powdered and extracted with methanol. The methanol fraction was fractionated with chloroform and crude mass was used in column chromatography which gave compound **41** [25].

Brevifoliol and some baccatin-VI derivatives possess a rearranged 11(15→1) *abeo*-taxane skeleton and not a baccatin type skeleton as originally reported in the taxoid, making *abeo*-taxanes an emerging major structural type of taxoid. The structure of rearranged compounds **43, 44, 46, 47, 51** and **61** were presented accordingly [26].

There are few molecules **39, 45, 48, 50, 52, 54, 56, 59, 60** and **62** which were reported by doing metabolic profiling of needles extract by Mass spectrometry [28].

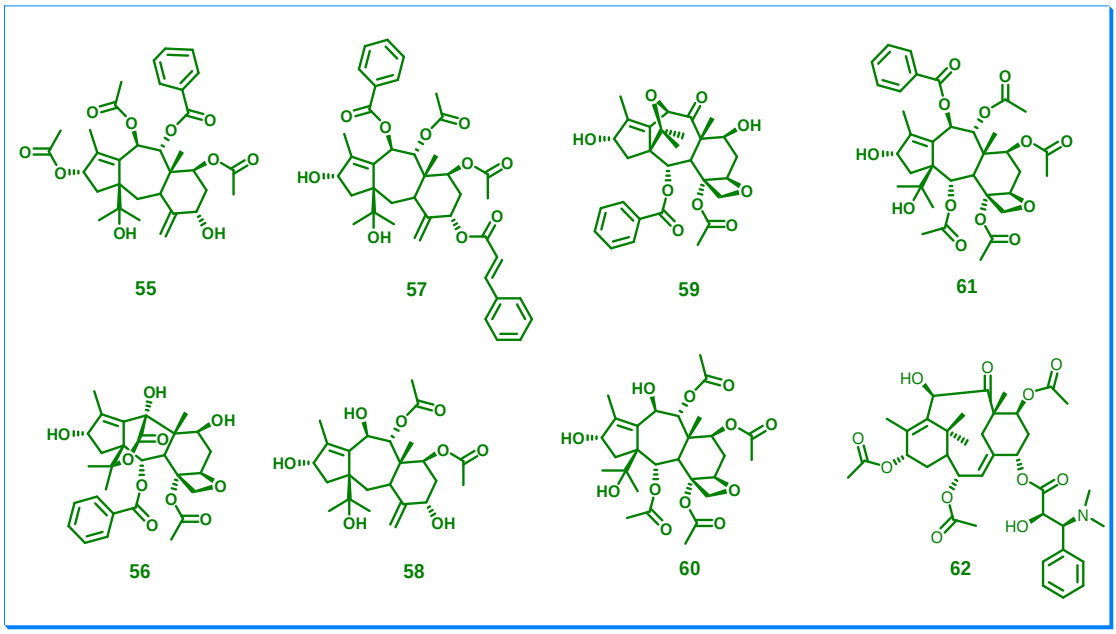


Figure (6): Taxanes isolated from leaves

1.4.2 Bark

T. wallichiana, bark was extracted with DCM and possessed six new taxoids, wallitaxanes (**65, 67, 68, 70, 71** and **73**), the wallitaxanes possessed core structure of taxol. Structure of all compounds was confirmed by the NMR spectroscopy. The isolated compounds were evaluated for their α-glucosidase inhibitory activity and cytotoxicity against the HeLa human cervical cancer cell line. In the present work, taxanes were found to exhibit α-glucosidase inhibitory activity for the first time and wallitaxanes A (**67**) showed the most potent activity, with an IC_{50} value of 3.6 µM [27].

T. wallichiana grows throughout the in different regions of the Himalayas in India. Needles of the plant were taken for the extraction by using methanol, and then partially purified methanolic extract was subject for the profiling of the taxoid by electrospray ionization tandem mass spectrometry (MS/MS). The study also revealed

the occurrence of several basic taxoids **63, 64, 66, 69** and **72** in these samples. MS/MS profiling by electrospray ionization was shown to be a fast and reliable technique for the analysis of taxoid samples [28].

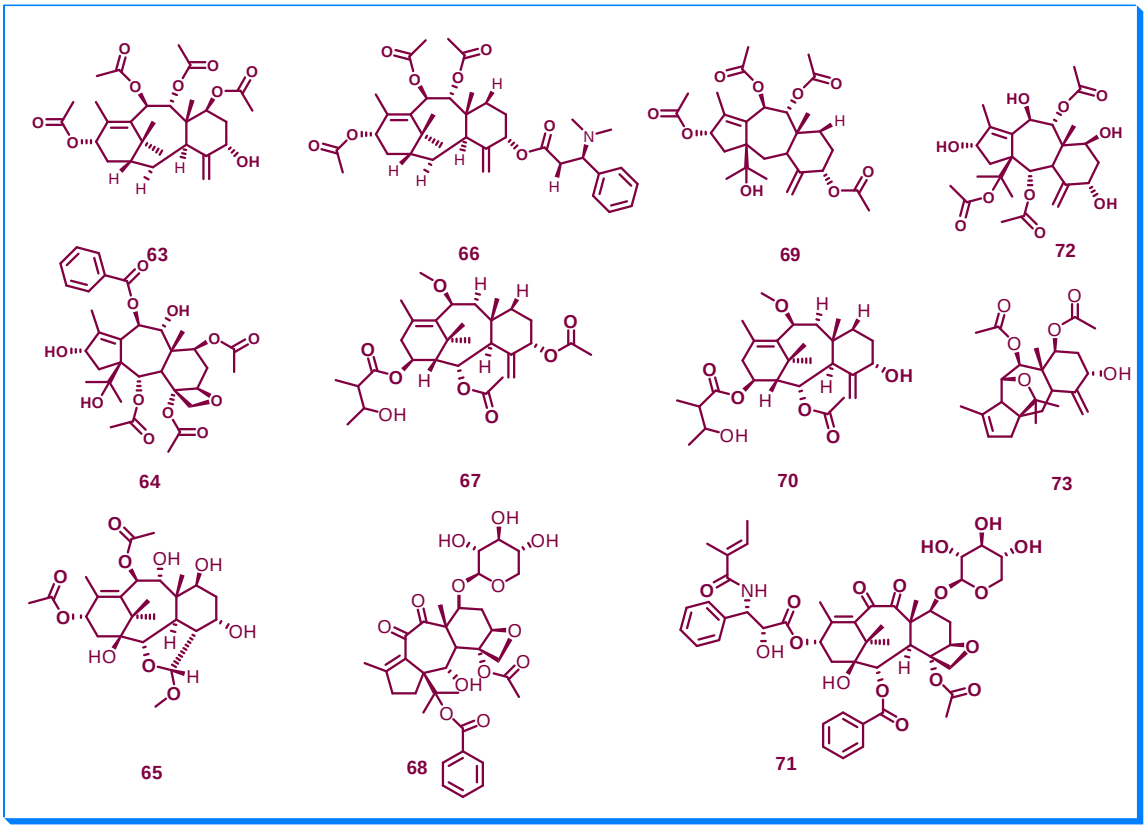


Figure (7): Taxanes isolated from bark

1.4.3 Roots

T. wallichiana plant growing in India and same species were growing in other countries taxus chinesis China, Vietnam and Philippine were called with same name but often few group named has *Taxus wallichiana*.var. mairei. From this about 41 compounds were isolated novel, first time and all other compounds **75-82** were isolated from this plant first time but these are already in the taxanes family. This new compound **74** screened on human breast cancer cell line MCF-7 IC_{50} 20.89 µg/ml. The new compound showed moderate cytotoxicity on above said cell lines [29].

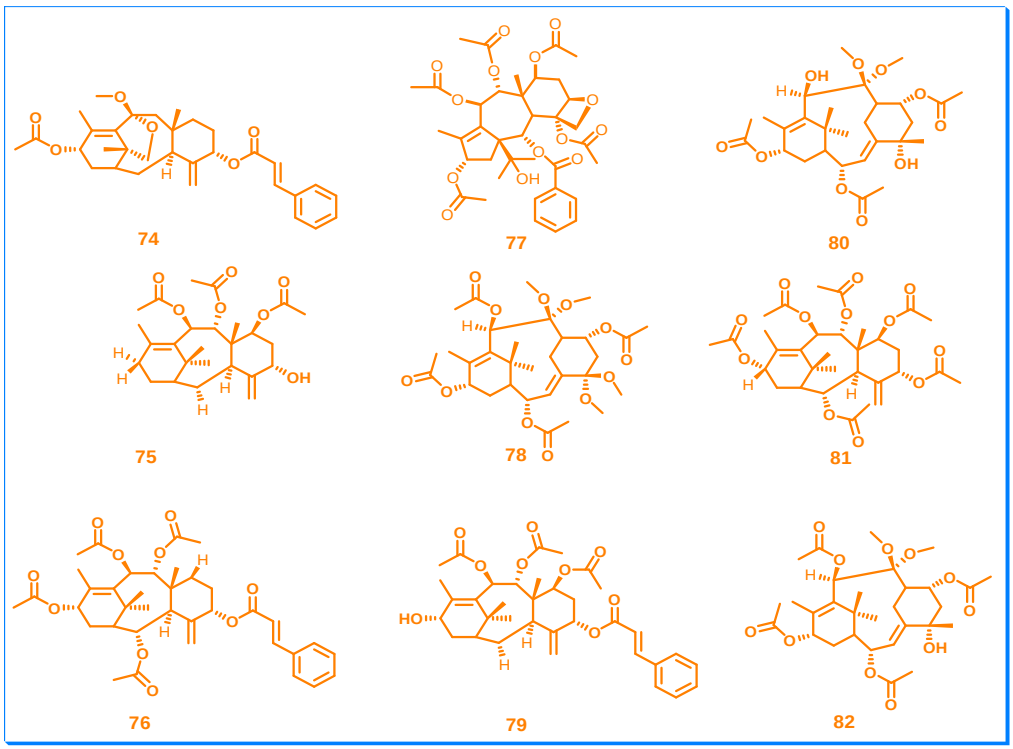


Figure (8): Taxanes isolated from roots

1.4.4 Bark, leaves, Twig and hardwood

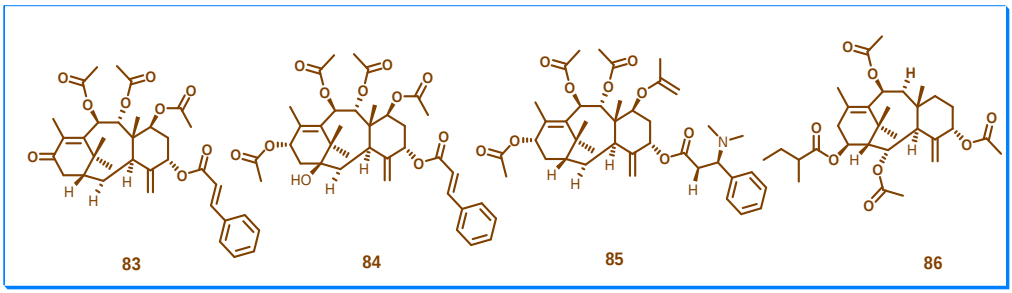


Figure (9): Taxanes isolated from aerial parts of the plant

Compound **83** was isolated from the Shrestha group from Nepal. This compound isolated from hexane soluble fraction of methanol extract [30]. Compound **84** and **85** was isolated from the bark of *Taxus wallichiana*. This bark was collected from the jilong Tibet P.R.China. The bark was dried and finely powdered and extracted with 95% ethanol and concentrated in rotary evaporator. Then dissolved in water, further fractionated with dichloromethane, ethyl acetate and n-butanol. Finally the DCM fraction was used in column chromatography which was yielded above said compound along with sitosterol, taxol and many other compounds [31].

Most of the time *T. wallichiana* needles were used in the chemical investigations because of the needles was collected from the plant without harming it. More over the plant was classified in the category of endangered. But there were no chemical investigation reports on hardwood of the *T.wallichiana*. Chattopadhyay group has carried out the chemical investigation on hardwood of the *taxus wallichiana* for systematic exploration. They brought hardwood from Himachal Pradesh and extracted with the methanol at room temperature by percolate method. The crude was extracted with chloroform, it was subjected to further purification by column chromatography which provided **86** and many other compounds, but all the compounds were already isolated from the taxus species previously [32].

1.4.5 Leaves stem and roots

T. wallichiana leaves, stems and roots were brought from the Shilong of the Meghalaya, India. The finely air dried above parts of plant material was finely grounded and extracted with the 95% of ethanol. All the fraction of crude mass were submitted for the anti-leukemic activity and found that the chloroform extract was good for the anti-leukemic activity. So chloroform crude was further fractionated to get the pure compounds **87** [33].

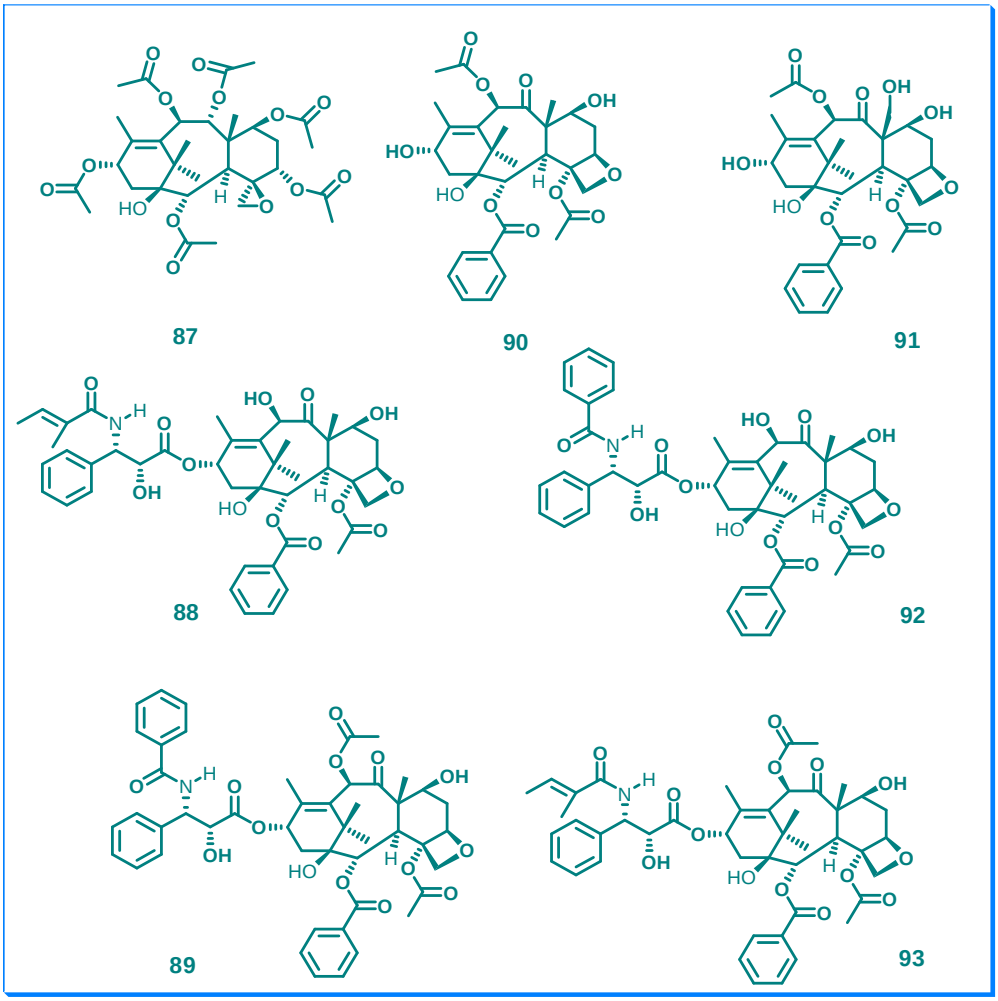


Figure (10): Taxanes isolated from leaves stem and roots

T. wallichiana leaves, stems and roots were extracted by percolation method. Then carried out cytotoxicity evaluation on KB cell line. The fraction of chloroform extract showed that significant activity. Chloroform extract were used in chromatographic fractionation which resulted the compounds of **88-93** [34].

1.5 Different sources for the taxanes

Taxanes can also be isolated from the family of taxaceae. There were other plant species also which produce taxol *Austrotaxus spicata.* Many taxanes were isolated from the plant source and only one natural taxanes has become active pharmaceutical ingredient towards anticancer and given many derivatives which have shown potent activity towards various cancer disease and marketed as drugs. But the taxol availability in plant was very less and its production was not sufficient to the market

demand. So there were many ways trial was going on to increase availability of the taxol by doing tissue culture. *In-vitro* tissue culture propagated plant and natural grown plant extract quantification, compared with the HPLC. The availability of taxol percentage was presented (0.85% and 0.055%) respectively [36]. Recently taxol also efficiently producing by the entophytic fungus which was isolated from the *T. wallichiana* var, marine bark. Almost 528 funguses were isolated from the above plant bark, but only one strain (*Phoma medicaginis*) was turned into high quantity of taxol producing in media [7]. Finally the best way to get the taxol is from the total synthesis and semi-synthesis. Similarly by using needles of the *T. wallichiana* growth rate has tried by using different light emitting bodies then the paclitaxel and baccatin were measured by using HPLC. The content of paclitaxel from callus derived from needles 0.00628% and the 10-Deacetyl baccatin-III content was 0.003665% then the paclitaxel content of callus derived from the stem was 0.00412% and 10-deacetyl baccatin was not detected [6].

1.6 *Taxus wallichiana* usage in various forms

1.6.1 Herbal formulation

T. wallichiana species are distributed in hill areas and their habitat is unique. This plant has many medicinal properties which were disclosed in the various folklore medicines. Moreover, it has been used from many years to cure various diseases like anticancer, antioxidant, antimicrobial antibacterial hepatoprotective, cold, cough, fever and anti-diabetic. Recently, it is reported that this plant roots with other plant leaves and seeds are used to prepare traditional Chinese medicine decoction (for manufacturing) for treating kidney-impairing-type diabetes and moreover it does not have any other side effects [35]. By using *T. wallichiana* leaves prepared healthy food and paste containing leaves 35-50, in combination with the other content like beer grains , tea, aloe and so on this has been used to moisturizing skin, preventing cancer and anti-aging [7].

1.6.2 Stem cell usage

Traditionally, *T. wallichiana* leaves, roots, bark, stem, twig and hardwood have been used as such in their original state or their extract in different ways to cure many chronic disorders in human being. High exploitation of the plant parts in the research as well as in traditional use and in herbal formulation. This plant has been declared an endangered. But now can be used *T. wallichiana* stem cells which were prepared and grown in a callus inducing culture medium where complete removing of toxic plant hormone from the cells. Now *T. wallichiana* cells without toxic hormone can be widely used as drug, food and cosmetic material [3]. This is the new way of usage and it will be direction to do same in the other medicinally important plant, stem cell can be prepared and used as such.

1.6.3 Marketed drugs

From this plant lot more taxanes were isolated. Out of them taxol has become very potential drug for treating cancer. In order to generate many derivatives, its core structure should be remaining same and only small changes in their functional groups, which can enable or enhance the activity of the derivative with comparatively parent drug. Sebaceous Carcinoma (SC) is eyelid tumor cancer, for this only the surgical treatment was present but, to date, no chemotherapy regimen has been established for Sebaceous Carcinoma with distant metastasis. They experienced a case of eyelid SC with multiple lung metastases that responded to combination chemotherapy with carboplatin and paclitaxel with 11-month progression-free survival (PFS). This patient also responded to second-line treatment with Docetaxel, another taxane, with 7-month PFS, resulting in at least 18-months of survival at the time of reporting. This report shows that taxanes-based chemotherapy may be effective for advanced SC, for which no standard therapy has been established [37]. The evolution of taxanes therapy for metastatic breast cancer (MBC) including the development of the novel delivery platform of nanoparticles albumin-bound (nab-paclitaxel). Combination of albumin and paclitaxel that forms particles of a mean 130 nm in diameter. The nab-paclitaxel formulation was solvent free and employs a novel delivery mechanism for paclitaxel to tumor. Nab-paclitaxel, the only solvent-free taxane indicated for the treatment of MBC. Although paclitaxel was initially designed to minimize the toxic effects of

taxane treatment and improve tolerability, it became evident that this formulation of paclitaxel was also more effective compared with standard Abraxane (cremophore ethoxylated is a formulation vehicle which used for the low soluble drugs formulation) CrEL paclitaxel for the treatment of MBC. Docetaxel was superior to CrEL-paclitaxel (solvent based drug delivery) efforts to improve on the tolerability of the solvent-based taxanes using a novel method for drug delivery [38].

At present, Taxanes are commonly prescribed to treat several cancers and have been shown to have an antitumor effect on lung cancer, gastric cancer, breast cancer, and ovarian cancer. Moreover, taxanes have been consistently used in combination with other systemic chemotherapeutic agents including 5-fluorouracil, cisplatin, bevacizumab, and S-115–17. Some meta-analyses have evaluated the use of paclitaxel and docetaxel against cancers such as advanced non-small-cell lung cancer, prostate cancer, and breast cancer [39]. Novel oral formulations of docetaxel and paclitaxel are DHP107 and Modra Doc001 respectively, which may provide an alternative route of dosing those medications. Ortataxel and milataxel have been used in Phase I studies but oral dosing is not yet well established. BMS-275183 has been used in several Phase I studies at different doses with established oral dosing. Tesetaxel was the most widely studied of the novel oral taxane and was currently being studied in Phase II trials both alone, as well as in combination regimens with capecitabine Clin Investing (London). 2013; 3:333–341 [40].

1.7. **Brevifoliol derivatives**

Generally nature is the best chemistry in this world which can make novel structure and highly selective activity which are highly useful for the peoples. There are many ways (medicinal chemistry, combinatorial chemistry and, derivatization) to generate novel structures and can be tested on various diseases to know their activity. But the synthetic compounds do not show the selectivity in their activity. In selectivity and unique nature of the natural product isolation and their derivatization occupied high impact and this plant fully utilized to isolate all the class of compounds like abeo-diterpenoid, taxoid, flavonoids, lignan's, steroids and essential oils [1]. Coupling reaction performed by using β-lactam, brevifoliol in DCM with pyridine and DMAP which yielded compound **95**. This derivative has not shown activity in the

microtubule binding assay. Thus compound has like taxol like link but its core has little bit modification, this maybe the reason that it was not showed the activity like taxol [41]

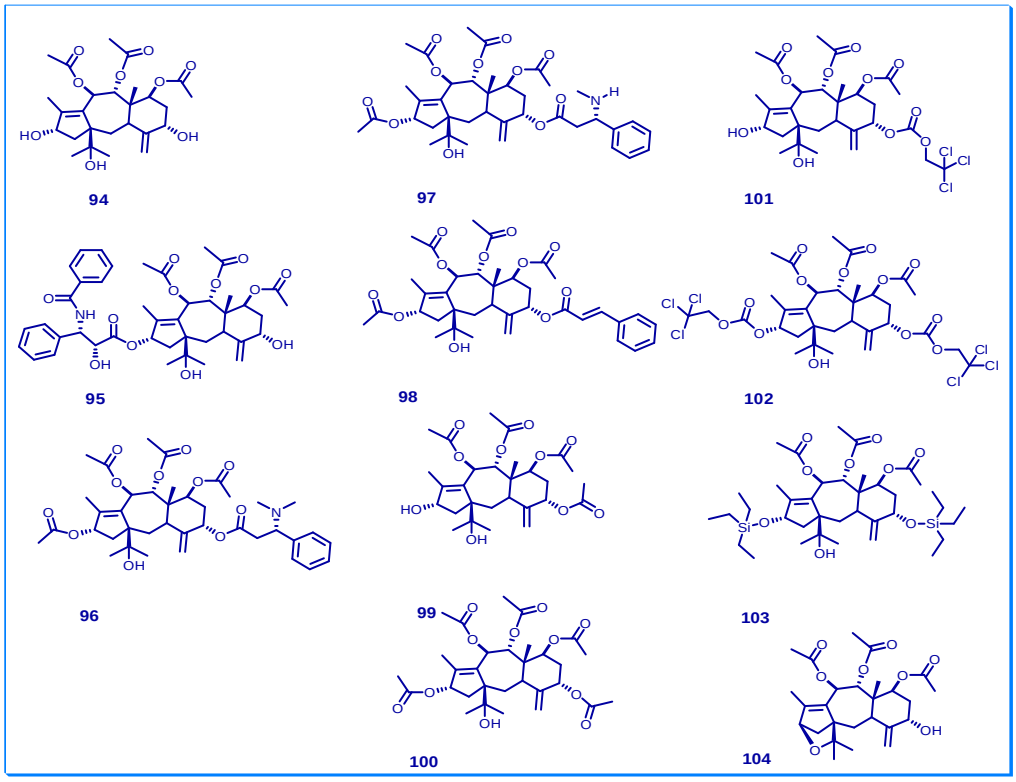


Figure (11): Brevifoliol and its derivatives

A series of brevifoliol natural diterpene derivatives **100-106** were prepared. Beauty of their derivatization was in introduction of acetyl, Troc, and TES groups at C-5 and C-13. All the derivatives were evaluated for their biological activity.

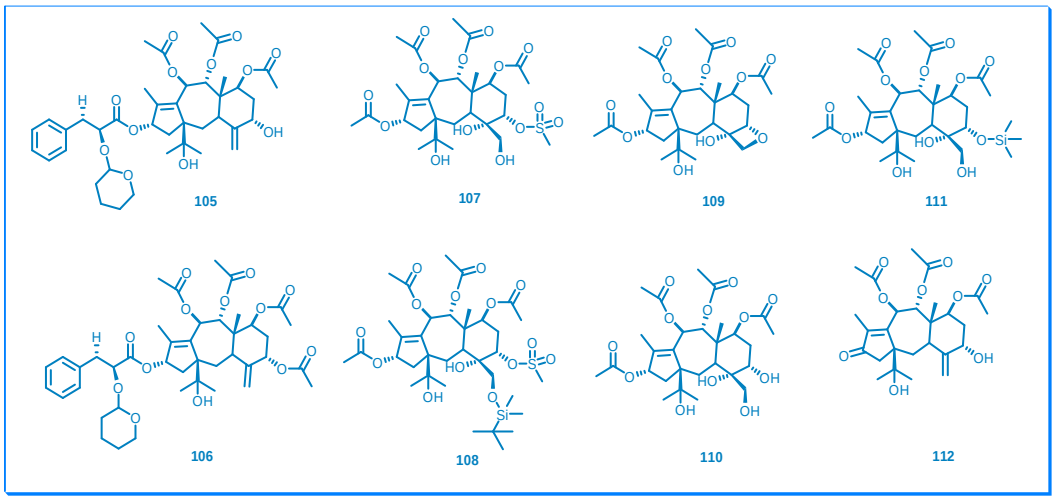


Figure (12): Brevifoliol and its derivatives

Most of the chemical derivatization can be performed to get highly active lead molecule by adding similar or relevant Pharmacophore which they target (disease) the activity being studies. Further the protection of functional groups in medicinal chemistry only like methylation, ethylating and benzylation so on. But here they performed unique functional groups for the derivatization [43].

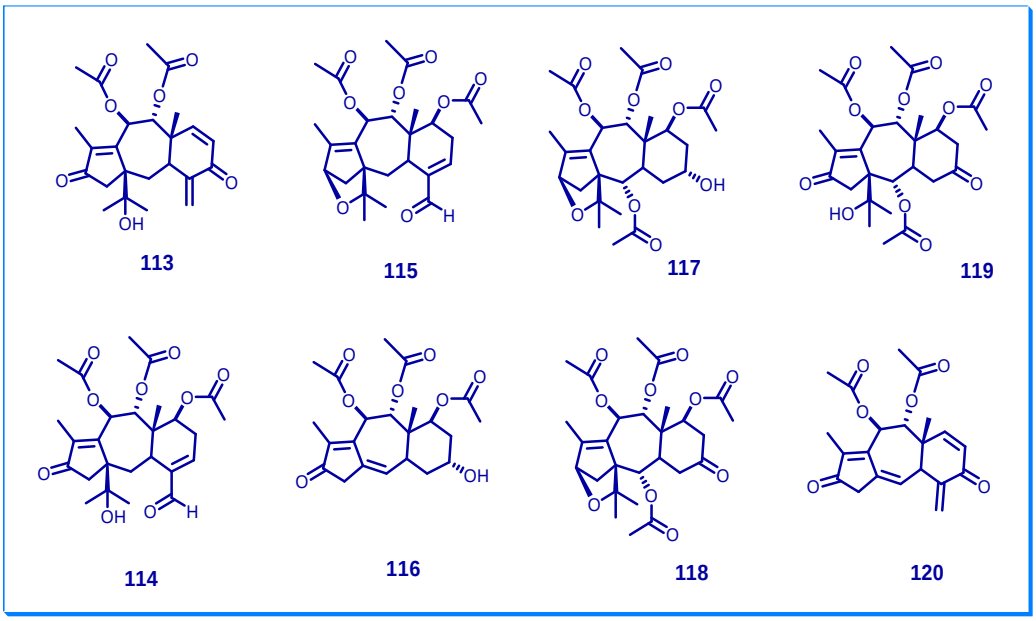


Figure (13): Brevifoliol and its derivatives

There were many derivatives prepared analogs **107-125** by using brevifoliol and deacetoxy brevifoliol as a starting substrates. Most of the derivatives have shown activity on the human non-small-lung cancer (A549). Compound-**117** was the most potent derivative [42].

A novel derivative of brevifoliol-**100** was isolated as oil from the chloroform soluble fraction of the methanol extract of the needles of *T.wallichiana*. Main difference was that brevifoliol and novel brevifoliol derivatives at C-13, C-5 and C-10 positions have acetylated group whereas parent brevifoliol had C-13 and C-5 positions as free hydroxyl group and C-10 position was benzoylated [44].

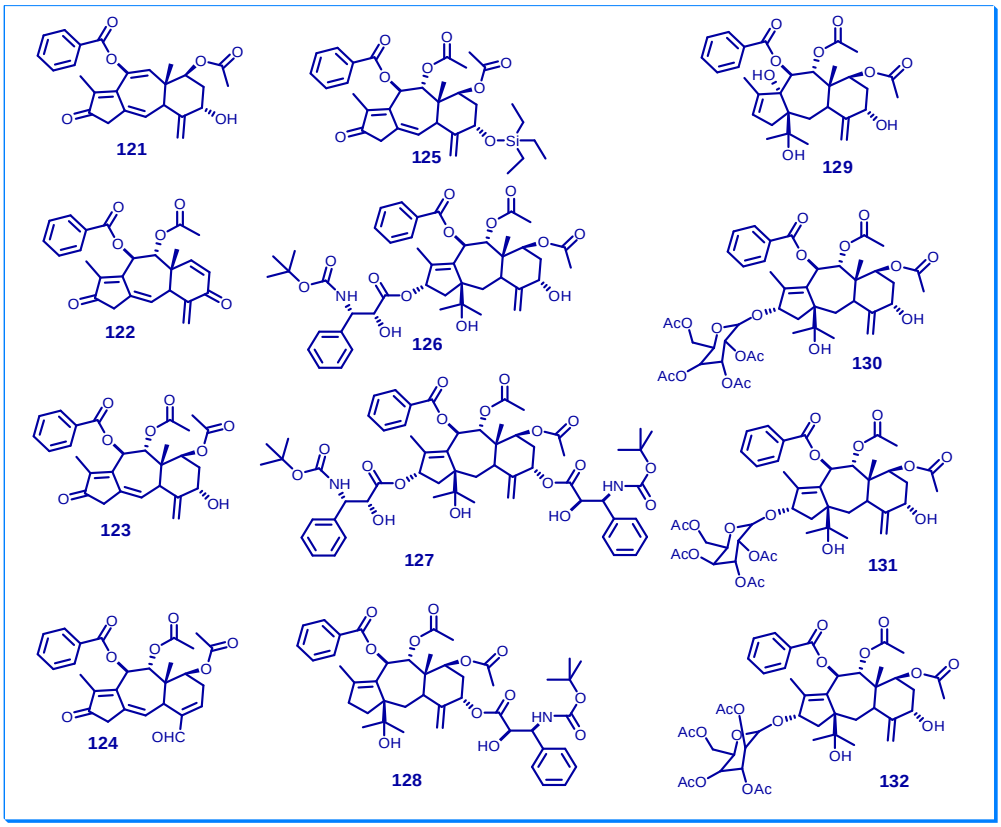


Figure (14): Brevifoliol and its derivatives

Natural diterpenoid brevifoliol has shown significant cytotoxicity against colon (Caco2), mouth epidermal (KB), breast (MCF-7) and liver (HepG-2) cancer cell lines. This was the basis and to start the derivatization of brevifoliol to get potent derivatives. Then prepared four derivatives **129-132.** All the four derivatives were tested for *in vitro* cytotoxicity against four human cancer cell lines. Derivatives **129**, **130** and **131** exhibited significant inhibition of cell growth [45].

Most of the brevifoliol derivatives (**94, 96-99** and **126-128**) were prepared and their structure was confirmed by the X-ray crystallography and their structure was presented in the article [46].

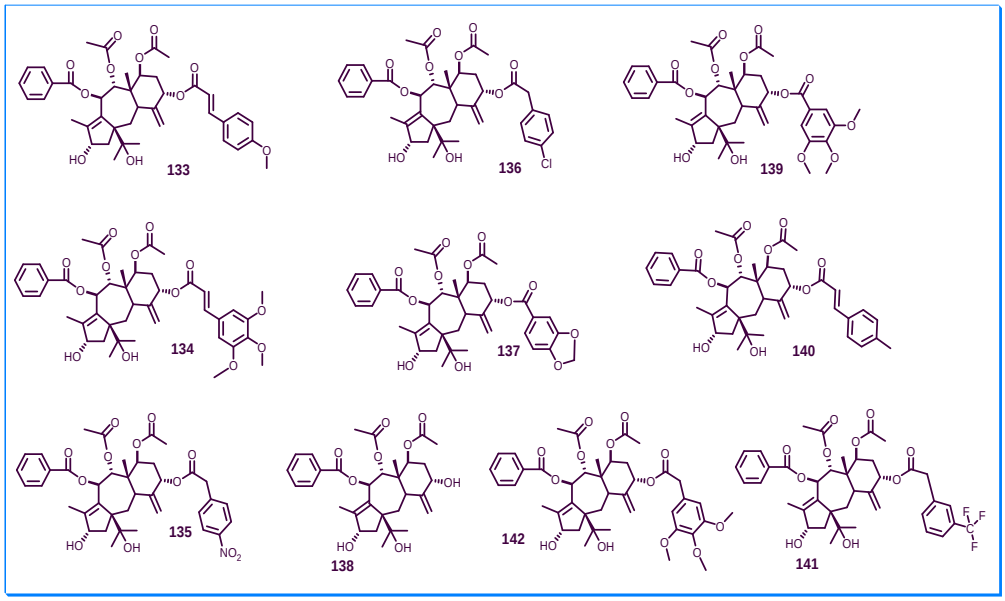


Figure (15): Brevifoliol and its derivatives

Recently we also isolated a few taxanes from the *Taxus wallichiana* plant and derived **18** derivatives **133-151** and all the derivatives are evaluated their anti tubercular activity on H37Ra strain. Out of 19 derivatives almost all the derivatives showed the above activity [47].

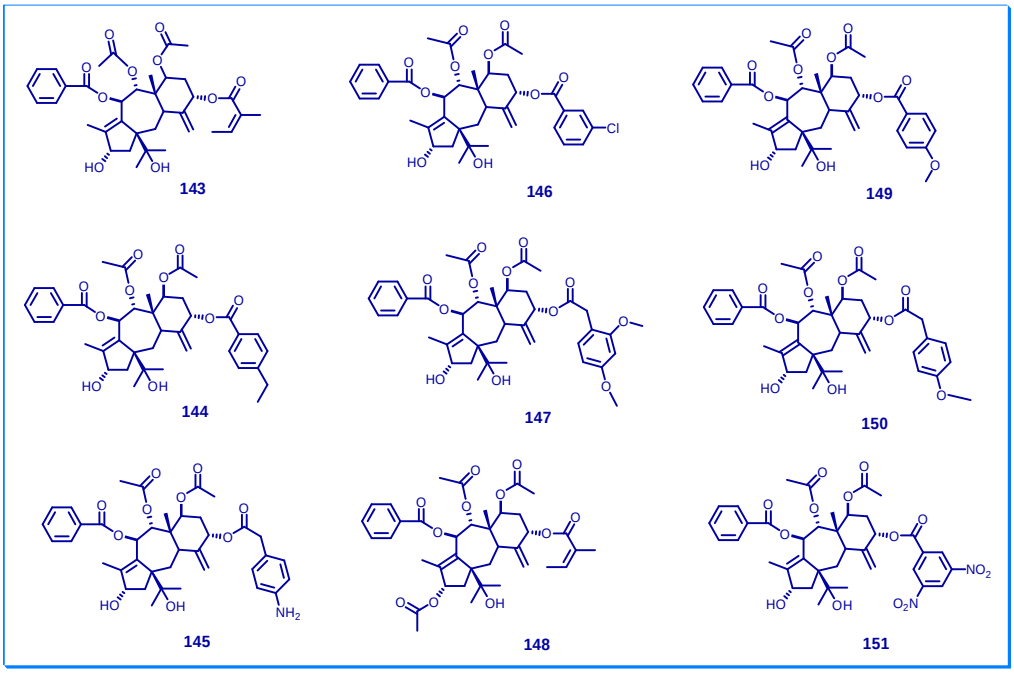


Figure (16): Brevifoliol and its derivatives

1.8 10-DAB derivatives

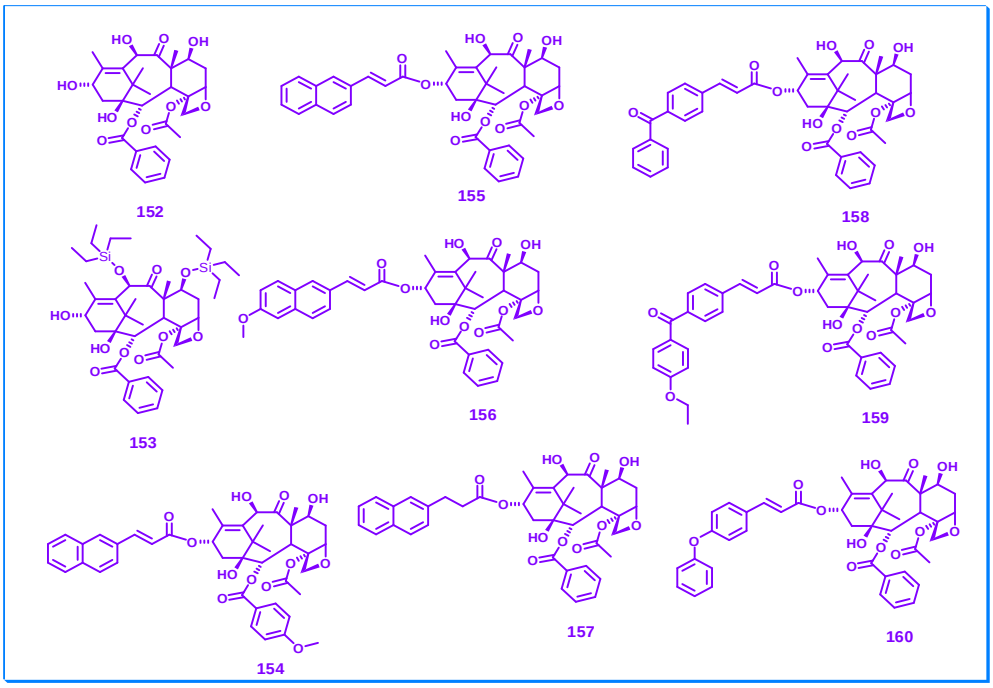


Figure (17): 10-deacetoxy baccatin and its derivatives

Screening of **120** taxanes rational optimization of selected compounds led to the discovery that the C-seco-taxanes as multidrug-resistance (MDR) reversal agents (C-seco-TRAs) and non cytotoxicity at the upper limit of solubility and detection (>80 µM), while maintaining MIC99 values of 1.25-2.5 µM against drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* (Mtb). Out of many taxanes the 10-deacetylbaccatin (10-DAB) was taken as starting substrate for further derivatization to get many derivatives to tackle the Mtb. By using the 10-DAB derivatives **153-168** were prepared and one derivative derived from the paclitaxel **163,** all the derivatives were evaluated the anti-tubercular activity [48].

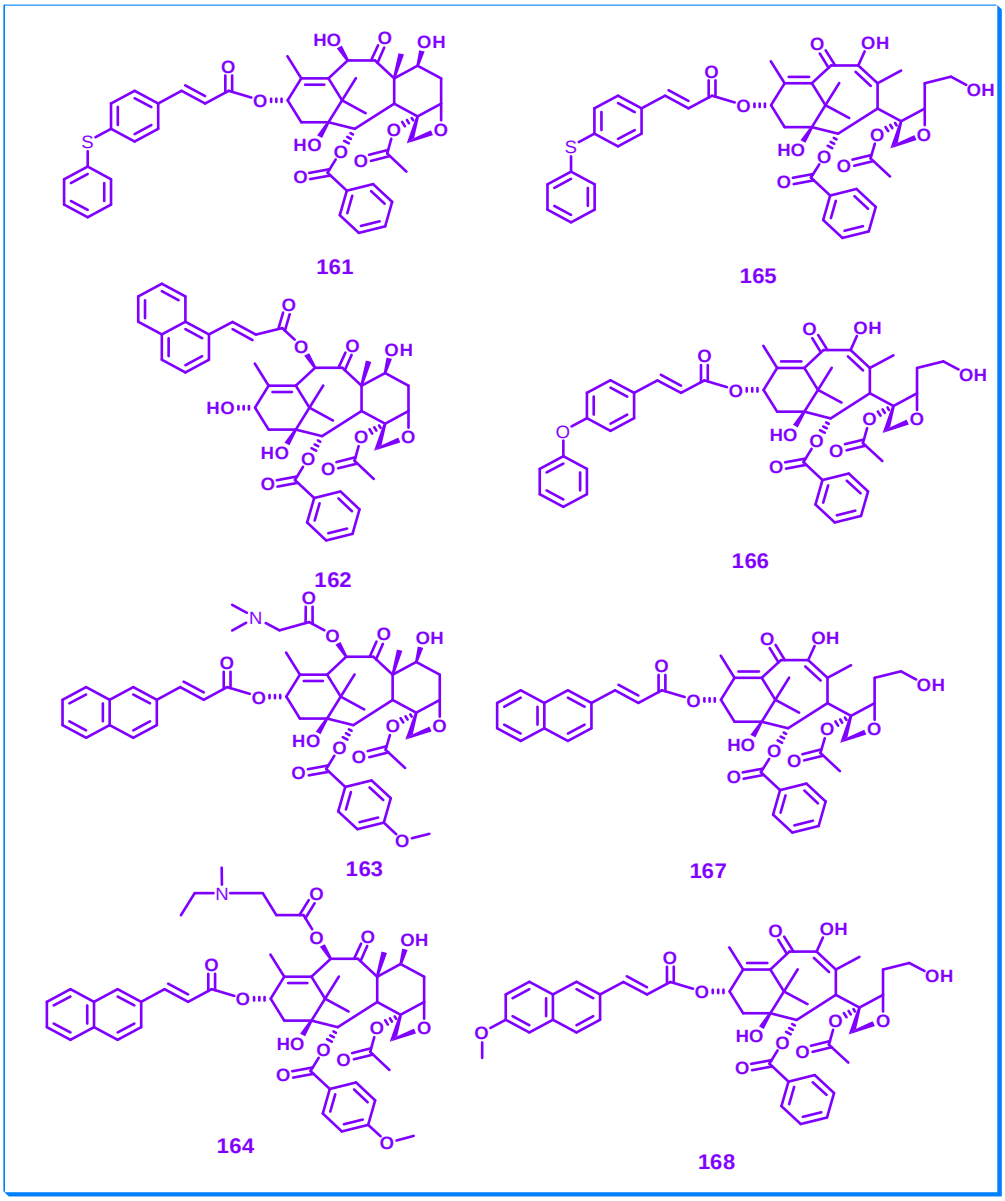


Figure (18): 10-deacetoxy baccatin and its derivatives

1.9 Taxol isolation, synthesis and mode of actions

Taxol is API which was isolated from the Pacific yew or *Taxus brevifolioa* . Its molecular formula $C_{47}H_{51}NO_{14}$ and it has 11 stereo centers.Taxol is a diterpenoid tetracyclic compound attached with phenyl isoserine side chain. Taxol is the brand name for the paclitaxel. Paclitaxel bind with tubulin protein and stabilizer the microtubuline proliferation process. Taxol have many side effects like nausea and vomiting, brittle hair, joint pain and skin rashes so on. Total synthesis was the hot topic in 1990s almost 30 research groups were involved for its synthesis in the

1992.Taxol is the most important molecule and it first total synthesis completed by the Dr. Robert Holton, Florida State University on: 12/21/1993. Secondly taxol total synthesis completed by Dr. K.C. Nicolou Scripps on: 1/24/1994. Both the total synthesis was linear synthesis. Method contains 51 steps 0.03 % overall yield. Total synthesis of taxol is not feasible and not economical. This is the commercial method developed by Bristol-Myers Squibb.

1.10 Taxol derivatives

Taxol was a naturally occurring diterpene, isolated from the bark of *Taxus brevifolia*. Taxol derivatives **169**, **179, 185, 186** were more potent than the taxol. Historically, parts of the yew tree were used for treatment of noncancerous conditions and leaves of *Taxus wallichiana* were used in Indian medicinal system (Ayurveda), with only one reported use in the cancer treatment [40, 49]. Paclitaxel isolated from this plant (In 1981, USA lead a research for the isolation of paclitaxel at 0.001% from a mixture of leaves, stems and roots of *T. wallichiana*) [50]. It showed very good activity towards many cancers. Docetaxel is a semi-synthetic analogue of paclitaxel. This drug is used to treat many types of cancers like non-small-cell lung cancer, breast cancers, stomach cancer, prostate cancer, advanced ovarian cancer, head and neck cancer as a chemotherapy agent. Docetaxel increases survival time in certain types of cancer. These taxanes like taxol and docetaxel drugs mostly bind with sub-unit tubulin, and then increasing polymerization of tubulin to stabilize the resultant microtubules, there is no further depolymerization. Cancer cells generally develop multidrug-resistant to chemotherapeutics [51]. To overcome this situation hunting for the new leads will be very important. Recently, Kingston et al, prepared new derivatives **170**-**177** of taxol and some of them possess higher activity (**170, 171, 176,** IC_{50}- 0.36, 0.9 0.36, nM) than the paclitaxel and docetaxel (IC_{50} 1.7, 1.0 nM) and are considered as second-generation taxanes [52].

Paclitaxel and docetaxel have P-GP based (MDR) and many drawbacks, significant side effects are there in clinical use of the above drugs. To overcome the side effects of these drugs, derivatization of the parent molecule should be carried out to get no or less side effect. Although many derivatives were prepared which had higher activity than the parent molecules but only one molecule **173** was the best and selected for

clinical studies due to its excellent oral bioavailability and did not show any notable neuron and cardio toxicity which provides a wider therapeutic window [53].

Docetaxel was approved by US FDA in 2004 as a first-line drug to treat metastatic Castration-Resistant prostate cancer (mCRPC), but after long-duration prostate cancer becomes resistant to Docetaxel. To overcome the situation cabazitaxel was approved by the US FDA as a second-line drug to treat docetaxel resistant patients, to increase their overall survival rate with cabazitaxel [54].

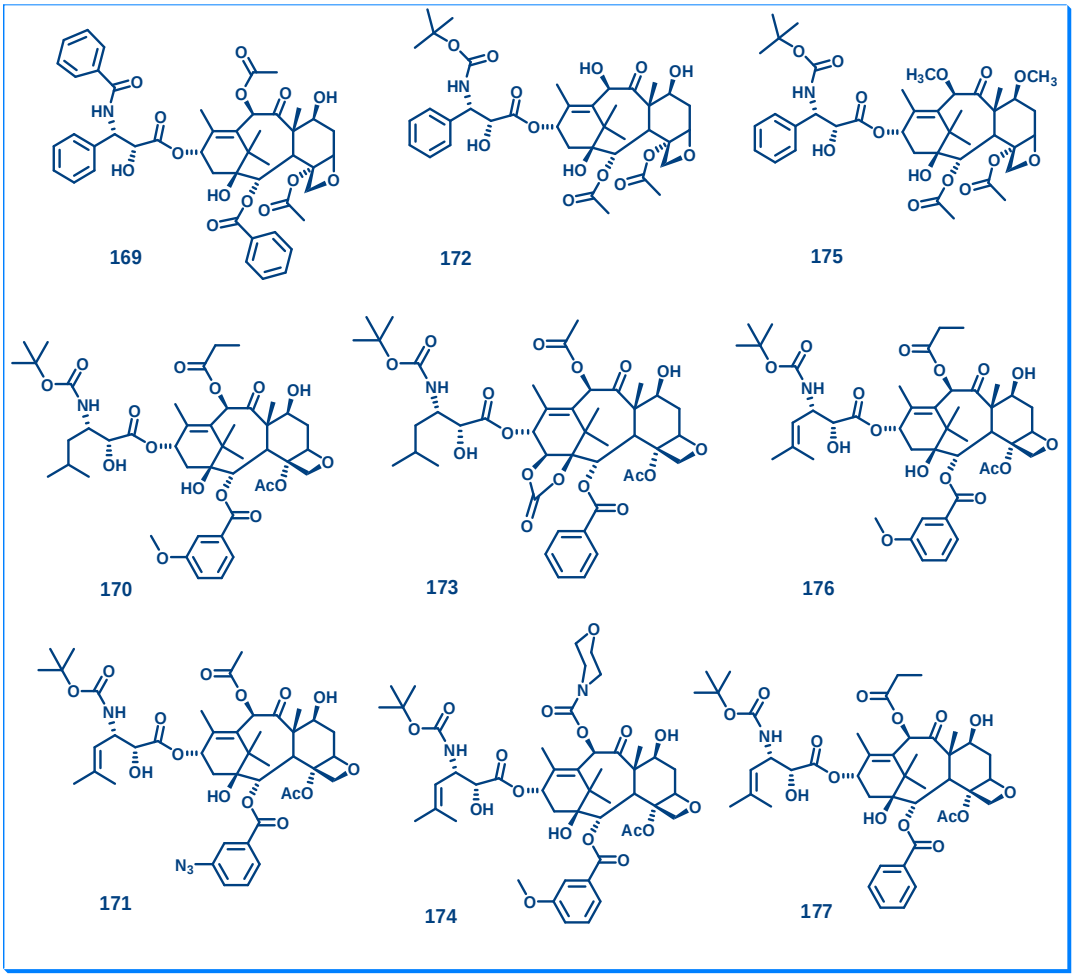


Figure (19): Taxol and its derivatives

182 and ortataxel both were novel derivatives of paclitaxel. Their activity can be comparable with their parent molecule. These two drugs were tested orally as well as intravenously against lung carcinoma, colon carcinoma, breast cancer and ovarian cancer respectively. Both the analogues showed equal activity with parent molecule. The milataxel was semi-synthetic analogue of docetaxel .It was used in KB-8-5, MX-1W and HCT-15. Routed as orally and intravenously and found better activity in oral

dose than the paclitaxel. Tesetaxel another taxanes analogue was more potent than the paclitaxel in oral dose against breast cancer, gastro cancer, colorectal cancer and other solid tumors. Its synthesis from taxol or docetaxel is not yet disclosed.

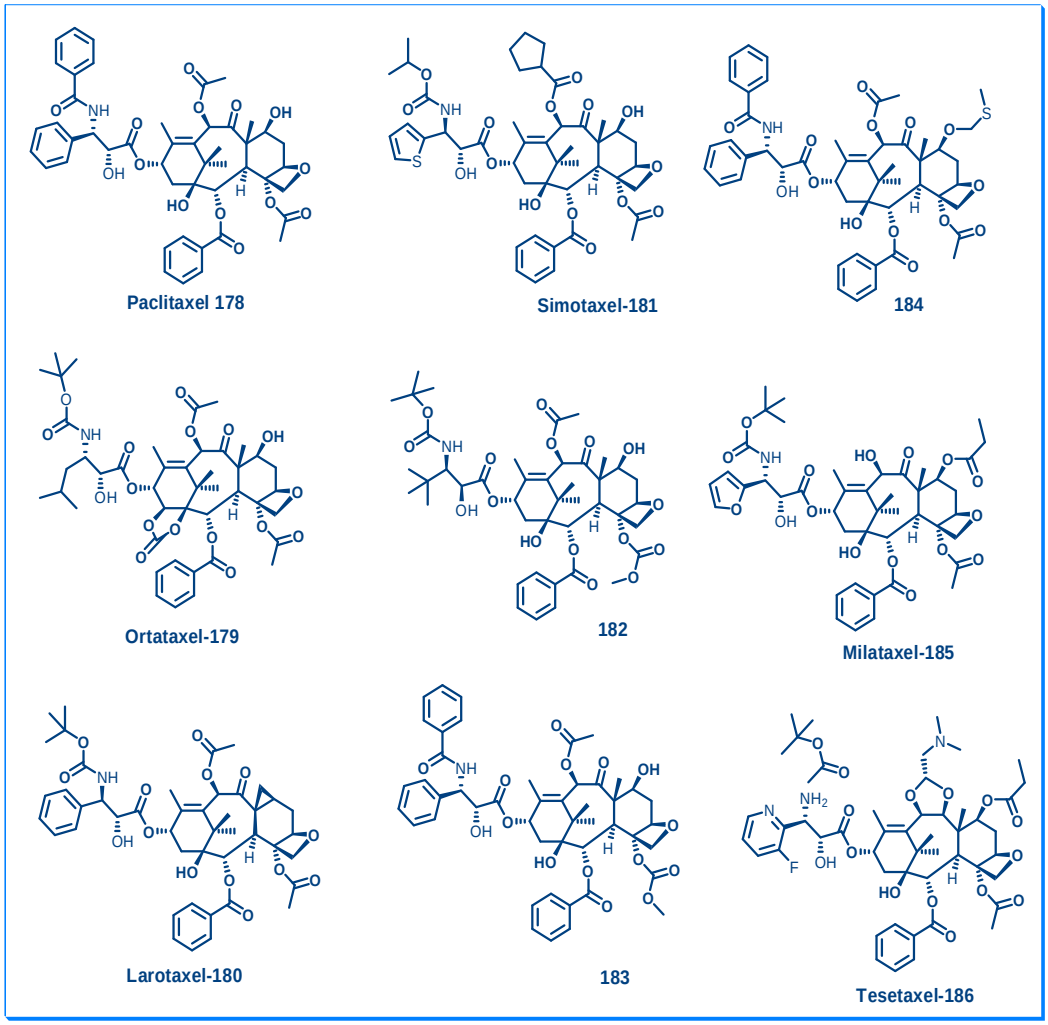


Figure (20): Taxol and its potent derivatives

Generally first line taxanes chemotherapy used to treat the breast cancer. Once the breast cancer becomes resistance to the first line taxanes then to treat the resistant or non resistant breast cancer can be treated with Larotaxel which is a novel taxoid with preclinical activity against taxanes-resistant breast cancer. Larotaxel has good activity, low toxicity, and a favorable therapeutic index in women with taxanes-pretreated MBC [55].

183 is a novel paclitaxel analogue which showed two fold higher activities than the parent molecules. Patients with advanced malignancies were treated with escalating doses of **183** on a weekly schedule as a 1-h i.v. infusion. Plasma sampling was

performed to characterize the pharmacokinetics of BMS-188797 [56]. Simotaxel and **184** were not yet disclosed that from which taxanes it was derived and used to cure the advanced tumor. **184** may have several advantages compared with paclitaxel in terms of toxicity, pharmacokinetics, pharmaceutics, and its administration warrants further clinical development [57].

1.11 Conclusion

According to the literature survey about 3500 taxanes have been isolated from the Taxaceae family. Among them only one taxol molecule was the most potent. By using taxol many derivatives has been prepared and derivatization yielded 18 derivatives. Some of them are already in the market as drugs and others are in the various phases of clinical trials.

1.12 Author's views

There are few derivatives which are having significant activity more than taxol. Those molecules can be derived further to get more lead molecules and can be used as second generation drugs. Plant parts are used in herbal medicine and more can be concentrated on tissue culture formulation for their better use.

1.13 References

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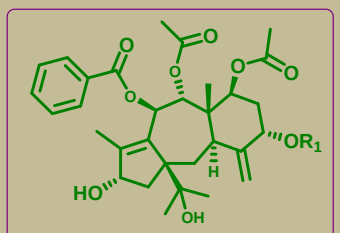
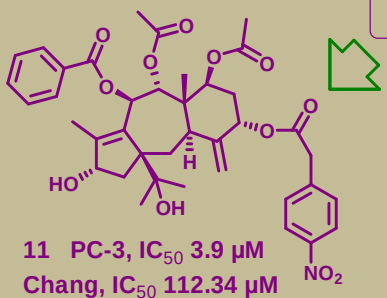
Chapter-2A

C5 Derived esters of brevifoliol as anticancer agents against prostatic adenocarcinoma

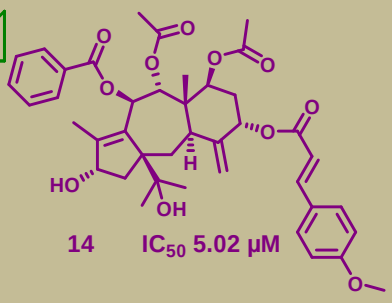
Significant active derivatives on prostate adenocarcinoma cells



1 Brevifoliol IC_{50} 185 µM



11 PC-3, IC_{50} 3.9 µM
Chang, IC_{50} 112.34 µM



14 IC_{50} 5.02 µM

2.1 Introduction

Cancer is a group of disease identified by the uncontrolled growth and spread of abnormal cells. Still its causes remain elusive. Depending on advancement and duration, it spreads to other organs known as metastasis. If abnormal cell growth uncontrollable then it leads to death. Cancer is one of the major grounds of morbidity and mortality worldwide. According to the WHO, cancer was the 2nd leading cause of death globally, and roughly accountable for 9.6 million deaths in 2018 [1]. Prostate cancer incidences occupied fourth place around the world. It can be screened with the prostate-specific antigen (PSA) blood test. Worldwide, there were approximately 1.28 million cases in 2018 [1]. It was the common cancer in men in USA. Prostate cancer cases were 60% more in blacks than white. However it causes remain unclear. Usually, prostate cancer grows slowly and firstly attacks to prostate gland. However, critical and spreading type prostate cancer must be treated early. Currently, flutamide, docetaxel, cabazitaxel, abiraterone, enzalutamide, apalutamide, mitoxantrone etc, are available as first generation drugs with many side effects [2]. On further advancement it may migrate to other organs (metastasis). Among the various risk factors to increase chances of prostate cancer, were increasing age, racial, family history, and obesity. Consequently it may cause metastasis, urinary incontinence, and erectile dysfunction. Treatments for metastatic disease may eventually be limited by the development of biological mechanisms of drug resistance [3]. Advanced or terminal stage is not always curable and needs palliative care. There is scope and need to generate an efficacious, safe and affordable drug against prostate cancer.

Yew plant (*taxus spp*, taxaceae) is a slow-growing evergreen medicinal plant. Long-time ago Native Americans used bark of *Taxus brevifolia* as a disinfectant, an abortifacient, and medicine for healing skin cancer. China has been this plant using for headaches, calming nerves, cold, cough and snakebites by local residents. In India, the Himalayan region population has used bark of *Taxus wallichiana* for making tea. From various species of the genus of Taxus a number of taxanes have been isolated from various species of taxanes like Himalayan yew (*Taxus wallichiana*), Japanese yew (*Taxus cuspidata*), pacific or western yew (*Taxus brevifolia*), English yew (*Taxus baccata*) and Canadian yew (*Taxus Canadensis*). So far no other plant has been reported to contain taxanes except of these in Taxus and *Austrotaxus* of Taxaceae.

The Austrotaxus is represented by only one specie *Austrotaxus spicata,* distributed in New Caledonia Oceania with limited chemical investigations. [4, 5]

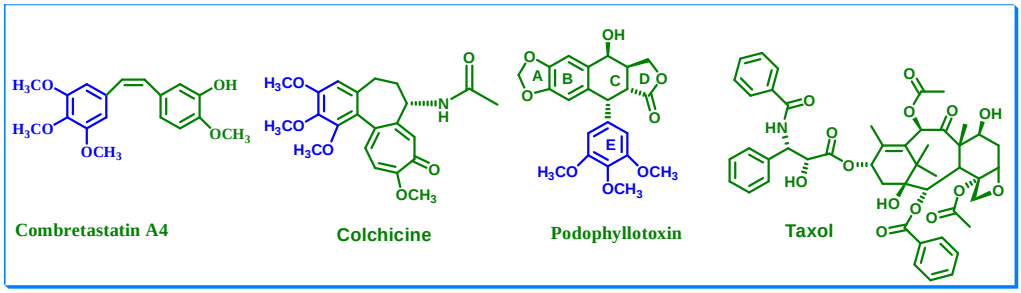


Figure (1): Few natural molecules as an anticancer drug

Among the 3500 natural taxanes, only paclitaxel (taxol) has shown significant activity towards cancer, but taxol production by bioengineering has not become applicable to scale-up, hence total synthesis of taxol was left as the best one of the alternative. In this regard six groups of chemists tried to carry out total synthesis and after two decades success was achieved with linear synthesis but in extremely low overall yield, because the construction of oxetane ring was very tough. Thus total synthesis of taxol became impractical for bulk scale commercial production. Another way to approach for taxol production its semi-synthesis from 10-deactyl baccatin and analogs [6].The 10-deactyl baccatin can be isolated from the needles, (renewable sources) and this is the current commercial method to produce taxol. However, the availability of yews is decreasing significantly due to over-exploitation for medicinal and commercial purposes. So the most challenging task is that we have to start designing, synthesis, and derivatization of biologically active natural products which can reduce the exploitation of nature and provide the library of the hit molecules.

2.1.1 Importance of taxanes and abeo-taxane in cancer

Nature is the best source for the new class of chemical entities and plants are the finest chemist in this world. Nowadays most of the drugs in the market are from medicinal plants. For example, taxol from *Taxus brevifolia,* is a naturally occurring diterpene which was isolated from the bark of *Taxus brevifolia*. Historically, parts of the yew tree were used for noncancerous conditions and leaves of *Taxus wallichiana* were used in Indian medicinal system (Ayurveda), with only one reported use in the cancer treatment. [7, 8]. Taxol and docetaxel are the most active against various

human tumors like breast cancer, advanced ovarian cancer [1, 2]. These taxanes like taxol and docetaxel drugs mostly bind with subunit tubulin, and then increasing polymerization of tubulin to stabilize the resultant microtubules, there is no further depolymerization. Cancer cells generally develop multidrug-resistant to chemotherapeutics. To overcome this situation hunting for new leads will be very important. Recently, Kingston *et al.* Prepared new six derivatives of taxol and some of them possess higher activity (SB-T-11033, SB-T-121303, SB-T-121304, IC_{50}-0.36, 0.36, 0.9 nM) than the paclitaxel and docetaxel (IC_{50} 1.7, 1.0 nM), they are considered as second-generation taxanes.

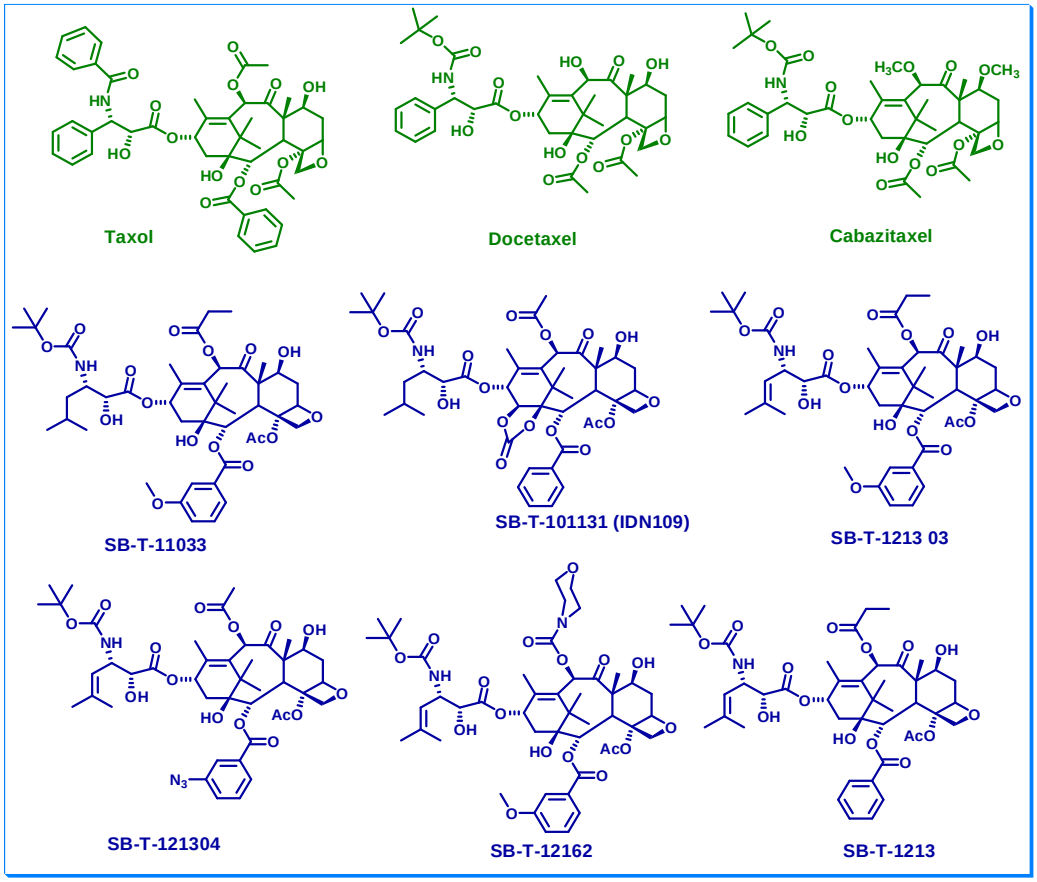


Figure (2): Taxane related drugs

Paclitaxel and docetaxel have P-glycoprotein based (MDR) and many drawbacks, like significant side effects are there in clinical use of the above drugs. To overcome the side effects of these drugs, derivatization of the parent molecule should be carried out to get no or less side effects. Although many derivatives were prepared which had higher activity than the parent molecules but only one molecule SB-T-110131 was the

best and selected for clinical studies due to its excellent oral bioavailability and did not show any notable neuro and cardio toxicity which provides a wider therapeutic window [9].

Docetaxel was approved by US FDA in 2004 as a first-line drug to treat metastatic castration-resistant prostate cancer (mCRPC), but after long-duration prostate cancer becomes resistant to docetaxel. To overcome the situation cabazitaxel was approved by the US FDA as a second-line drug to treat docetaxel resistant patients to increase their overall survival rate with cabazitaxel.

2.1.2 Outline of hypothesis

Phytomolecules play very important role in the drug discovery and development process [10]. The drugs used for the cancer treatment are about 49% of the phyto-molecules or directly derived from them. Medicinal plants are valuable sources of novel bioactive phyto-molecules which can produce novel structure with novel activity. There are many more new taxoids in taxus species which may possess impressive anticancer activity. But their very low abundance and complex system limit them to use as such. However, taking some structural learning from these taxoids, some semi synthetic drugs can be developed like docetaxel and cabazitaxel [11]. This paucity of active natural product mostly hampers elucidation of their target studies or extensive biological studies.

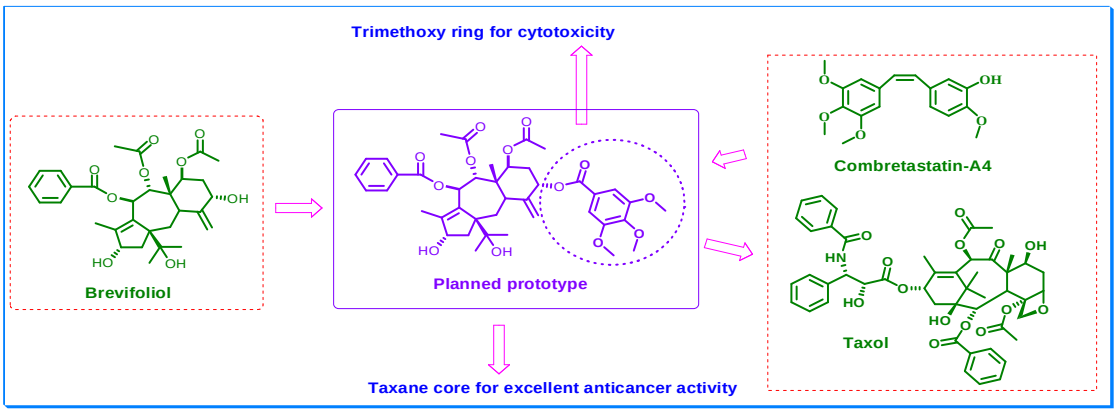


Figure (3): Outline of hypothesis

Brevifoliol is a rearranged Abeo-taxoid. Brevifoliol is a major taxoid which was high in percent in Himalayan yew (*T. wallichiana*). Brevifoliol itself possessed poor cytotoxicity against several human cancer cell lines (IC_{50}>100µM). We planned to introduce some important fragments at its C5 position to enhance its efficacy. The rationale behind synthesizing the C5 analogues of brevifoliol was for inducing selectivity towards certain tumors and increasing its cytotoxicity. Previously, several researchers have explored the cytotoxicity of C13 ester derivatives of brevifoliol. Synthesized mainly C13 esters and two C5 esters of brevifoliol [12]. C13 ester derivatives possessed potential anticancer activity while the both C5 ester possessed low activity. But, prostate cancer cell line was not used by this group. In another study, prepared several C5 and C13 ester derivatives of brevifoliol. Our curiosity was to explore C5 position of brevifoliol (Which is not explored much), using diverse fragments to enhance cytotoxicity of parent molecule.

Since, several derivatives of brevifoliol have been synthesized and some of them showed significantly increased cytotoxicity in different cancer cell lines [13-15], Brevifoliol was selected as synthon for chemical transformation into new derivatives and their derivatives were evaluated for their anticancer potential followed by *in-silico*, cytotoxicity and *in-vivo* studies.

2.2 Results and discussion

2.2.1 Isolation of brevifoliol

68 Kg of fresh needles of *T. wallichiana* were collected from Jageswar, dist Almora, Uttarakhand, India. Plant material was shade dried, powdered and extracted with the increased polarity of organic solvents as shown in the Figure (10). The purified brevifoliol of 1.64 g from above plant material was used for starting substrate for derivatization.

2.2.2 Synthetic strategy

Purified brevifoliol (1.64 g) was further derivatized by using steglich esterification chemical reaction at C5 hydroxyl as shown in 2.6.5 **Scheme 1**. Eighteen derivatives were prepared and submitted for their anticancer activity evaluation.

2.3 Biological evaluation

2.3.1 Cytotoxicity evaluation by MTT assay.

All the ester analogues of the brevifoliol (**2-19**) were evaluated for their anticancer activity against four human cancer cell lines i.e. breast (MCF-7), prostate (PC-3), lung (A459), colon (COLO-205) and a liver normal cell line (CHANG) by MTT assay. All results were presented in Table (1). Among eighteen derivatives seventeen derivatives showed cytotoxicity on MCF-7. Out of seventeen, twelve derivatives exhibited cytotoxicity on prostate cancer-3 cells. Finally only one derivative i.e. derivative **11** showed potent activity (IC_{50}=3.89 µM), against PC-3 cell line with a higher selectivity index is 28.88. However, derivative **14** (IC_{50}=5.02 µM), and selectivity index is 29.88 almost near to the compound **11.**

Table (1): Cytotoxicity evaluation of brevifoliol derivatives against human cancer cell lines

S.No	Compound Codes	Cytotoxicity IC_{50} in µM					Selectivity index IC_{50}(PC-3)/IC_{50} (CHANG)
		MCF-7	COLO-205	PC-3	A549	CHANG	
1.	**1**	149.58	136.96	Inactive	132.46	>150	---
2.	**2**	Inactive	23.91	61.39	Inactive	>150	>2.44
3.	**3**	Inactive	33.23	Inactive	Inactive	>150	---
4.	**4**	Inactive	89.58	Inactive	Inactive	>150	---
5.	**5**	Inactive	Inactive	71.80	Inactive	>150	>2.09
6.	**6**	Inactive	Inactive	49.34	Inactive	>150	>3.04
7.	**7**	38.68	Inactive	20.54	Inactive	>150	>7.30
8.	**8**	Inactive	58.59	29.78	33.76	134.25	04.51
9.	**9**	Inactive	Inactive	28.92	33.28	48.15	01.66
10.	**10**	Inactive	Inactive	21.32	Inactive	>150	>7.04
11.	**11**	Inactive	Inactive	**3.89**	Inactive	**112.34**	**28.88**
12.	**12**	Inactive	76.05	32.08	56.78	66.60	2.08
13.	**13**	Inactive	128.54	19.52	36.48	144.60	7.41
14.	**14**	Inactive	47.29	**5.02**	Inactive	>150	**29.88**
15.	**15**	Inactive	56.04	Inactive	Inactive	>150	---
16.	**16**	Inactive	62.34	22.47	Inactive	>150	>6.68
17.	**17**	Inactive	23.67	Inactive	Inactive	---	---
18.	**18**	Inactive	24.96	Inactive	Inactive	---	---
19.	**19**	Inactive	Inactive	Inactive	Inactive	---	---
20.	Doxorubicin	3.90	2.71	5.19	3.08	30.09	**5.79**

* IC_{50}>150 µM was considered as inactive;

2.3.2 Formation of soft agar colony assay of compound 11

It is a partially quantitative measure of the structural modification of cell colonies induced by chemicals. These alterations are connected with certain phenotypic changes such as loss of contact inhibition (cells can grow one over another) and anchorage independence (cells form colonies in soft agar). Derivative **11** inhibited growth of colonies of PC-3 prostate cancer cells by 22.42% at 2 µg/mL When concentration was increased to 10 µg/mL suppression of PC-3 cell line was 47.41%

and similarly further the concentration of the compound increased at the 50 µg/mL the suppression also increased by 69.18%. It clearly indicates that the potential antiproliferative action of derivative **11** was concentration-dependent.

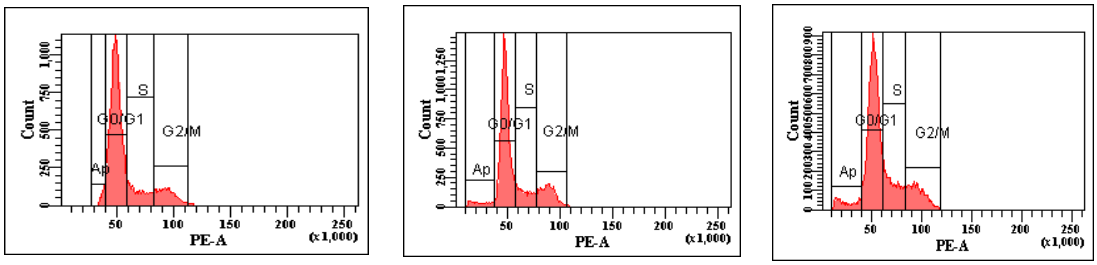
Table (2): Effect of derivative **11** on colony formation in PC-3 prostate cancer cells in soft agar after 24 hours incubation (no. of seeded cells=$5x10^4$ cells/mL, area of 60mm plate=2826mm^2).

Condition	Concentration In µg/mL	Avg. % live cells#	PC-3live cells (%dead cells)	PC-3 IC_{50}in µg/mL
Control	---*	100	0	
Compound **11**	2	77.58	22.42±3.77**	10.15
	10	52.59	47.41±3.73**	
	50	30.82	69.18±0.52**	

* Number of colonies=10675±333; # n=2; **p<0.01 (Dunnett test);

2.3.3 Effect of compound-11 on cell cycle phases of PC-3

In MTT assay, derivative **11** showed very good cytotoxicity against PC-3 cells. Cell cycle analysis was carried out to know the effect of compound **11** on arresting of the PC-3 cells in different phases of the cell cycle. In the case of PC-3 cells treated with compound **11**, poor S phase arrest was observed at IC_{50} for 24 h but very good G2/M phase arrest was there. While at $2xIC_{50}$, there was a nominal G2/M phase arrest and very good S phase arrest Figures (4A) and (4B). At low concentration compound **11** induced G2/M Phase arrest, but at higher concentration S phase arrest was observed. It clearly indicates that cell cycle arrest by compound **11** was induced by multiple mechanisms, which is considered a better approach**.**



Control Compd **11** (IC_{50}) Compd **11** (2 x IC_{50})

Figure (4A): Effect of compound **11** on cell cycle analysis of prostate cancer cells

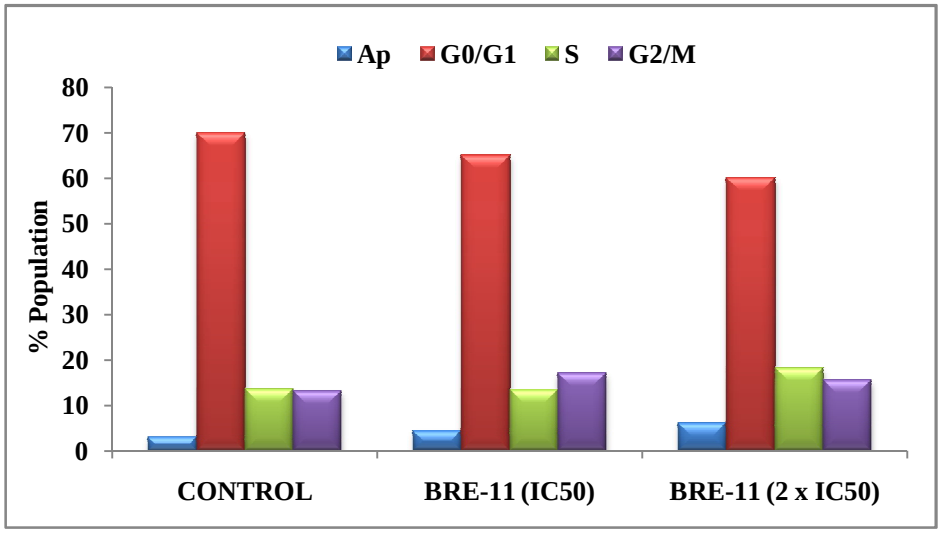
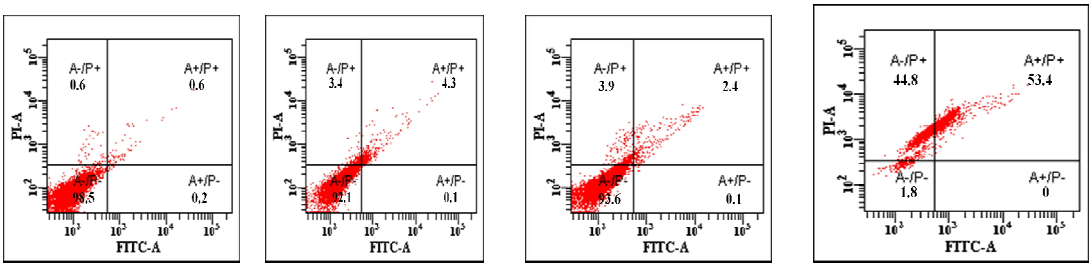


Figure (4B): Graphical presentation of cell cycle analysis in prostate cancer cells affected by compound **11**

2.3.4 Effect of compound 11 in apoptosis induction by annexin V-FITC assay.

Derivative **11** at its half IC_{50} (1.95 µM) activated late apoptosis by 4.3% and necrosis by 3.4%. It did not show early apoptosis at IC_{50} (3.89 µM). At higher concentration, the apoptosis was decreased to 2.4%, while necrosis increased to 3.9% Figure (5). At higher concentration apoptosis was reducing and necrosis was increased. It suggests that the usage of the derivative **11** at half IC_{50} is good with respective to anticancer drug doxorubicin.



(a) Control (b) Compd. **11** (1.95 µM) (c) Compd. **11** (3.89 µM) (d) Doxorubicin (1 µM)

Figure (5): Effect of derivative **11** in PC-3 cells in Annexin V-FITC assay.

2.3.5 *in silico* studies of derivatives 11 and 14 on caspase-3 and caspase-9.

The potent molecules of the series compound **11**, compound **14** and reference drug doxorubicin were docked with caspase-3 protein (cysteine-aspartic acid protease) caspase-3 plays a central role in cell apoptosis. The binding compatibility score obtained from above test showed potent activity of both compounds **11** and **14** to be correlated to that of the reference anticancer drug doxorubicin Table (3A). All the three compounds reside the same binding pocket of caspase 3 (seven common amino acids) and exhibited binding energy of -8.0 -7.7 and -8.1 kcal/mol Table (3A). All the above compounds were also docked with caspase 9 (ten common amino acids), these compounds exhibited docking energy of -7.9 -7.7 and -7.4 kcal/mol reported in Table (3B). Among the compound **11** and **14** docking scores were almost similar but the compound **11** showed little higher score in the caspase-3 pathway. Hence the compound **11** was taken further for evaluation of *in-vivo* efficacy.

In docking studies, only in compound **11**, C13-OH showed some interactions with amino acid residue TRP B206, while C15-OH did not show any interaction. There was no interaction with C13-OH or C15-OH in case of compound **14**. However, the phenyl ring of C5 esters in **14** and **11** had π-π stacking with the aromatic amino acids of caspase 3 and 9. Docking studies showed binding affinities of compound **11** and **14** comparable to standard drug doxorubicin. However, in wet lab experiment compound **11** showed moderate activities at 15.6 µM (26.6% activation of caspase 3).

Table (3A): Molecular interactions of compound **11**, and **14** with caspase 3 (PDB ID: 3KJF)

S. No	Compound	Docking Energy in kcal/mol	Residues within the region of 4Å radius	H-bond forming residues bond length in Å
1	Compound **11**	-8.0	**HIS A:121**, **CYS A:163**, TYR B:204, **SER B:205**, **TRP B:206**, **ARG B:207**, , ASN B:208, SER B:209, **SER B:249**, **PHE B:250**, SER B:251, PHE B:252 PHE B:256,	ARG B:207 (2.3), SER B:209 (2.8)
2	Compound **14**	-7.7	THR A:62, SER A:63, **HIS A:121**, **CYS A:163**, TYR B:204, **SER B:205, TRP B:206**, **ARG B:207**, SER B:209, **SER B:249**, **PHE B:250**, SER B:251, PHE B:252, ASP B:253, , PHE B:256,	ARG B:207 (2.2)
3	Doxorubicin	-8.1	THR A:62, ARG A:64, SER A:120, **HIS A:121**, GLN A:161, ALA A:162, **CYS A:163**, , **SER B:205**, **TRP B:206**, **ARG B: 207**, ASN B:208, TRP B:214, PHE B:247, GLU B:248, **SER B:249**, **PHE B:250**	ARG B: 207 (2.2), TRP B:214 (2.3)

Table (3B): Molecular interactions of compound **11,** and **14** with caspase 9 (PDB ID: INW9)

S. No.	Compound	Docking Energy kcal/mol	Residues within the region of 4Å radius	H-bond forming residues bond length in Å
1	Compound **11**	-7.9	SER B:242, **HIS B:243**, **LEU B:244, GLN B:245, PHE B:246,** ARG A:258, GLU B:261, ASN B:265, **LYS A:299**, CYS A:303, GLY A:304, **GLY A:305**, **GLY A:306**, **PHE B:319**, **GLN B:320**, **TYR A:324**, LEU B:335, THR B:337, PRO B:338,	GLY A:306 (2.3), SER B:242 (2)
2	Compound **14**	-7.7	**HIS B:243**, **LEU B:244**, **GLN B:245**, **ARG A:258**, ASN B:265, PHE B:267, ASN B:268, GLY B:269, GLY B:276, GLY B:277, LYS B:280, **LYS A:299**, **GLY A:305**, **GLY A:306**, PRO B:318, **PHE B:319**, **GLN B:320**, **TYR A:324**, LEU B:335, THR B:337, PRO B:338, SER B:339, ILE B:341	---
3	Doxorubicin	-7.4	SER B:242, **HIS B:243**, **LEU B:244**, **GLN B:245**, **ARG A:258**, GLU B:261, LYS A:299, HIS A:302, CYS A:303, GLY A:304,, **GLY A:305**, **GLY A:306**, PRO B:318, **PHE B:319**, **GLN B:320**, **TYR A:324**	ARG A:258 (2.4), GLN B:245 (2.1), TYR A:324 (2.4)

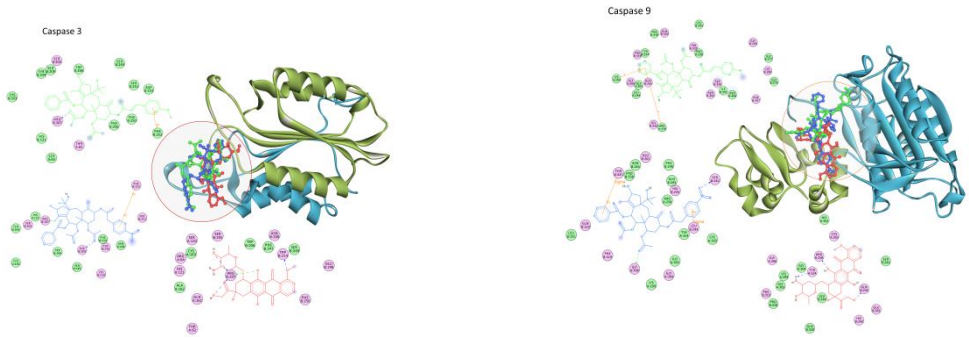


Figure (6): A compounds **11** (Blue), **14** (Green), and doxorubicin (Red) docked in the same binding pocket of caspase 3 PDB ID: 3KJF and **(B)** caspase 9 PDB ID: INW9.

According to Lipinski's rule of five the drug candidate will be poorly bioavailable if following condition is there (more than 5, H-bond donors, more than 5, H-bond acceptors, molecular weight more than 500 and then log P over 5).

If any lead compound follows these rules then it can exhibit better solubility and permeability. We determined Lipinski's rule of five for derivative **11** and derivative **14** both the compounds showed two deviations in rule of five on two of the properties (Molecular weight and H-bond donors) Table (4). Anyhow, it may not be a mandatory rule. Because in last three years 21% of new oral drugs were approved by FDA due to high efficacy for the treatment of cancer [16].

Table (4): The studied compounds computationally calculated parameters for drug-likeness (oral bioavailability) through Lipinski's rule of five.

Compound	Molecular weight (≤500)	LogP (≤5)	H-bond donors (≤5)	H-bond acceptors (≤10)	Rule of 5 violations allowed (≤1)
Compound **11**	720.782	4.28	3	13	2
Compound **14**	716.813	4.91	2	11	2
Doxorubicin	541.546	0.43	7	11	3

*One deviation/violation is acceptable

2.3.6 Activation of caspase-3 pathway by derivative 11

Caspase-3 is protein (cysteine-aspartic acid protease) that plays a vital role in cell apoptosis through both (mitochondrial) and extrinsic (death ligand) pathways. Derivative **11** did not activate caspase-3 pathway at IC_{50}, but at $4xIC_{50}$, there was a

very good effect on caspase-3 activation (26.6%) but too less than the standard anticancer drug (Doxorubicin). The standard drug doxorubicin showed higher effect (36.7% and 51.4%) at 1 µM and 2 µM but the derivative **11** activated caspase-3 pathway moderately in prostate cancer cells Table (5).

Table (5): Activation of caspase-3 by doxorubicin and derivative **11**

S. No.	Derivative	Concentration (µM)	% Activation of Caspase-3
1.	**11**	3.90	0.00
2.	**11**	15.60	**26.61**
3.	Doxorubicin	1.00	36.73
4.	Doxorubicin	2.00	51.37

2.3.7 In-vivo efficacy of compound 11 by Ehrlich Ascites Carcinoma (EAC)

Compound **11** was further evaluated for *in-vivo* efficacy by Ehrlich ascites carcinoma (EAC) in experimental mice. Four different i.p. doses were 25, 50, 75, and 100 mg/kg. Clinical drug 5-fluorouracil was taken as positive control. Compound **11** reduced EAC tumor by 36.67, 44.51, 47.35, and 55.85% at 25, 50, 75, and 100 mg/kg intraperitoneal doses respectively Table (7). In the EAC experiment, compound **11** exhibited moderate activity. There was no mortality and no reduction in body weight of animals Table (6). Anyhow, the activity of derivative **11** was much less comparatively with the standard drug 5-fluorouracil.

Table (6): Effect of derivative **11** on body weight of mice bearing Ehrlich Ascites Carcinoma

Bodyweight (g)					
Sample	**Dose**	**Day 1**	**Day 5**	**Day 9**	**Day 12**
Control	NS(0.2mL), i.p.	23.42	26.12	32.12	35.04
Derivatives **11**	25 mg/kg, i.p.	23.40	26.40	30.52	30.88
	50 mg/kg, i.p.	23.60	25.94	30.10	31.20
	75 mg/kg, i.p	22.80	25.62	30.62	31.24
	100 mg/kg, i.p.	22.80	25.04	29.82	30.52
5-Fluorouracil	20 mg/kg, i.p.	21.50	24.02	21.05	20.70

Table (7): *In-vivo* efficacy of compound **11** against Ehrlich Ascites Carcinoma

Sample	**Dose(mg/kg) i.p.**	**Tumor volume (mL)Mean±SE**	**Tumour weight (g)Mean±SE**	**Tumour Cell Count ($1x10^7$) Mean±SE**	**Tumour Growth Inhibition (%)**
Control	NS(0.2mL),i.p.	7.50±0.74	7.81±0.70	57.75±9.74	0
Compound **11**	25 mg/kg, i.p.	5.10^{**} ±0.98	5.28^{**} ±0.99	36.75^{**} ±2.89	36.67
	50 mg/kg, i.p.	3.90^{**} ±1.05	4.30^{**} ±0.94	32.05^{**} ±1.47	44.51
	75 mg/kg, i.p.	5.40^{*} ±1.22	5.72^{*} ±1.19	30.40^{**} ±3.65	47.35
	100 mg/kg, i.p.	6.92±1.60	7.05±1.58	25.50^{**} ±2.19	**55.85**
5-Fluorouracil	20 mg/kg, i.p.	1.51^{**} ±0.50	1.70^{**} ±0.52	9.56^{**} ±4.74	83.54

**p<0.01 ; *p<0.05 (Dunnett test);

2.3.8 In-vivo acute oral toxicity of compound 11

Safety profile of the derivative **11** was assessed by above experiment in *Swiss albino* mice at four different doses i.e. 5, 50, 300 and 1000 mg/kg once orally. No remarkable effects were noticed in all the studied parameters like illness, death, observational parameters, body weight, organ weight, serum biochemical and most of the hematological parameters up to the dose level of 1000 mg/kg body weight Table (8) and Figure (5). But the parameters like RBC (million/mm^3) and SGOT were slightly affected at 1000 mg/kg dose. However there was no such effect at other doses 5, 50, and 300 mg/kg once orally. There was notable changes in differential leukocyte cell count as well as eosinophil and lymphocyte in the treated groups compared to control Figure (6). Since differential leukocyte counts are very important parameters, further studies are required to evaluate the probable hematotoxicity of the compound in higher doses and is suggestive of further studies at a sub-acute and chronic level. In view of significant *in vitro* activity of the lead derivative, it is suggested for modification of the test compound so as to reduce its toxicity but retain its biological activity.

Table (8): Effect of derivative **11** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on body weight, hematological and serum biochemical parameters in *Swiss albino* mice (Mean±SE; n=6; *, P<0.05 compared to control).

Parameters	**Dose of compound 13 at mg/kg body weight as a single oral dose**				
	Control	**5 mg/kg**	**50 mg/kg**	**300 mg/kg**	**1000 mg/kg**
Body weight (g)	35.64±0.73	33.77±2.43	33.51±1.41	32.06±1.47	31.02±13.87
Haemoglobin (g/dL)	11.01±0.83	11.79±0.31	11.94±0.73	10.95±0.66	12.13±0.72
RBC (million/mm^3)	5.40±0.56	3.78±0.20	4.09±0.52	5.07±0.39	**3.76±0.32**
WBC(thousands/mm^3)	6.81±1.24	4.91±0.45	5.20±0.60	4.50±0.51	7.95±1.11
ALP (U/L)	298.35±29.67	235.31±50.26	339.00±19.80	342.76±23.53	309.35±68.60
SGOT (U/L)	41.02±6.37	44.98±7.19	53.04±8.91	43.82±6.06	**52.14±5.00**
SGPT (U/L)	35.20±6.65	31.36±4.84	24.65±4.25	28.45±6.82	25.40±5.30
Creatinine (mg/dL)	1.61±0.35	1.71±0.62	3.04±0.61	1.82±0.35	2.03±0.45
Triglycerides (mg/dL)	67.99±9.46	52.85±3.03	81.81±10.66	61.3±7.59	57.16±5.73
Bilirubin(mg/dL)	0.67±0.03	0.87±0.07	0.72±0.10	0.67±0.06	0.64±0.05
Cholesterol (mg/dL)	55.87±6.97	39.10±7.63	34.26±3.36	57.84±8.69	54.65±3.23
Albumin(g/dL)	3.66±0.33	3.04±0.33	2.19±0.13	3.25±0.20	4.02±0.66
Protein(mg/ml)	1.60±0.24	2.17±0.14	2.22±0.09	1.95±0.10	1.99±0.15

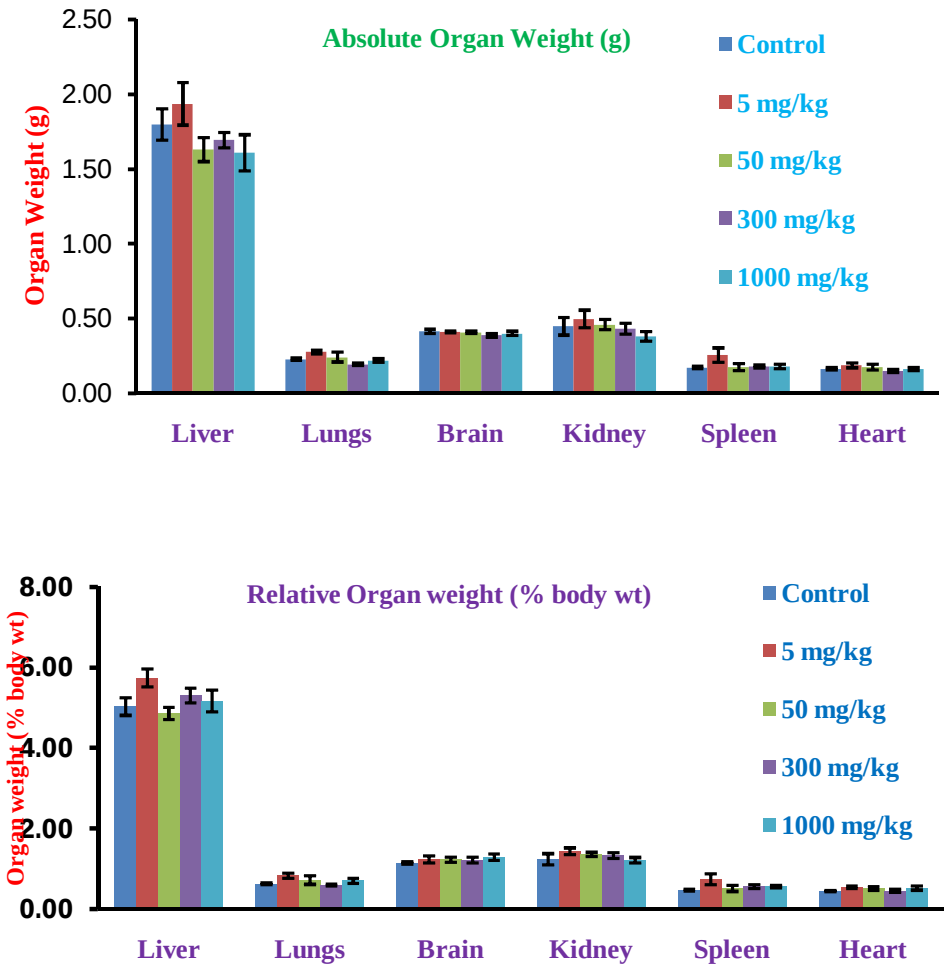


Figure (7): Effect of compound **11** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weight in *Swiss albino* mice (Mean±SE; n=6, *, P<0.05 compared to control).

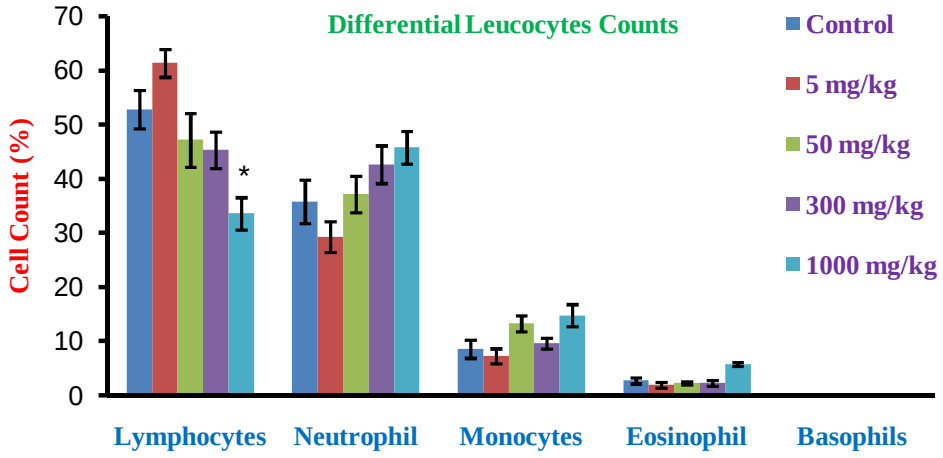


Figure (8): Effect of Compound **11** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in *Swiss albino* mice (Mean±SE; n=6, *, P<0.05 compared to control).

2.3.9 UPLC analysis of brevifoliol, potent derivatives 11 and 14

The purity profile of the brevifoliol, lead compounds **11** and **14** was assessed on reverse-phase UPLC (ACQUITY UPLC H-Class Bio System, Waters USA) using C-18 (BEH 130Å, 1.7×50 mm, 1.7 µm, Waters, USA) at 35±0.1°C constant column temperature. The analysis was performed by using gradient elution programme. The mobile phase composition was Acetonitrile (0.1% HCOOH), **A** and water (0.1% HCOOH), **B** at a flow rate of 0.30 ml/min. The composition of the mobile phase was changed from 10% A to 90% B and run time for the complete analysis was 5 min. The volume injection was 3.0 µL. The purity of the compound is reported based on peak area normalization method. Purity of the analyzed compounds *i.e*. brevifoliol (**1**) and potent compounds **11** and **14** were found to be 96.7, 97.9 and 98.1% respectively.

2.4 Discussion

Throughout the world prostate cancer cases are growing. Depending on age and family history increase the chances of prostate cancer [17]. Derivatives **11** and **14** showed very good activity (IC_{50}=3.89 µM and 5.02 µM) in PC-3 cell line respectively. PC-3 and DU-145 are hormone (Androgen receptor, AR) independent and LNcaP is an AR hormone-dependent PC-3 cell line [18]. The soft agar colony assay revealed that the compound **11** inhibited PC-3 cell colonies in concentration-dependent manner. This assay is a gold standard assay for cellular transformation or tumorigenicity *in-vitro*, but it is unsuited for high throughput screening. The growth in soft agar is strongly correlated to tumorigenicity in animals, typically mouse xenografts [19].

The derivative **11** affected cell cycle of PC-3 cell in both phases (S-phase and G2/M phase). But at its IC_{50} the effect at S-phase was poor. However, at double IC_{50} this effect was quite high with significant apoptosis induction. S-phase arrest in cell cycle analysis may be due to the induction of apoptosis through the caspase pathway which was moderately effective in caspase-3 activation assay. Caspase are cysteine aspartic proteases playing an important role in apoptosis and inflammation [20]. Among these, caspase-9 as initiator caspase and caspase-3, as executioner caspase is activated in both intrinsic and extrinsic caspase cascade pathways to initiate apoptosis [21].

Avoidance of programmed cell death is considered one of the important hallmarks of cancer [22]. Therefore, the activation of apoptosis is the best way to treat cancer. Compound **11** initiated G2/M phase arrest, which could not be understood, it needs to be further studied. There may be other pathways which causes later phase of cell cycle division of prostate cancer cells. It would be worth indicating here that brevifoliol (**1**) as such used in tubulin kinetics experiment did not show anti-tubulin activity. In general G2/M phase arrest indicates anti tubulin effect of the drug [23].

Ehrlich ascites carcinoma is an undifferentiated carcinoma, hyper diploid, 100% malignancy rapid proliferation, and shorter life span. More over it resembles human tumors cells and most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate. Nonmalignant somatic cells are more sensitive to breakable but EAC cells are difficult to break [24]. Compound **11** showed moderate efficacy in EAC as compared to standard drug and it reduced 55.85% of EAC tumor at 100 mg/kg dose.

For Investigational New Drugs (INDs) safety studies are mandatory which is known as Pharmacovigilance.This study always indicate that adverse drug reactions (ADRs) to experimental animals. ADR is also responsible for the 4^{th} and 6^{th} cause of death in the USA [25]. Derivative-**11** did not show any toxicity to the experimental animals in acute oral toxicity experiment.

2.4.1 Structure activity relationship

To generate structure activity relationship, prepared the derivatives on C-5 position by using brevifoliol as a starting material. All the derivatives were screened against four human cancer cell lines. Out of eighteen derivatives only one derivative was not active on all the cell lines (**19**) reason may be that there is no free hydroxyl group at C-13 position of brevifoliol and derivative (**17**) was active on only one cell line that was COLO-205 and inactive against all other cell lines because benzylic methylene group not present on it. Brevifoliol and its five derivatives were inactive against PC-3 cell line (**1**, **3, 4**, **15**, **17** and **18**). Twelve derivatives were active on PC-3 cell line among them four derivatives namely angelic acid, 3-chlorophenyl benzoic acid, 3, 5-dinitrophenyl benzoic acid and 2, 4-dimethoxy phenyl acetate (**2, 5, 6** and **8**) were

shown mild activity. Then the derivatives contain the functional group like 3, 4, 5-trimethoxy phenyl acetate and 3-trifluoromethyl phenyl acetate (**9** and **12**) were showed moderate activity on PC-3 cell line. Derivatives which have the 4-methoxy phenyl acetate, 4-chlorophenyl acetate and 4-methyl cinnamyl, were very good active on PC-3 cell lines (**7, 10** and **16**). The compound which contains 4-methoxy cinnamyl (**14**) showed very good activity and another compound **11** contained 4-nitrophenyl acetate shown excellent activity on PC-3 cell line. The derivative with nitro group with phenyl acetic acid and 4-methoxy cinnamic acid were most suitable derivatives on PC-3 cell line.

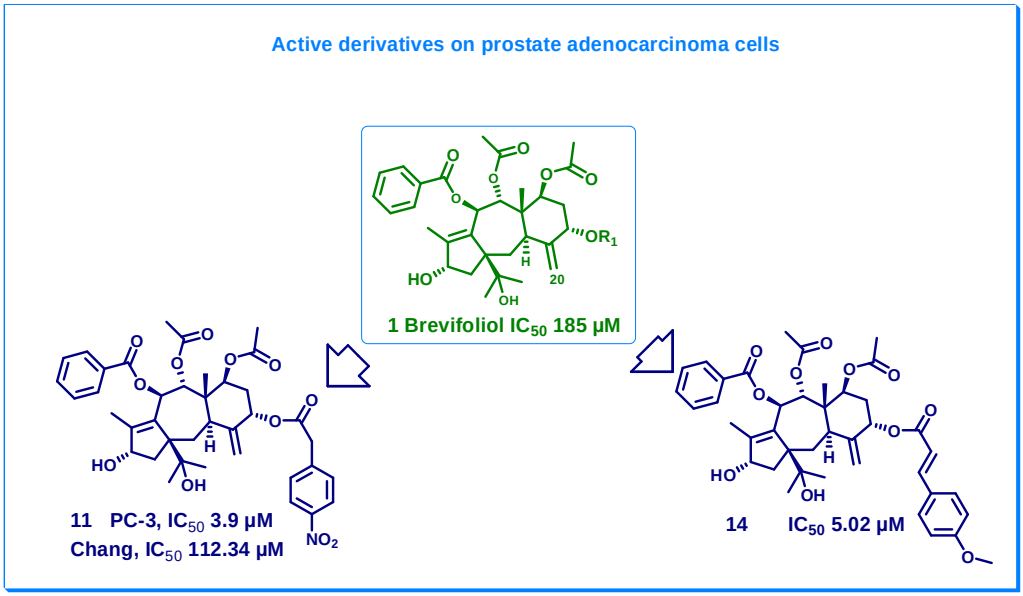


Figure (9): Active derivatives on prostate adenocarcinoma cells

2.5. Conclusion

In this study, we have synthesized eighteen derivatives at the C5 position of the brevifoliol. To our delight two of the compounds i.e. compound **11** and **14** showed good activity against prostate cancer. Depending on their merit further compound-**11** showed antiproliferative activity by apoptosis induction in prostate cancer cells *via* activation of caspase cascade pathway. Derivative **11** was moderately effective in the reduction of EAC in rodents. Compound **11** was safe and well-tolerated in *Swiss albino* mice up to 1000 mg/kg dose.

2.6. Experimental section

2.6.1 General methods

All the chemicals and reagents were purchased from the Sigma Aldrich, Merck India Limited, Avra Synthesis, India and used without further refined. TLC-GF_{254} aluminum sheets Merck pre-coated silica gel TLC used for reaction monitoring and for detection under UV cabinet (254 nm and 365 nm) and subsequently charred with 2% ceric sulfate in 10% sulphuric acid (aqueous) with heating. Compounds were refined through silica gel (60-120 and 100-200 mesh) and conformed by ^{1}H and ^{13}C NMR recorded on Bruker Avance III NMR-500MHz, Chemical shifts are given in δ ppm values with (TMS) as reference. $^{1}H–^{1}H$ coupling constant (J) values are given in Hz. ESI mass spectra were recorded on Shimadzu LC-MS and (HRMS) on Agilent 6545-Q-TOF in methanol. The purity of compounds **11** and **14** was determined by ACQUITY UPLC H-class Biosystem, Waters Corporation, USA. Melting points were determined on Stanford Research System, USA. E-Z Melt automated melting point apparatus in open glass capillaries and was uncorrected.

2.6.2 Isolation of brevifoliol

T. wallichiana needles of 68 Kg were collected form Jageshwar, dist Almora, Uttarakhand, India. It was authenticated at CSIR-CIMAP, Lucknow and assigned the repository Ref. no. T026. It was properly shade dried. Finally and got the powder 13Kg dried material after (Sieve no. 16) sieving. 13Kg powdered plant material was used for extraction purpose as shown in Figure (10). Ethyl acetate fraction (82.81g) was brevifoliol rich, from it (40 g) was used for purification through silica gel column (600 g, 60-120 mesh) and eluted with $CHCl_3$, acetone/chloroform (up to 1-10%), MeOH / $CHCl_3$ (up to 1-8%). Pure brevifoliol (0.79 g) was obtained at 6-8% MeOH/ $CHCl_3$ and process was repeated for the rest of the ethyl acetate fraction to get sufficient quantity of brevifoliol for further chemical modifications.

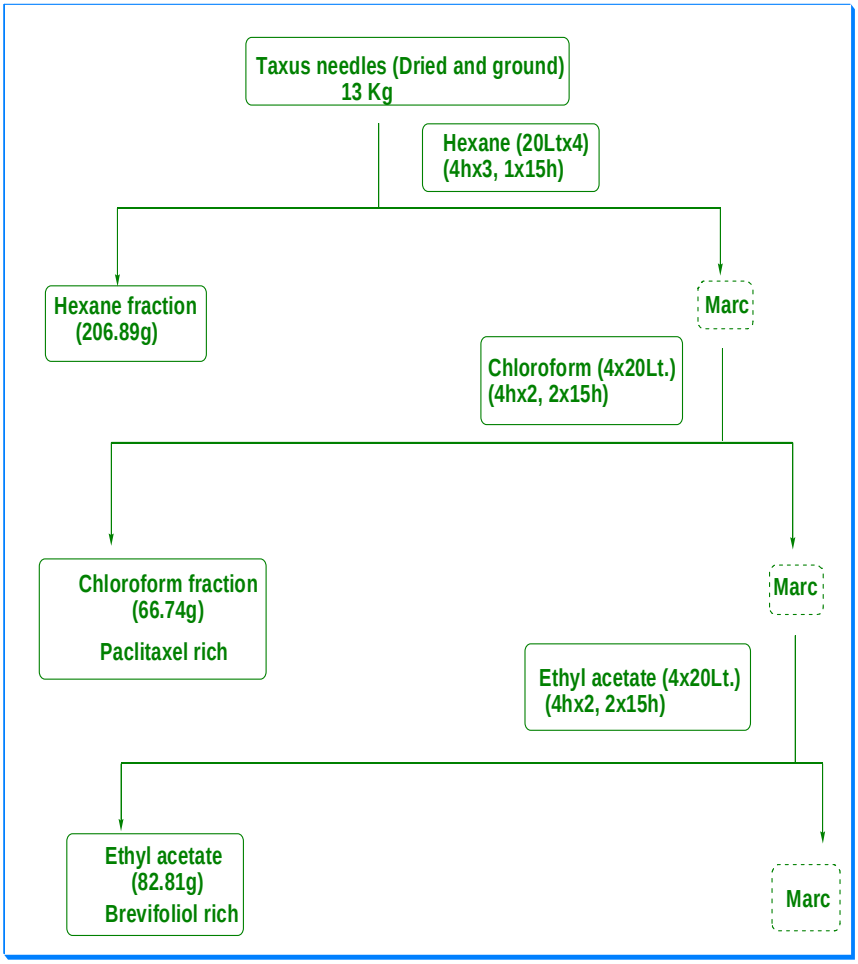


Figure (10): Isolation procedure for brevifoliol

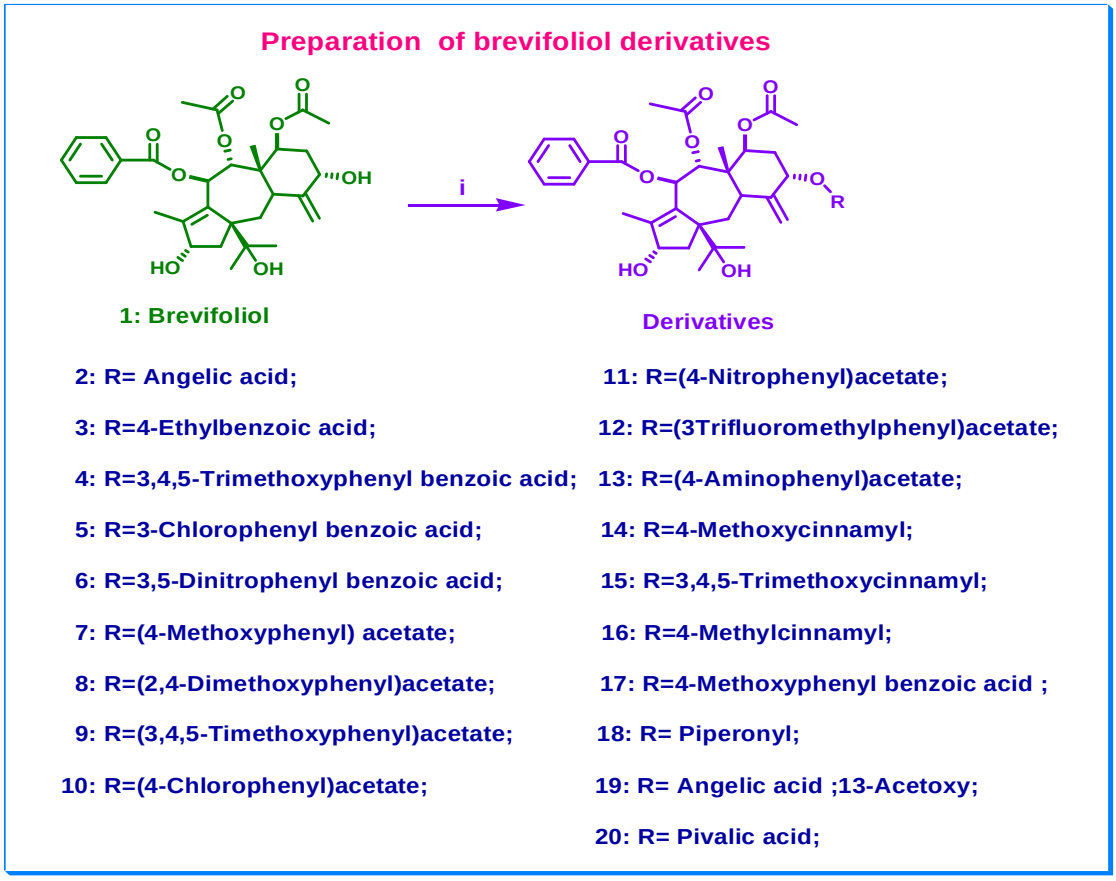
2.6.3 Synthetic strategy

Natural taxane brevifoliol was our starting substrate which was isolated from the needles of *T.wallichiana*. Purified brevifoliol (1.64 g) was obtained by column chromatography. Brevifoliol was derivatized through steglich esterification reaction particularly at C5-OH. The time duration and reagent molar ratio limits the esterification only at C5-OH. However brevifoliol structure naturally existing in the form of twist-boat/chair form and this stereochemistry was playing a major role in product formation at C-5 and C-13 position in the ratio of 3:1. Many derivatives were prepared by using various aryl benzoic acid, phenyl acetic acid, cinnamic acids and other aliphatic acids were (angelic acid, pivalic acid and 5-angelic acid, 12-acetate) used as shown in the scheme-1. All the derivatives were refined through column chromatography and structures were confirmed by NMR spectroscopy.

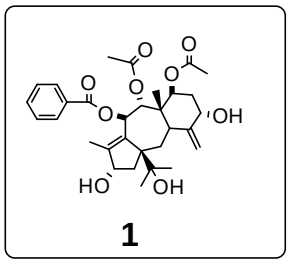
2.6.4 General synthetic procedure for the preparation of brevifoliol ester derivatives (2-18 and 20)

Scheme-1

To a stirred solution of 3, 4, 5-trimethoxybenzoic acid (30 mg, 0.14 mmol) N, N-Dicyclohexylcarbodiimide (18.5 mg 0.089 mmol) and DMAP (11 mg 0.09 mmol) in DCM (5 mL) were added at ambient temperature and stirring continued at RT for 30 min. Then brevifoliol added to the reaction and stirring continued for 3.5h until TLC showed the complete consumption of brevifoliol. After completion the reaction mixture was diluted with 10 mL of dichloromethane washed with water and pooled DCM layer was dried with Na_2SO_4 and reduced on rotary evaporator to get the unrefined product which was further purified by column chromatography (silica gel 60-120) to get the final yellowish gummy compound at 8-10% Acetone/chloroform) in 85% yield.



Scheme-1: Reagents and conditions: Carboxylic acid, DCC, DMAP, CH_2Cl_2, RT, 4-8 h, 85% to 95%.



1

Brevifoliol (1):-

Yield: 1.64 gm (1.98%), white amorphous;

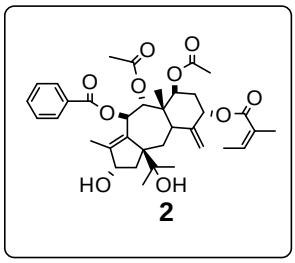
1.98% in EA basis and 0.013% in DM basis (Dry Mass)

Melting point: 202-204ºC;

Electrospray mass for $C_{31}H_{40}O_9$ (MeOH): 557 $[M+H]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.41 and 2.35 (bs, 2H, CH_2), 1.83 (s, 3H, CH_3), 1.86 (bm, 2H, CH_2), 2.05 (s, 3H, OAc), 2.12 (s, 3H, OAc), 1.49 and 2.46 (m, 2H, CH_2), 2.77 (bd, 1H, CH), 4.38 (bs, 2H, CH and CH), 4.36 (s, 1H, CH), 5.16 (s, 1H, CH), 5.56 (bm, 1H, CH), 6.03 (bs, 1H, CH_2), 6.52 (bs, 1H, CH_2), 7.42 (m, 2H, CH aromatic), 7.54 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 12.02, 13.11, 21.32, 21.45, 24.86, 26.91, 29.15, 36.12, 45.08, 47.37, 62.56, 70.26, 70.39, 70.92, 72.55, 75.31, 75.94, 76.09, 112.04, 128.75, 129.37, 129.44, 133.26, 134.03, 149.14, 151.55, 164.38, 169.97, 170.54.



2

Brevifoliol-5-O-yl-angelic acid ester (2):-

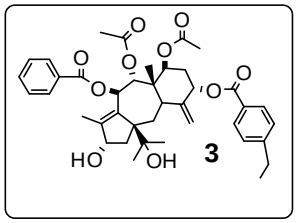
Yield: 84%; Yellowish gum;

Electrospray mass for $C_{36}H_{46}O_{10}$ (MeOH): 661 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.90 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.34 (s, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.86 (bm, 2H, CH_2), 2.02 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.48 (m, 2H, CH_2), 2.37 (bt, 1H, CH), 2.76 (bd, 1H, CH), 4.47 (bs, 1H, CH), 4.88 (s, 1H, CH), 5.08 (s, 1H, CH_2), 5.40 (s, 1H, CH_2), 5.43 (d,3H, CH_3), 5.59 (bm, 1H, CH), 6.06 (bs, 1H, CH), 6.50 (d, 1H, CH), 6.87 (q, 1H, CH) 7.32 (m, 2H, CH aromatic), 7.52 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 12.17, 14.09, 14.47, 20.34, 20.73, 21.40, 24.70, 25.23, 32.71, 39.17, 49.31, 59.80, 69.80, 70.40, 70.39, 71.01, 73.97, 75.75, 76.83, 114.06, 128.30, 128.72, 129.45, 133.24, 133.28, 138.22, 147.55, 153.66, 154.85, 164.18, 166.56, 169.87, 169.92, 175.10.

Brevifoliol-5-O-yl-4-ethyl benzoic acid ester (3):-



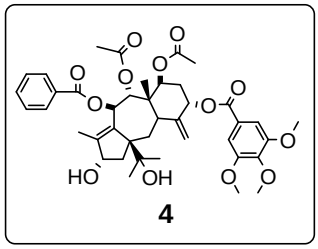
Yield: 66%; Yellowish gum;

Electrospray mass for $C_{40}H_{48}O_{10}$ (MeOH): 711 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.27 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.76 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.00 (s, 2H, CH_2), 1.32 (s, 3H CH_3), 4.39 (bs, 1H, CH), 4.95 (s, 1H, CH_2), 5.35 (s, 1H, CH_2), 5.62 (bm, 1H, CH), 6.10 (bs, 1H, CH), 6.50 (d, 1H, CH), 7.43 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 7.96 (d, 2H, CH aromatic), 8.02 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.73, 13.00, 15.27, 20.76, 21.40, 24.46, 24.94, 29.70, 32.26, 33.93, 39.11, 44.91, 47.84, 57.25, 69.77, 70.38, 74.52, 75.83, 76.79, 114.53, 126.92, 128.02, 128.76, 129.48, 129.48, 129.90, 133.28, 134.41, 147.54, 148.45, 154.64, 164.21, 165.26, 169.88, 170.03, 171.50.

Brevifoliol-5-O-yl-(3, 4, 5-trimethoxyphenyl) benzoic acid ester (4):-



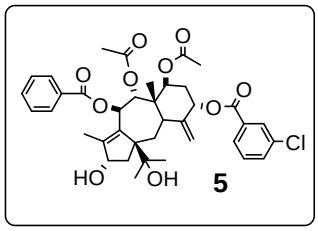
Yield: 83%; Yellowish gum;

Electrospray mass for $C_{41}H_{50}O_{13}$ (MeOH): 773 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.34 (s, 3H, CH_3), 1.96 (bm, 2H, CH_2), 2.03 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.74 and 2.40 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.71 (bd, 1H, CH), 3.87 (s, 6H, 2xOCH_3), 3.93 (s, 3H, OCH_3) 4.76 (bs, 1H, CH), 5.02 (s, 1H, CH), 5.44 (s, 1H, CH_2), 5.54 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.80 (d, 1H, CH), 7.32 (s, 2H, aromatic), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 14.11, 20.75, 21.40, 22.70, 24.92, 29.70, 31.92, 33.90, 44.97, 49.22, 56.34, 60.95, 69.75, 70.13, 70.36, 75.30, 75.82, 106.80, 114.07, 118.90, 124.91, 128.77, 129.46, 129.72, 133.26, 133.33, 147.81, 150.56, 150.88, 153.06, 153.24, 164.30, 164.80, 165.94, 169.93, 170.03, 170.15.

Brevifoliol-5-O-yl-(3-chlorophenyl) benzoic acid ester (5):-



5

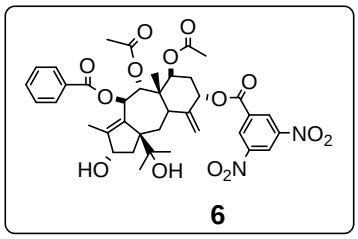
Yield: 86%; Yellowish gum;

Electrospray mass for $C_{38}H_{43}O_{10}Cl$ (MeOH): 717 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.24 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.76 (bm, 2H, CH_2), 2.05 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.40 (m, 2H, CH_2), 2.38 (bt, 1H, CH), 2.86 (bd, 1H, CH), 4.46 (bs, 1H, CH), 4.98 (s, 1H, CH), 5.36 (s, 1H, CH_2), 5.66 (s, 1H, CH_2), 6.03 (bs, 1H, CH), 6.48 (d, 1H, CH), 7.42 (m, 2H, CH aromatic), 7.54 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 7.87 (d, 1H, CH aromatic), 7.96 (d, 1H, CH aromatic), 8.01 (d, 1H, CH aromatic), 8.17 (d, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.80, 20.76, 21.40, 24.53, 22.39, 24.94, 29.35, 33.94, 39.06, 47.36, 49.77, 69.71, 70.43, 70.39, 71.01, 72.55, 75.74, 76.80, 115.01, 128.33, 128.77, 129.48, 129.48, 133.30, 134.51, 138.57, 145.03, 148.94, 151.30, 164.28, 165.11, 169.92, 169.97, 170.01.

Brevifoliol-5-O-yl-(3, 5-dinitrophenyl) benzoic acid ester (6):-



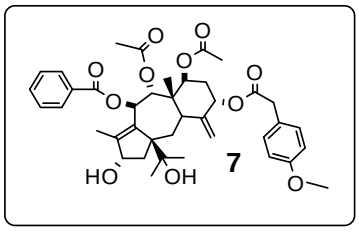
6

Yield: 83%; Yellowish gum;

Electrospray mass for $C_{38}H_{42}N_2O_{14}$ (MeOH): 770 $[M+H_3O]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.27 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.72 (bm, 2H, CH_2), 2.05 (s, 3H, CH_3), 2.14 (s, 6H, 2xOAc), 1.49 and 2.40 (m, 2H, CH_2), 2.41 (bt, 1H, CH), 2.77 (bd, 1H, CH), 4.37 (bs, 1H, CH), 4.40 (s, 1H, CH), 4.80 (s, 1H, CH_2), 5.15 (s, 1H, CH_2), 5.56 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.53 (d, 1H, CH), 7.41 (m, 2H, CH aromatic), 7.54 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 8.73 (s, 1H, CH aromatic), 9.20 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 12.90, 14.11, 20.74, 21.46, 22.68, 25.60, 29.18, 36.13, 39.63, 45.09, 47.26, 62.55, 70.45, 72.43, 75.97, 76.80, 114.06, 122.30, 128.76, 129.36, 129.43, 129.50, 133.27, 134.15, 135.35, 149.30, 151.58, 156.16, 164.39, 165.42, 169.80, 169.79, 170.50.



Brevifoliol-5-O-yl-(4-methoxyphenyl) acetate (7):-

Yield: 79%; Yellowish gum;

Electrospray mass for $C_{40}H_{48}O_{11}$ (MeOH): 727 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.71 (s, 2H, CH_2), 3.78 (s, 3H, OCH3), 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 7.86 (d, 2H, CH aromatic), 8.18 (d, 2H, CH aromatic).

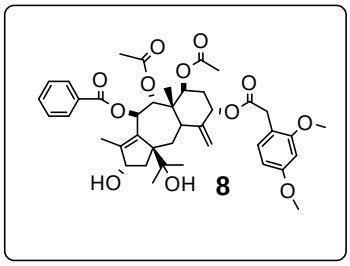
^{13}C NMR (125MHz, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 39.06, 45.08, 47.37, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 114.06, 128.33, 128.75, 129.37, 129.44, 133.24, 133.26, 134.03, 149.14, 151.55, 154.54, 164.38, 169.91, 170.36.

Brevifoliol-5-O-yl-(2, 4-dimethoxyphenyl) acetate (8):-

Yield: 81%; Yellowish gum;

Electrospray mass for $C_{41}H_{50}O_{12}$ (MeOH): 757 $[M+Na]^+$.

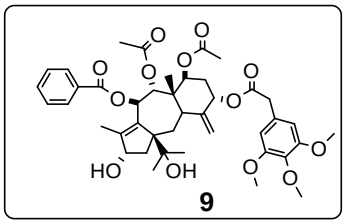
^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H,



CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.66 (s, 2H, CH_2), 3.75 (s, 6H, 2xOCH_3) 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.84 (m, 2H, aromatic), 7.85 (m, 2H, CH aromatic), 8.16 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 45.08, 47.37, 39.01, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 104.41, 106.57 112.04, 128.72, 128.75, 129.33, 129.37, 129.44, 133.26, 134.03, 149.14, 151.55, 154.29, 154.23, 164.38, 169.91, 170.00, 171.38.

Brevifoliol-5-O-yl-(3, 4, 5-trimethoxyphenyl) acetate (9):-



9

Yield: 76%; Yellowish gum;

Electrospray mass $C_{42}H_{52}O_{13}$ (MeOH) : 787 $[M+Na]^{+.}$

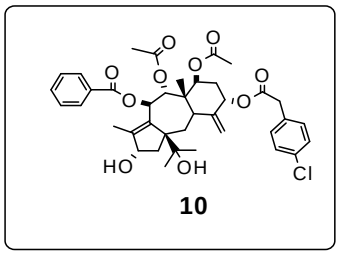
^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.65 (s, 2H, CH_2), 3.8 (s, 3H 3xOCH_3), 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 8.12 (s, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 38.97, 45.08, 47.37, 56.05, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 128.75, 129.37, 129.44, 130.43, 133.16, 133.26, 134.03, 149.14, 151.55, 153.16, 164.38, 169.91, 170.54, 171.40.

Brevifoliol-5-O-yl-(4-chlorophenyl) acetate (10):-

Yield: 87%; Yellowish gum;

Electrospray mass for $C_{39}H_{45}ClO_{10}$ (MeOH): 731 $[M+Na]^+$.



10

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.70 (s, 2H, CH_2), 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.21 (m, 2H, CH aromatic), 7.25

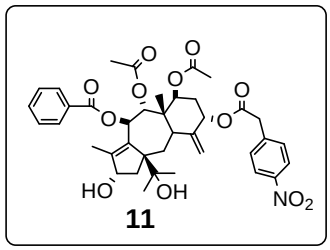
(m, 2H, CH aromatic), 7.40 (m, 2H, CH aromatic), 7.84 (m, 1H, CH aromatic), 8.16 (m, 2H, CH aromatic).

^{13}C (125NMR, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 45.08, 47.10, 47.37, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 128.75, 129.37, 129.44, 129.47, 130.60, 130.94, 133.26, 134.03, 149.14, 151.55, 164.38, 169.91, 170.54, 171.10.

Brevifoliol-5-O-yl-(4-nitrophenyl) acetate (11):-

Yield: 82%; Yellowish gum;

Electrospray mass for $C_{39}H_{45}NO_{12}$ (MeOH): 742 $[M+Na]^+$.

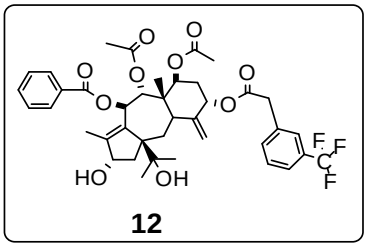


11

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.32 (s, 3H, CH_3), 1.87 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.05 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.97 (bd, 1H, CH), 3.82 (s, 2H, CH_2), 4.37 (bs, 1H, CH), 4.36 (s, 1H, CH), 4.76 (s, 1H, CH_2), 5.11 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.41 (m, 2H, CH aromatic), 7.51 (m, 1H, CH aromatic), 7.82 (m, 2H, CH aromatic), 7.84 (m, 2H, CH aromatic), 8.12 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ11.91, 13.83, 20.67, 21.40, 22.36, 24.84, 29.13, 34.81, 38.94, 47.18, 48.88, 55.66, 70.55, 72.10, 75.84, 76.09, 111.46, 124.10, 128.70, 129.32, 129.36, 130.23, 130.28, 133.14, 146.90, 149.61, 153.70,154.30, 164.20, 169.97, 170.12.

Brevifoliol-5-O-yl-(3-(trifluoromethyl)phenyl) acetate (12):-



12

Yield: 74%; Yellowish gum;

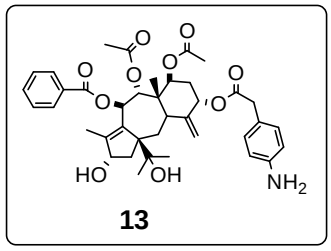
Electrospray mass for (MeOH): 765 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.76 (s, 2H, CH_2), 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH),

7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic),7.81 (m, 2H, CH aromatic), 7.83 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic), 8.12 (s, 1H, CH, aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 38.97, 45.08, 47.37, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 124.00, 126.00, 126.34, 128.75, 129.94, 129.37, 129.44, 133.26, 133.29, 134.03, 135.22, 139.84, 149.14, 151.55, 164.38, 169.91, 170.54, 170.82.

Brevifoliol-5-*O*-yl-(4-aminophenyl) acetate (13):-



13

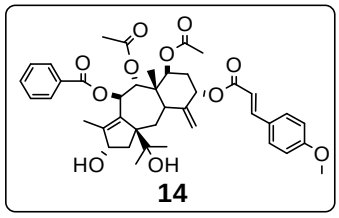
Yield: 49%; Yellowish gum;

Electrospray mass for $C_{39}H_{47}NO_{10}$ (MeOH): 712 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$):): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.85 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.6 (bs, 2H, NH_2), 3.91 (s, 2H, CH_2), 4.37 (bs, 1H, CH), 4.41 (s, 1H, CH), 4.81 (s, 1H, CH_2), 5.16 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 6.61 (m, 2H, CH aromatic), 7.0 (m, 2H, CH aromatic), 7.40 (m, 2H, CH aromatic), 7.85 (m, 1H, CH aromatic), 8.15 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.11, 14.96, 20.75, 21.40, 22.39, 24.86, 29.15, 36.12, 38.00, 45.08, 47.37, 62.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 115.66, 124.24, 128.75, 129.37, 129.44, 130.44, 133.26, 134.03, 149.14, 149.86, 151.55, 164.38, 169.91, 170.54, 172.10.

Brevifoliol-5-O-yl-(4-methoxyphenyl)-prop-2-enoic acid ester (14):-



14

Yield: 46%; Yellowish gum;

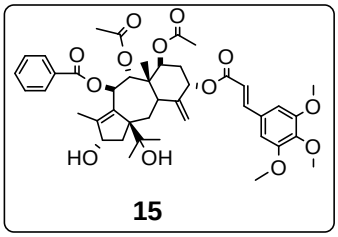
Electrospray mass for $C_{41}H_{48}O_{11}$ (MeOH): 739 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.94 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.77 (bd, 1H, CH), 3.82 (s, 3H, OCH_3), 4.91 (s, 1H, CH), 5.32 (s, 1H, CH_2), 5.52 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 6.67 (d, 1H, CH), 6.80 (s, 2H, CH

aromatic), 6.88 (d, 2H, CH aromatic), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.57 (d, 1H, CH), 7.87 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.00, 14.11, 20.78, 21.41, 22.70, 24.94, 29.70, 33.94, 39.02, 47.51, 49.08, 55.32, 63.03, 69.83, 70.35, 70.85, 71.01, 72.55, 74.00, 76.80, 114.30, 115.90, 117.00, 127.22, 128.77, 129.48, 129.88, 133.91, 136.27, 139.28, 144.90, 145.71, 154.34, 161.37, 164.23, 166.18, 169.97, 170.04.

Brevifoliol-5-O-yl-(3, 4, 5-trimethoxyphenyl)-prop-2-enoic acid ester (15):-



15

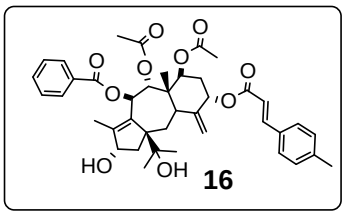
Yield: 83%; Yellowish gum;

Electrospray mass for $C_{43}H_{52}O_{13}$ (MeOH): 779 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.76 (bm, 2H, CH_2), 2.02 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.49 and 2.43 (m, 2H, CH_2), 2.40 (bt, 1H, CH), 2.87 (bd, 1H, CH), 3.87 (s, 9H, 2xOCH_3), 4.50 (bs, 1H, CH), 4.93 (s, 1H, CH), 5.32 (s, 1H, CH_2), 5.52 (s, 1H, CH_2), 5.52 (bm, 1H, CH), 6.40 (bs, 1H, CH), 6.52 (d, 1H, CH), 6.67 (d,1H, CH) 6.80 (s, 2H, CH aromatic), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.57 (d, 1H, CH), 7.87 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.93, 12.94, 14.10, 20.76, 21.40, 22.39, 24.84, 27.05, 29.35, 29.68, 31.91, 33.81, 49.04, 44.88, 47.79, 56.22, 61.00, 63.26, 69.80, 70.70, 74.10, 75.52, 76.09, 105.39, 114.06, 117.68, 128.80, 129.38, 129.48, 129.94, 133.32, 134.40, 139.27, 145.60, 150.88, 153.40, 153.45, 164.38, 169.94, 170.02.

Brevifoliol-5-O-yl-(4-methylphenyl)-prop-2-enoic acid ester (16):-



16

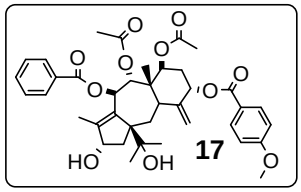
Yield: 74%; Yellowish gum;

Electrospray mass for $C_{41}H_{48}O_{10}$ (MeOH): 723 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 1.95 (bm, 2H, CH_2), 2.00 (s, 3H, CH_3), 2.03 (s, 6H, 2xOAc), 1.72 and 2.32 (m, 2H, CH_2), 2.32 (s, 3H, CH_3), 2.93 (bt, 1H, CH), 2.94 (bd, 1H, CH),

4.50 (bs, 1H, CH), 4.72 (s, 1H, CH), 5.26 (s, 1H, CH_2), 5.50 (s, 1H, CH_2), 5.55 (bm, 1H, CH), 6.03 (bs, 1H, CH), 6.52 (d, 1H, CH), 7.30 (d, 1H, CH), 7.37 (dd, 2H, CH aromatic), 7.40 (dd, 2H, CH aromatic), 7.42 (m, 2H, CH aromatic), 7.49 (d, 1H, CH), 7.62 (m, 1H, CH aromatic), 7.85 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.82, 12.82, 20.61, 21.26, 22.39, 24.60, 26.95, 29.51, 33.76, 38.84, 44.65, 47.15, 62.76, 70.00, 70.83, 73.87, 75.50, 76.21, 106.50, 113.65, 116.65, 128.10, 128.60, 129.18, 129.30, 129.34, 131.70, 133.10, 133.13, 143.00, 145.00, 149.00, 154.24, 164.10, 166.84, 169.80, 169.87, 170.20.



17

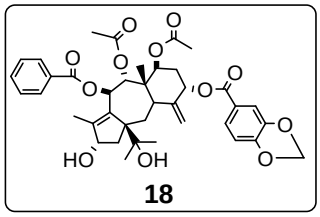
Brevifoliol-5-O-yl-4-methoxybenzoic acid ester (17):-

Yield: 53%; Yellowish gum;

Electrospray mass for $C_{41}H_{48}O_{11}$ (MeOH): 713 [M-(CH_2CO) +K]$^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.87 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.26 (bs, 2H, CH_2), 1.34 (s, 3H, CH_3), 1.97 (bm, 2H, CH_2), 2.03 (s, 3H, CH_3), 2.07 (s, 6H, 2xOAc), 1.51 and 1.78 (m, 2H, CH_2), 2.36 (bt, 1H, CH), 2.78 (bd, 1H, CH), 3.81 (s, 3H, OCH_3), 4.93 (s, 1H, CH_2), 5.32 (s, 1H, CH_2), 5.62 (bm, 1H, CH), 6.10 (bs, 1H, CH), 6.46 (d, 1H, CH), 6.91 (dd, 2H, J= 6.5Hz, CH aromatic), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.86 (m, 2H, CH aromatic), 8.00 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.77, 12.99, 20.75, 21.39, 24.51, 24.94, 26.24, 27.13, 29.67, 30.82, 32.33, 33.91, 39.05, 49.63, 55.43, 57.26, 69.80, 70.44, 74.41, 75.84, 76.70, 106.58, 113.72, 114.36, 122.40, 128.32, 128.75, 129.09, 129.46, 131.93, 133.27, 148.75, 154.52, 154.78, 161.79, 163.64, 164.22, 165.26, 169.90, 170.02, 170.94.



18

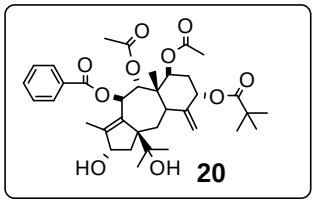
Brevifoliol-5-O-yl-(3, 4-methylendioxyphenyl)-prop-2-enoic acid ester (18):-

Yield: 43%; Yellowish gum;

Electrospray mass for $C_{39}H_{44}O_{12}$ (MeOH): 727 [M+Na]$^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.28 (bs, 2H, CH_2), 1.33 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 2.06 (s, 6H, 2xOAc), 1.57 and 2.45 (m, 2H, CH_2), 2.34 (bt, 1H, CH), 2.36 (bd, 1H, CH), 4.32 (bs, 1H, CH), 4.81 (s, 1H, CH_2), 4.92 (s, 1H, CH), 5.00 (bm, 1H, CH), 5.80 (s, 1H, CH_2), 6.00 (s, 2H, CH_2), 6.07 (bs, 1H, CH), 6.60 (d, 1H, CH), 6.79 (s, 1H, CH aromatic), 7.35 (m, 2H, CH aromatic), 7.51 (s, 1H, aromatic), 7.52 (s, 1H, aromatic), 7.53 (m, 1H, CH aromatic), 7.72 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.70, 14.10, 19.18, 22.68, 24.51, 25.40, 29.16, 37.11, 38.00, 49.62, 57.60, 65.56, 70.26, 70.39, 71.01, 72.55, 75.31, 76.09, 112.04, 128.75, 129.37, 130.76, 130.89, 132.35, 139.30, 147.80, 150.00, 154.54, 162.13, 167.70, 170.63.



20

Brevifoliol-5-O-yl-pivalic acid ester (20):

Yield= 65%; gummy**;**

Electrospray mass for $C_{36}H_{48}O_{10}$ (MeOH): 621 $[(M-CH_3CO+Na]^+$.

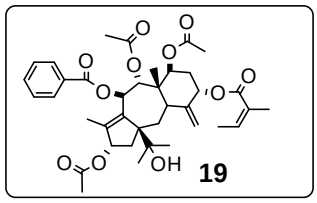
^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.18 (s, 3H, CH_3), 1.25 (s, 9H, 3xCH_3), 1.29 (bs, 2H, CH_2), 1.32 (s, 3H, CH_3), 1.45 and 2.51 (m, 2H, CH_2), 1.70 (bm, 2H, CH_2), 2.03 (s, 3H, CH_3), 2.06 (s, 6H, 2xCH_3), 2.32 (bt, 1H, CH), 4.26 (s, 1H, CH), 4.91 (t, 1H, CH), 4.91 (s, 1H, CH_2), 5.01 (s, 1H, CH_2), 5.77 (bm, 1H, CH), 5.84 (d, 1H, CH), 6.57 (d, 1H, CH), 7.44 (t, 2H, CH aromatic), 7.53 (d , 1H, CH aromatic), 7.85 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 11.41, 19.73, 22.69, 24.86, 28.96, 29.37, 35.20, 37.11, 38.56, 47.93, 49.32, 61.03, 68.27, 69.65, 73.47, 74.57, 75.62, 76.34, 114.07, 128.84, 129.06, 129.62, 135.19, 136.26, 149.79, 152.05, 164.31, 168.00, 177.56.

2.6.5. Synthesis of Brevifoliol-13-O-acetyl-5-O-yl-angelic acid ester (19):-

To round bottom flask appended acetic anhydride (30 mg, 0.14 mmol), DCC (18.5 mg 0.089 mmol) and DMAP (11 mg 0.09 mmol) in DCM (5 mL) was added at room temperature. After 30 min, derivative-2 appended and the stirring continued for 14h where TLC showed the complete consumption of brevifoliol-5-*O*-yl-angelic acid ester

(2). The reaction mixture was diluted with the 10 mL of DCM and this solution was transferred into separating funnel and washed with the water to separate the DCM layer. Then the aqueous layer further extracted with 2 x 10 mL dichloromethane. The pooled DCM layer was dried with anhydrous Na_2SO_4 and thickened on rotary evaporator to obtained primitive compound .The residue was purified by column chromatography (silica gel mesh size 60-120) to get the final yellowish gummy compound at (6-8% Acetone/chloroform) in 80% yield.



19

Yield: 80%; Yellow gum;

Electrospray mass for $C_{38}H_{48}O_{11}$ (MeOH): 703 $[M+Na]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.29 (bs, 2H, CH_2), 1.42 (s, 3H, CH_3), 1.44 and 2.50 (m, 2H, CH_2), 1.66 (s, 3H, CH_3), 1.80 (d, 3H, CH_3), 1.87 (bm, 2H, CH_2), 1.97 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.06 (s, 6H, 2xCH_3), 2.35 (bt, 1H, CH), 4.23 (bs, 1H, CH), 4.90 (s, 1H, CH), 5.26 (s, 1H, CH_2), 5.49 (bm, 1H, CH), 5.50 (s, 1H, CH_2), 6.04 (bs, 1H, CH), 6.60 (d, 1H, CH), 6.88 (q, 1H, CH), 7.42 (m, 2H, CH aromatic), 7.53 (m, 1H, CH aromatic), 7.86 (m, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 12.16, 14.06, 14.99, 19.69, 20.32, 20.69, 21.34, 24.87, 29.14, 35.19, 38.16, 44.93, 47.96, 62.84, 69.96, 72.00, 73.97, 75.31, 75.88, 76.59, 114.03, 128.74, 129.48, 133.30, 135.25, 139.23, 149.79, 152.00, 166.68, 169.91, 169.92, 170.59, 175.16.

2.7 Biological assays

2.7.1 Anti-cancer activity evaluation by MTT assay

The MTT assay test was carried by as perform previously communicated method [26]. *In vitro* cytotoxicity was performed against the five human cancer cell lines, MCF-7 (breast adenocarcinoma), COLO-205 (colon carcinoma), PC3 (Prostate carcinoma), and A549 (lung carcinoma), and a healthy liver cell line Chang. The cell lines were originally American type of culture collection (ATCC), USA, procured from NCCS, Pune and sub-cultured at CSIR-CIMAP Lucknow. Dulbecco modified eagle medium was used for cell grown in the presence of 10% fetal bovine serum and antibiotic-antimitotic solution in a CO_2 incubator (Thermo Scientific, USA) under 95% relative humidity and 5% CO_2 at the 37˚C. Doxorubicin was used as standard

drug for all experiments. In 5% CO_2 incubator, $2*10^3$ cells/well were incubated for 24 hours to facilitate them to maintain properly to the 96 well polystyrene micro plates (Grenier, Germany).

The stocks of brevifoliol derivatives **2-18** were prepared with DMSO (DMSO, Merck, Germany). At least five concentrations were appended into the wells. The analogue and media were replaced with fresh media after the 4 h incubation and further CO_2 incubation continued for 24 h at 37ºC. To avoid the toxicity from DMSO to cells, its concentration always was retained below 1.25%. Afterwards from 0.5 mg/mL 10 µL MTT was appended to each well, further incubated at 37°C for 4 h. Then to dissolve the Formazan crystals, each well is added 100 µL of it and thoroughly mixed till it disappeared. The plates were recorded on Spectra Max 190 Micro plate reader (Molecular Devices Inc. USA) at 570 nm within 1h of DMSO addition. The cytotoxicity effect of the derivative was estimated as;

$$\% \textbf{ inhibition in cell growth} = \frac{1 - (\text{Absorbance of drug} - \text{treated cells})}{(\text{Absorbance of untreated cells})} \text{ X } 100$$

At 50% inhibitory concentration (IC_{50}) Dose-dependent curves were used to determine.

2.7.2 Formation of soft agar colony assay

This experiment was performed in 24 well plates, in this plate human prostate cancer PC-3 cells (5 X 10^4 per mL) were seeded with or without derivatives **11** to see its effect in terms of colony formation within 24 hours [27]. This plate having two layers, base jelly layer was prepared with media and agar (0.72% and 4 mL). Upper layer with cells, agar and media (0.36% and 3 mL).These plates were incubated in carbon dioxide batter for 15-20 days or till the visible of the colonies. After visibility of colonies stained with the crystal violet (0.04% in 2% ethanol) and further incubated for an hour at RT. Then all the colonies were counted by using inverted microscope and pictures were recorded respectively.

2.7.3 Analysis of cell division cycle of derivative-11

Prostate cancer has different stages in its cell cycle. To know exactly where the derivative protecting or stopping the PC-3 cancer cell cycle in order to control the PC-3 cancer growth. To perform above test 6-well cultured plates were needed and 4 $x10^5$ PC-3 cancer cells were seeded in each well and left at 37ºC in 5% CO_2 for overnight for PC-3 cancer cell growth. After growth of PC-3 cancer cell treated with derivative **11** at several time points and cells were collected by trypsinization and fixed with ice-cold 70% ethanol for 30 min at 4°C. The pellets were re-suspended in a propidium iodide solution, after washing with PBS. Triton X 100 and RNase (20 mg/mL, 0.1% and 1 mg/mL) in PBS respectively. After scattering cells in all phases of cell cycle, software "Cell Quest" was used for calculations. This procedure performed as described earlier [28].

2.7.4 Necrosis Vs Apoptosis induction by derivative 11 by Annexin V-FITC assay

This assay test performed by using Flow cytometry as per previously communicated method [29]. This experiment was performed in 6-well plate and each well seeded with PC-3 cancer cell 1 X 10^6 cells/mL and left for overnight for its growth. Then treated with deriavatie-**11** at two different concentrations (1/2 x IC_{50} value and IC_{50}) for 24 hours. All the adherent cells were gathered, kept at 4ºC after centrifuged at 5000 rpm for 5 min and washed with cold PBS twice. Pellets were dissolved in 100 µL 1X binding buffer and incubated as per protocol described in BD Bioscience Kit. Then added 5 µL FITC-annexin-V and 5 µL propidium iodide (PI) for 15 min. Samples were maintained with 500 µL 1x binding buffer and stained cells were analyzed by FACS Diva software of flow cytometry within 1 h.

The Fluorescein Isothiocyante is derivative of Fluorescein (Annexin V-FITC) which binds to phosphatidylserine present on membrane surface in case of apoptotic cells, whereas PI labeled the cellular DNA in necrotic cell. Depending on their four combinations allow the recognized among viable cells (annexin-V-ve, PI-ve), early apoptotic cells (annexin-V+ve, PI-ve), late apoptotic cells (annexin-V+ve, PI+ve), and necrotic cells (annexin-V+ve, PI-ve).

2.7.5 Molecular docking studies of the derivatives 11 and 14

In silico docking studies of potent analogues **11** and **14** were carried out by Auto Dock Vina software [**30**]. The 3D crystallographic protein structures of caspase-3 PDB ID: **3KJF** [**31**] and caspase-9 PDB ID: **INW9** [32] were downloaded from the RCSB PDB database in the PDB format. Protein PDB structures and compounds (**11**, **14**, and doxorubicin) were made ready for docking study. During docking ligand and protein, structures were kept flexible. Among all the top 10 generated docking poses of all docked analogues were checked by using Discovery Studio 3.5 Version visualizer (Accelrys USA), and selected best docking pose. Further, all the compounds were screened by Lipinski's rule of five which is used to determine the chemical and physicochemical properties which make them orally active drug in human beings.

2.7.6 Caspase-3 inhibition assay of derivative 11

The above test was conducted in 96-well plates, pre-coated with caspase-3 monoclonal antibody, supplied with the kit. In each well 100 µL of standards and samples was pipette. Then the plates were sealed and incubated at 37ºC for 1.5 h, washed twice with buffer (300 µL). And biotinylated antibody (100 µL) was added which was specific to caspase-3 then incubated for 1 hour. Plate was washed to remove unbound biotinylated antibody. Then 100 µL of enzyme-conjugate solution was appended and plates were hatched in incubator for half an hour at 37ºC, cleaned, and 100 µL of colour reagent was appended to every well followed by the adjoin of colour reagent C within half an hour. Thickness of the directly proportional to the amount of bound caspases-3, the intensity of colour measured at 450 nm expeditiously. This test was performed twice and doxorubicin drug was used as positive control. The concerted of casapase-3 in the samples was calculated by plotting the absorbance of the samples to the equation derived from the standard curve of caspases-3. Even though we also calculated the activation percent and analyzed the data by comparing it with the control value (untreated). The test was carried out in caspase-3 human ELISA kit according to the instructions given by My BioSource (Catalog # MBS260710) [33, 34**].**

2.7.7. *In-vivo* efficacy by Ehrlich ascites carcinoma of derivative 11

Above test conducted for analogues **11** in Non-inbred *Swiss albino* mice from our institutional animal house as per Khwaja *et al*. [26]. EAC cells were injected to the out bred mice through serial intraperitoneal passage and housed under standard husbandry conditions. Animals were provided with pelleted feed and autoclaved water (M/s Ashirwad Industries, Chandigarh, India and ad libitum*)* respectively. The Institutional Animal Ethics Committee (IAEC) approved this *via* CIMAP/IAEC/2016-19/32 dated 09-02-2017.

To perform the *in-vivo* efficacy experiment non-inbred female *Swiss albino* mice were needed. These mice were injected on day 0, EAC cells (1×10^7) intraperitoneal which were taken from the peritoneal cavity of Swiss mice harbouring 8-10 days old ascites carcinoma. Next day, the entire animals were randomly divided into 6 groups. Out of six groups, Group-I termed as vehicle control group were given normal saline (0.2 ml i.p.), other five groups termed as a treatment groups were given test derivative **11** at 25, 50, 75, and 100 mg/kg i.p. doses. The group-VI was given 5-fluorouracil at a dose of 20 mg/kg, i.p. from day 1-9 and it served as a positive control. On day 12, all the animals were sacrificed and collected ascetic fluid from peritoneal cavity of each mouse for the determination of tumour growth. Percent tumour growth inhibition (%TGI) was determined by using below formula.

$$\%TGI = \frac{(\text{Average number of tumor cells in control group} - \text{Average number of tumor cells in test group})}{\text{Average number of tumor cells in control group}} \times 100$$

2.7.8 Acute oral toxicity study of derivative 11 in *Swiss albino* mice

Derivative of **11** was the best molecule of the series. Owing to its excellent PC3 activity in *in-vitro* model, its toxicity of the derivative **11** was evaluated in *Swiss albino* mice for its further proceeding into drug candidate. This test was carried out as per Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). For this study, 30 mice (15 male and 15 female) were taken and divided into four groups, each group contained 3 male and 3 female mice. All the mice weights were between 20-25 g. The mice were kept at 22 ± 5^0C with humidity

control and automatic dark and light cycle of 12 h. The animals were fed with the standard mice feed and provided *ad libitum* drinking water. The group-1 mice were kept as control and groups-2, 3, 4 and 5 were kept for test. The animals were acclimatized for 7 days in the experimental environment prior to the actual test. The test compound was suspended in few drops of DMSO with food grade sesame oil and given at 5, 50, 300 and 1000 mg/kg body weight to animals of groups 2, 3, 4 and 5 respectively once orally. Control animals received only vehicle.

Test mice were observed for mortality and any signs of ill health at hourly interval on the day of administration of drug and there after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, piloerection respiratory patterns etc. Also changes in gait, posture and response to handling were also recorded. In addition to observational study, body weights were recorded. Blood and serum samples were collected from all the animals on 7^{th} day for above test. The samples were analyzed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, tissue and serum protein, malonaldehyde and reduced GSH activity. The animals were sacrificed and necropsed for any gross pathological changes. Weights of vital organs like heart, liver, kidney etc were also recorded [35].

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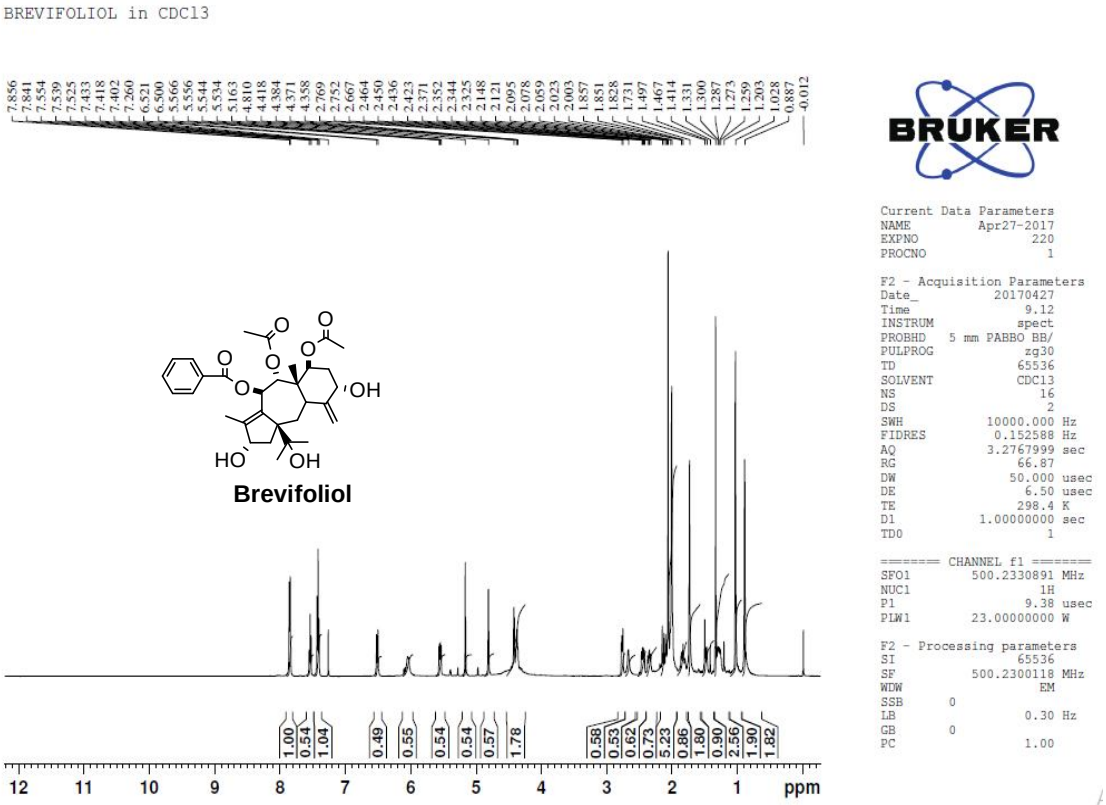
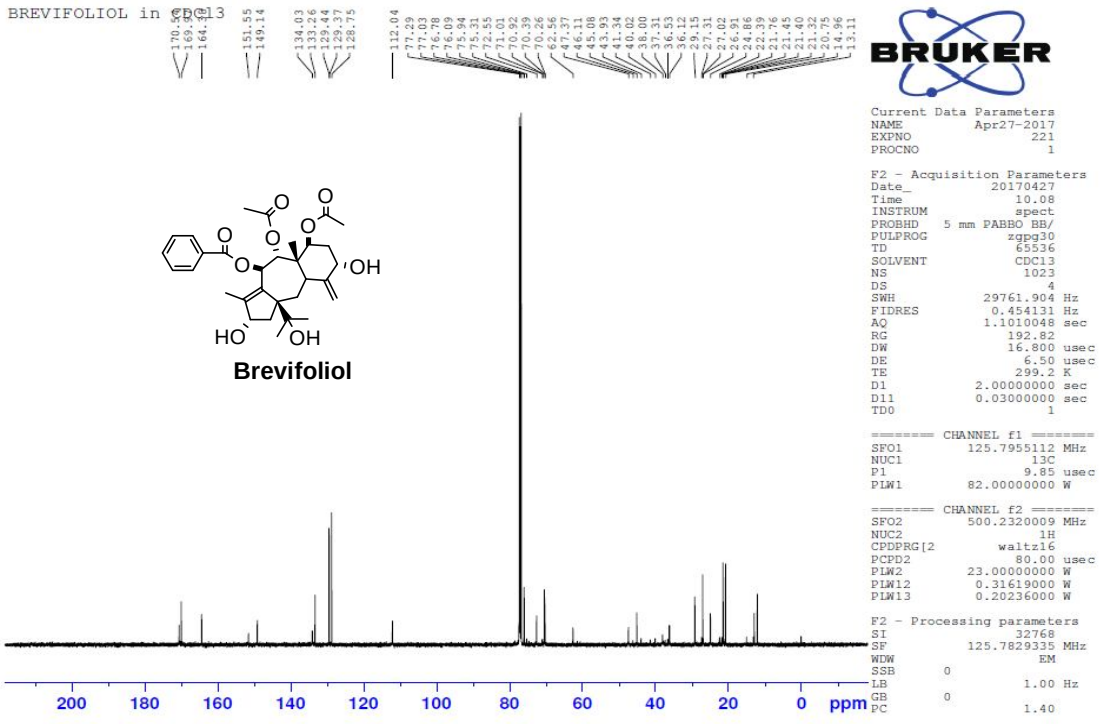
2.9 Spectra of final compounds

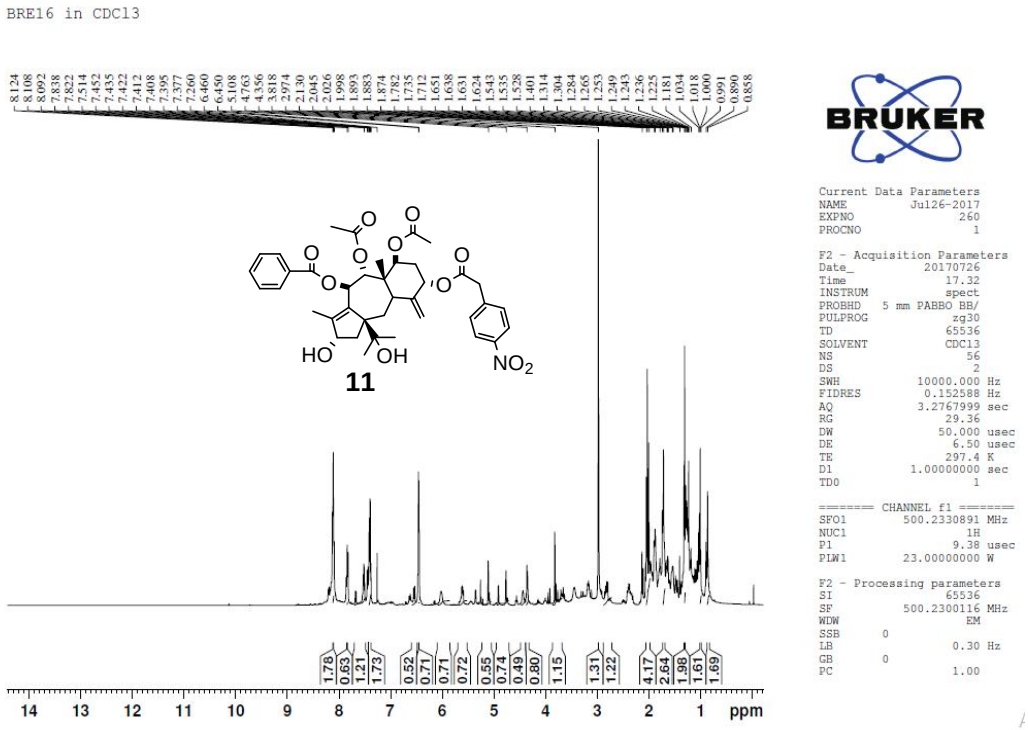
Brevifoliol: ^{1}H NMR



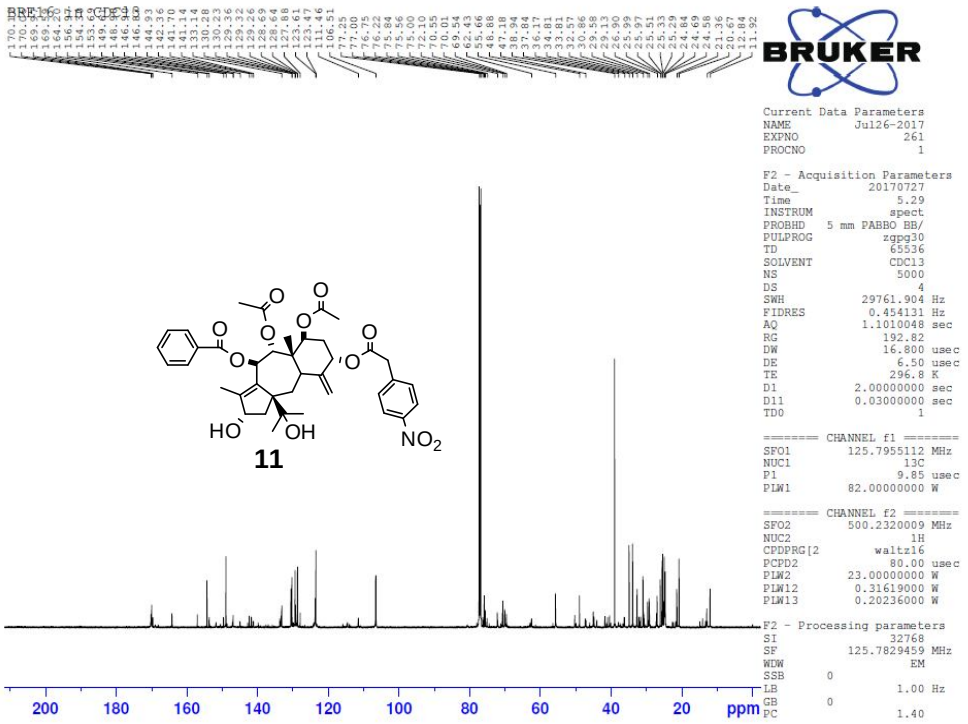
Brevifoliol: ^{13}C NMR



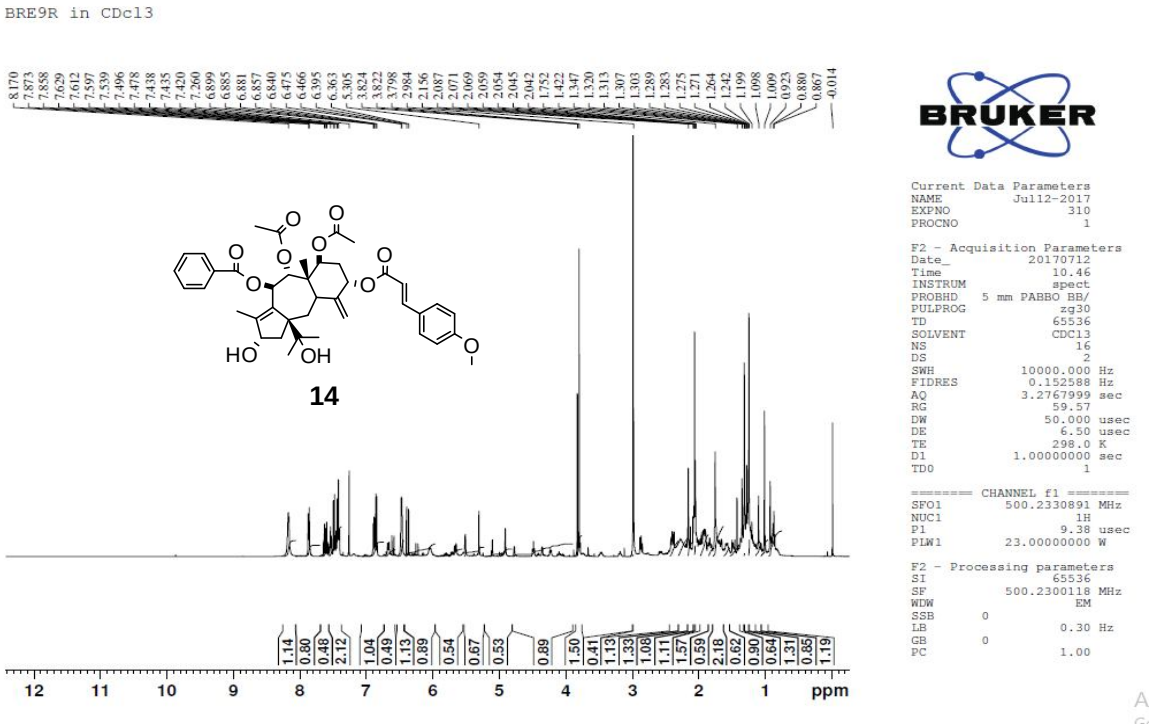
Compound 11:[1] H NMR



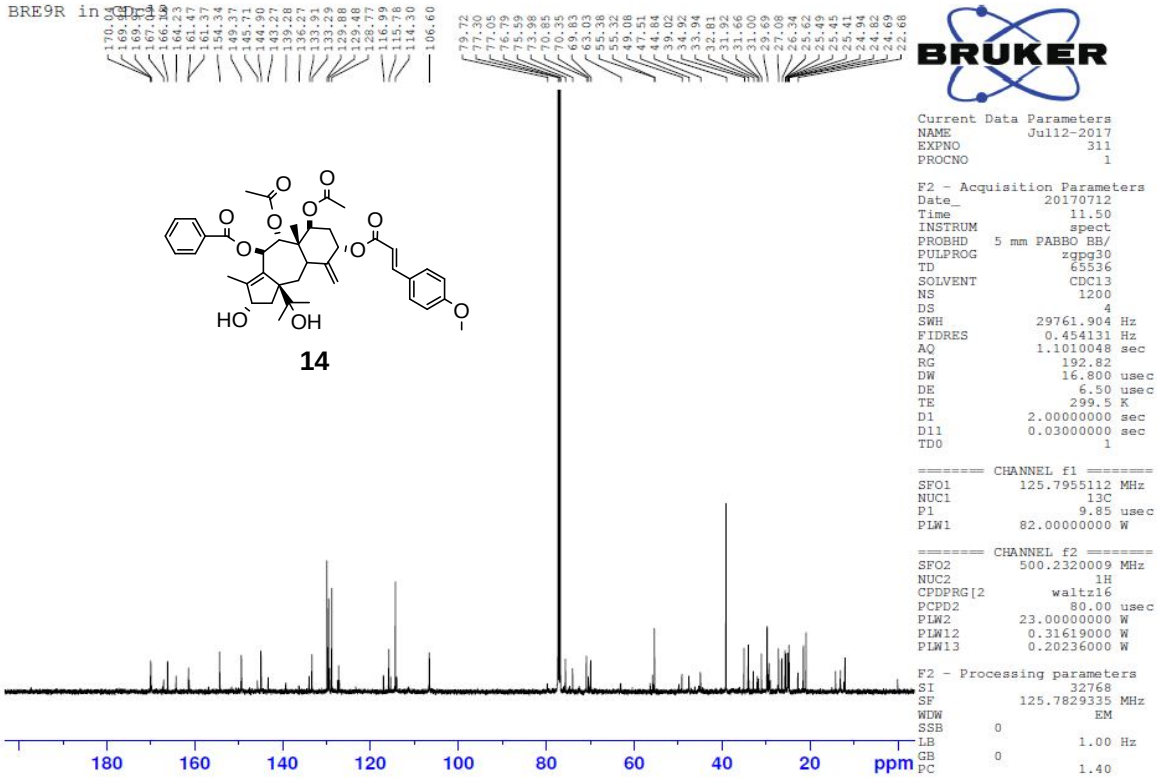
Compound 11: [13]C NMR



Compound 14: ^{1}H NMR



Compound 14: ^{13}C NMR



Compound 11: **Electrospray mass for $C_{39}H_{45}NO_{12}$ (MeOH) :** 742 $[M+Na]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

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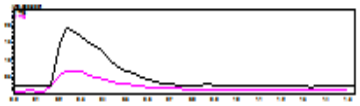
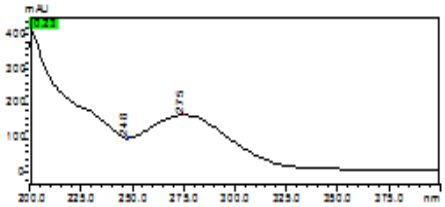
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

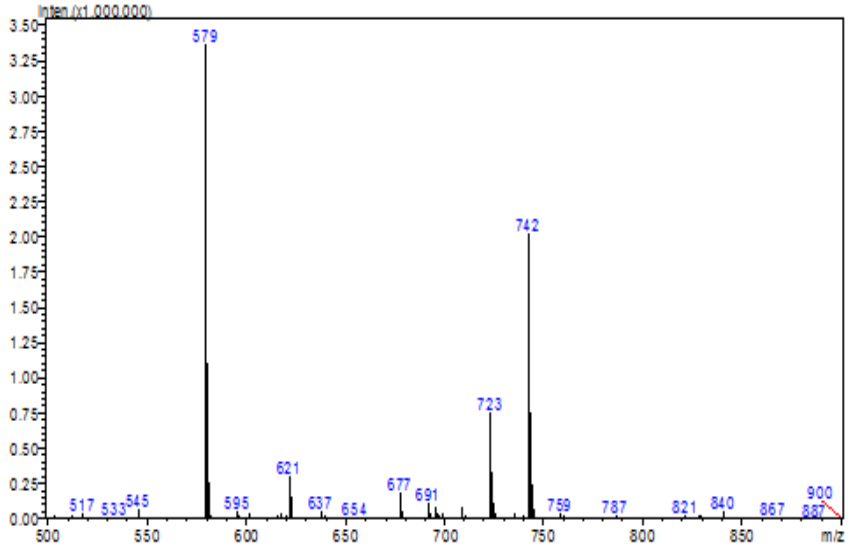
Mass chromatogram



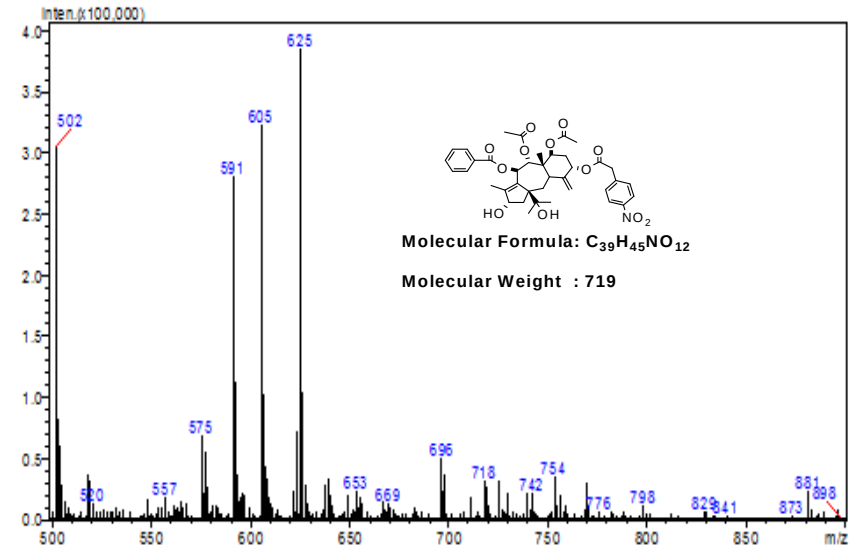
UV-Spectra



ESI+



ESI-



Molecular Formula: $C_{39}H_{45}NO_{12}$

Molecular Weight : 719

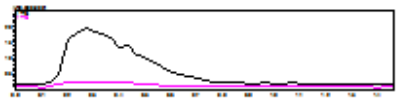
Compound 14: Electrospray mass for $C_{41}H_{48}O_{11}$ (MeOH): 739 $[M+Na]^+$

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

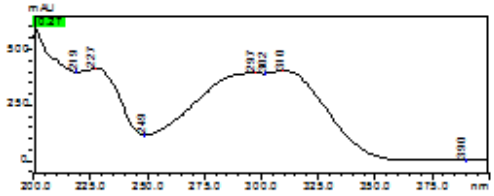
Sample Code : ASN_BRE9R
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

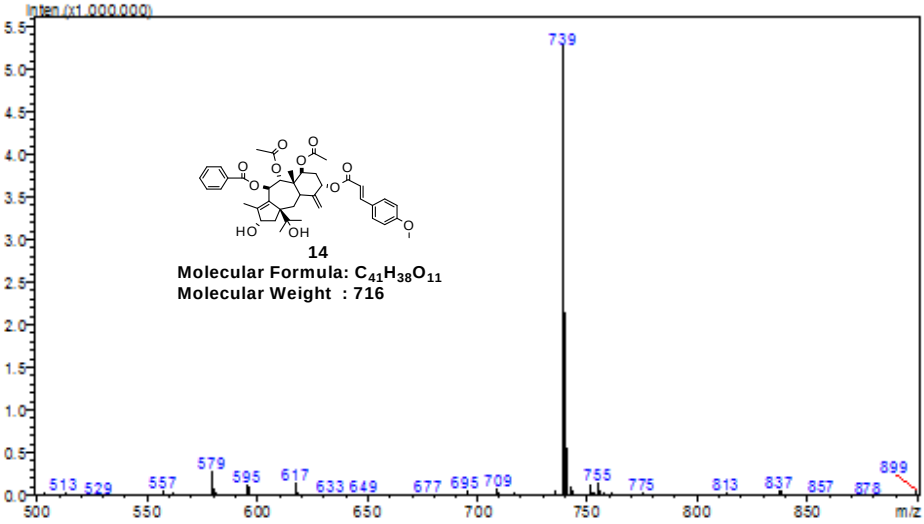
Mass chromatogram



UV-Spectra

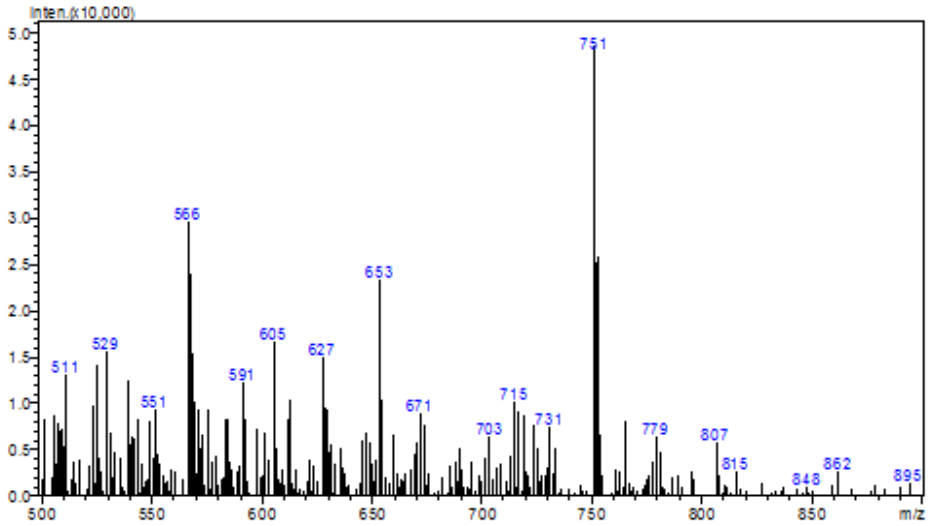


ESI+



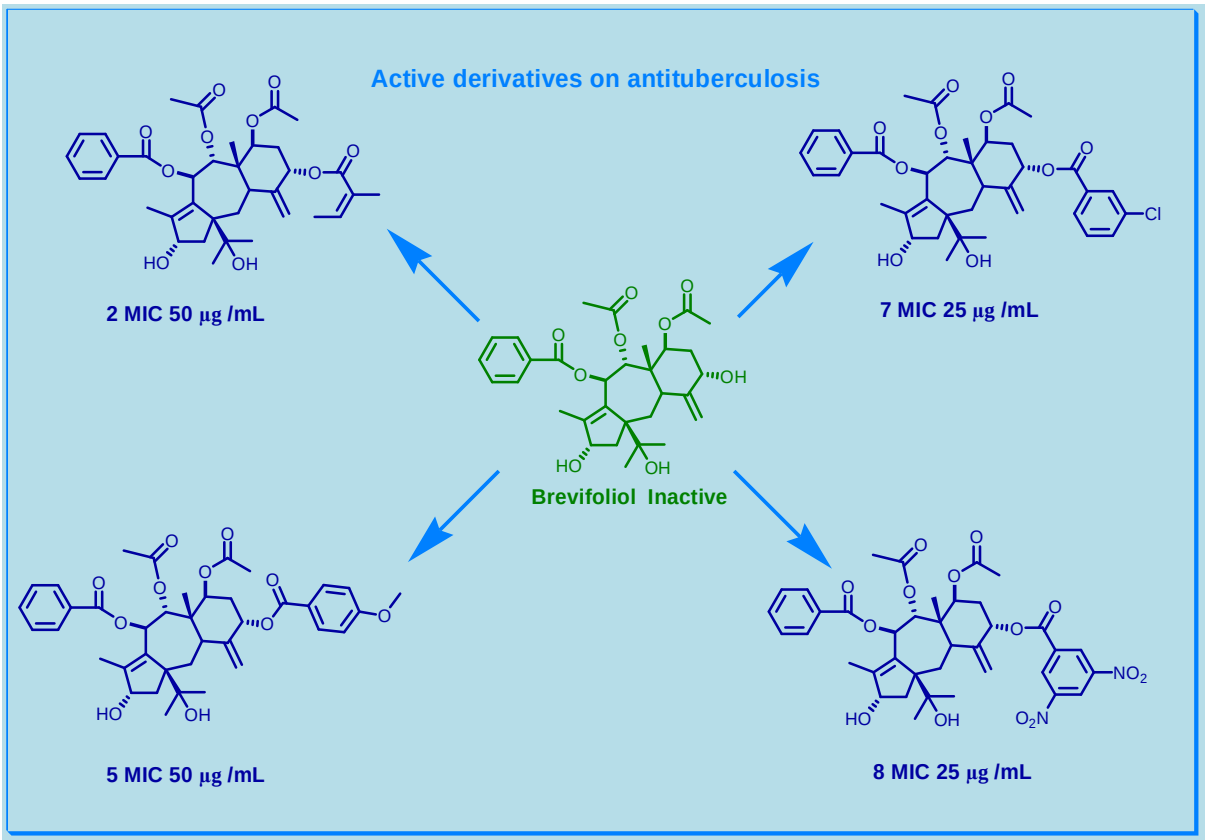
14
Molecular Formula: $C_{41}H_{38}O_{11}$
Molecular Weight : 716

ESI-



Chapter-2 B

Brevifoliol and its analogs: A new class of antitubercular agents


Active derivatives on antituberculosis
2 MIC 50 µg /mL
7 MIC 25 µg /mL
Brevifoliol Inactive
5 MIC 50 µg /mL
8 MIC 25 µg /mL

3.1 Introduction

Tuberculosis (TB) is one of the major causes of impermanence globally. It is caused by *Mycobacterium tuberculosis* (Mtb). It is transferable disease. Nowadays these bacterial strains have become resistant to many of the first and second line antitubercular drugs. Day by day global HIV cases have increased in TB patients that boosted the incidences of TB. There were many MDR cases which have to be taken special concern to reduce impermanence rate globally. Hence, special care to be taken to overcome MDR in order to get novel anti-TB drugs which can have higher activity to reduce consumption, better activity, potential permeability and rapid recovery from the disease.

Phytomolecules give new direction to reduce TB, because of their structural diversity and adaptation [2]. Hence, the moto of derivatization is that to get number of hits which may provide lead molecules with increased selectivity [1, 3]. Over the past few decades taxanes from the genus *Taxus* (yews), have exhibited broad range of biological properties. A detailed literature search revealed that paclitaxol, 10-deacetylbaccatin (10-DAB) and their derivatives possess significant antitubercular activity [4]. This boosted us to search antitubercular property of similar taxanes available in Himalayan yew i.e. *Taxus wallichiana*. Brevifoliol is an abeo-taxane and rearranged taxoid having an 11 (15→1) abeo-taxane system isolated from above plant [5, 6] and belongs to the same group of Taxol [7]. Himalayan yew needles relatively contain high content of brevifoliol as compared to paclitaxel. It would be worth mentioning that taxol is obtained from the bark (which is a destructive mode of harvesting) of the plant while brevifoliol is isolated from the dried needles of the plants, which is a renewable, sustainable and a non-destructive mode of harvesting. Although, several derivatives of brevifoliol were synthesized and some of them showed significantly increased cytotoxicity in different human cancer types [8-11], but to the best of our knowledge none of the brevifoliol derivatives have been investigated for their antitubercular activity [12-17]. Brevifoliol was derivatized into 18 new derivatives [18] evaluated for their *in-vitro* anti-tubercular activity against Mtb H37Ra strain. Further, *in-silico* and cytotoxicity studies were also carried out for significantly active derivatives.

3.2 Taxanes as an antitubercular agent

Taxane diterpene was most important class of compounds which occurs in taxane family. Almost 3500 numbers of taxane were isolated from this family mainly derived from yew trees and out of 3500 taxanes only one turned into successful drug that was paclitaxel. By keeping this in view 120 taxanes were found effective and active against the tuberculosis disease. Out of many taxanes 10-DAB also selected as an antitubercular agent. Further it was modified on various positions. All the derivatives were evaluated for antituberuclosis activity against MDR and drug sensitive *M. tuberculosis*. It was found that all the derivatives were active against both the sensitive and resistant strains which prompted us to prepare more number of derivatives to get good antitubercular agent. [19-21].

3.3 Basis of hypothesis

Taxoids are diterpenoid having lypophilic basic core. The cell wall of mycobacterium is made of mycolic acid which is highly lypophilic in nature. Generally, polar and hydrophilic compounds are unable to interact with mycobacterium cell wall, hence inactive. So moderate level of hyphophilicity is desirable in the molecule. Primarily several taxoids exhibited potent antitubercular activity. Hence, we planned modification on brevifoliol to several novel derivatives at C-5 position to get some antitubercular agents.

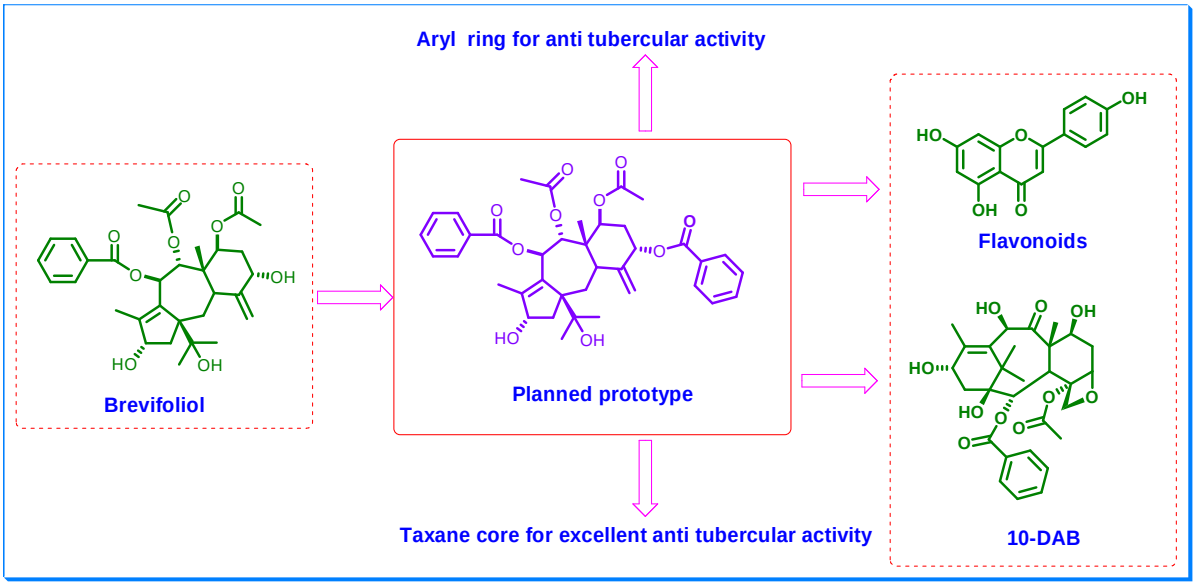


Figure (1): Background of hypothesis

3.4 Result and discussion.

3.4.1 Isolation of brevifoliol

Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India has been a pioneer in the isolation of taxanes such as paclitaxel, 10-deacetylbaccatin (10-DAB) and brevifoliol from the Himalayan yew *Taxus wallichiana* [8, 22, and 5]. In this study brevifoliol was our starting substrate which was refined from the needles of *Yew* and its structure was confirmed by ^{1}H, ^{13}C NMR and Mass spectroscopic data [23, 8]. Brevifoliol isolation procedure is same as chapter-2A Figure **(**10**).**

3.4.2 Synthetic strategy

It has been reported in an *in-silico* study that the presence of benzoate and acetate groups in brevifoliol is important as the removal of these groups makes brevifoliol inactive [23]. Hence, we targeted several esters on hydroxyl group of brevifoliol at C-5 position. Hence, brevifoliol was semi-synthetically converted into eighteen C-5 ester derivatives (**1**–**18**) using Steglich esterification as represented in Scheme 1. Diverse aryl benzoic acids, phenyl acetic acid and cinnamic acids were used for esterification with starting substrate. The structures of products were confirmed by ^{1}H, ^{13}C NMR and Mass Spectrometry. Synthesis of brevifoliol derivatives by using steglich esterfication reaction is shown in the scheme-1 (2.6.5 Synthesis of brevifoliol-5-O-yl-(3, 4, 5-trimethoxy) benzoic acid ester) in chapter-2A.

3.4.3 **Evaluation of antitubercular activity by using agar dilution assay**

All the brevifoliol analogues were evaluated for *in-vitro* anti-tubercular potential against Mtb H37Ra avirulent strain by using agar-based proportion assay [24] and results were shown in Table (1). According to Table (1), there were many analogues exhibiting activity in the range of 25 to 100 µg/mL. Out of them **7** and **8** were the most active (MIC 25 µg/mL) from this series. Then followed by the derivatives **2** and **5** (MIC 50 µg/mL) and derivatives **6, 11** and **15** (MIC 100 µg/mL) while the starting material brevifoliol was inactive. Further, Structure-Activity Relationship (SAR) for brevifoliol derivatives (**1-18**) against the H37Ra avirulent strain was derived.

Table (1): MIC of brevifoliol-derivatives against *M.tuberculosis* H37Ra

Compounds			**Test Concentrations (µg/mL)**					
		Active at (µg/mL)	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**
Compound codes Chapter-2A	Brevifoliol	Not Active	++	++	++	++	++	++
20	**1**	Not Active	++	++	++	++	++	++
2	**2**	**50**	--	--	++	++	++	++
19	**3**	Not Active	++	++	++	++	++	++
3	**4**	Not Active	++	++	++	++	++	++
17	**5**	**50**	--	--	++	++	++	++
4	**6**	**100**	--	++	++	++	++	++
5	**7**	**25**	--	--	--	++	++	++
6	**8**	**25**	--	--	--	++	++	++
7	**9**	Not Active	++	++	++	++	++	++
8	**10**	Not Active	++	++	++	++	++	++
9	**11**	**100**	--	++	++	++	++	++
10	**12**	Not Active	++	++	++	++	++	++
11	**13**	Not Active	++	++	++	++	++	++
12	**14**	Not Active	++	++	++	++	++	++
13	**15**	**100**	--	++	++	++	++	++
14	**16**	Not Active	++	++	++	++	++	++
15	**17**	Not Active	++	++	++	++	++	++
16	**18**	Not Active	++	++	++	++	++	++
Std Drug INH		**-- (0.025)**						
No drug Control		++++++						

3.4.4 Structure-Activity Relationship (SAR)

From the Table (1), it is evident that *t*-butyl ester derivative **1** of brevifoliol having three tertiary methyl groups was inactive, while the angelic acid ester derivative **2** having two *trans* olefin methyl was active (MIC 50 µg/mL). However, when the C-13 hydroxyl (-OH) of **2** was acetylated as in derivative **3**, the activity was lost. This suggests that for the antitubercular activity free hydroxyl group (-OH) at C-13 is essential. Since, activity was observed in olefinic methyl derivative **2**, R was substituted with 4-ethyl phenyl as in derivative **4,** but there was no change in activity with respect to starting material brevifoliol, but when this *p*-ethyl (+I) group of **4** was replaced with enhanced electron-donating, *p*-methoxy (-OMe) group as in **5,** the activity got enhanced (MIC 50 µg/mL) due to mesomeric effect. On the other hand when this *p*-ethyl group of **4** was replaced with 3, 4, 5 or *m*, *p*, *m*-trimethoxy (-OMe) group as in **6,** the activity got reduced to half (MIC 100 µg/mL) to that of derivative **5,** clearly suggesting that electron-donating group (-OMe) at meta position has a negative role on activity.

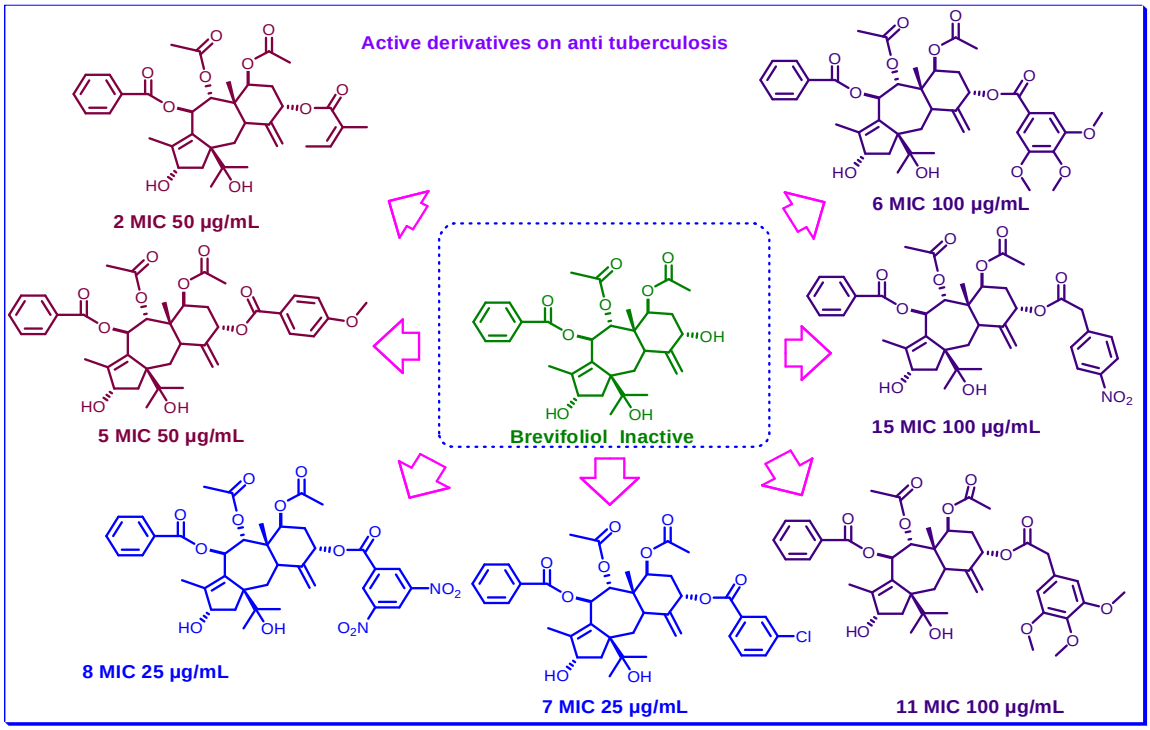


Figure (2): Antitubercular taxoids

Similarly, substitution of phenyl ring with electron-withdrawing (-Cl / -NO_2) group at *Meta* and *para* position as in derivatives **7** and **8** increased the activity (MIC 25 μg/mL) by two folds to that of derivative **5.** However, when R was replaced with benzyl group having different type of substitutions on its *ortho*, *Meta* and *Para* positions such as in derivatives **9** to **15,** the activity was lost or was very poor. This may be the presence of –CH_2- group between the ester's –C=O and phenyl ring making these benzyl substitutions ineffective or very less effective. Further, with analogy to olefinic methyl (angelic acid) derivative **2,** three cinnamoyl derivatives **16**, **17** and **18** were prepared, but all of them were found inactive. From the above, it may be concluded that phenyl esters of brevifoliol having an electron-withdrawing group at their *meta* position(s) were more active and we must focus our future strategies in this direction.

3.4.5 Molecular docking studies for potent derivatives 7 and 8

In in-*vitro* studies reference drug was taken as isoniazid. To know the LibDock score and binding energy to understand the potent derivatives lignad-protein interaction. According to molecules docking score the derivative given higher score has character to have high ligand-protein interactions. This has shown the drug likeness of derivatives. This all the LibDock score composition with response to control compound isoniazid results are shown in Figure (3). Among eighteen derivatives, three different target estimates, the compounds only docked with inhA and dfrA and failed to dock with katG. Results of the docking studies revealed that compounds are more inclined to bind with target inhA rather than target dfrA. The docking results of compound **7** and **8** are compared with the standard drug (isoniazid) showed in Table (2). Docking studies yielded numerous poses like various orientations and conformations within the area of active site. LibDock (docking) score higher i.e. it has chance to have higher chance of ligand-protein interactions. The docking results are shown in Table (2).

Further, the representation of best-docked conformation of brevifoliol ester **7** and **8** in binding site pocket of inhA has been provided in Figure 4. Here ligand-protein docked complexes exhibited binding site residues, H-bonds, and Pi interaction. The candidate compound **7** when docked with inhA gave a docking score of 152.68 and binding energy of -208.62 kcal/mol and formed three H-bonds with SER94, MET98, and

SER94. Similarly, when compound **8** docked with inhA, it gave a docking score of 113.55 and binding energy of -175.46 kcal/mol and formed single H-bonds with GLN100 and Pi-interaction with PHE97. Apart from the above docking results were also endorsed with various scoring parameters such as -PLP1, -PLP2, results are given in Table (2)**.** The molecular docking results and the way of binding pattern indicated that compound **7** and **8** bound with same binding pocket inhA and binding score shown higher than standard drug. 2D chart of docking for the known drug isoniazid, and the two active derivatives **7** and **8** showed interaction of amino acid residues Figure (4).

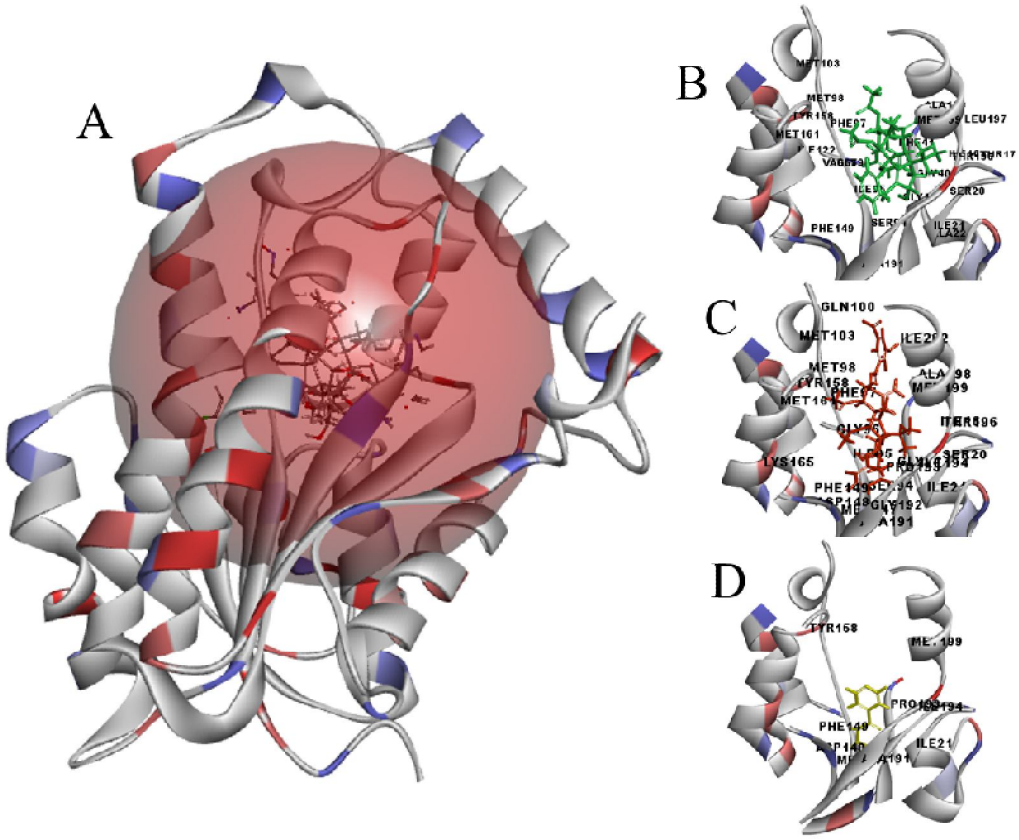
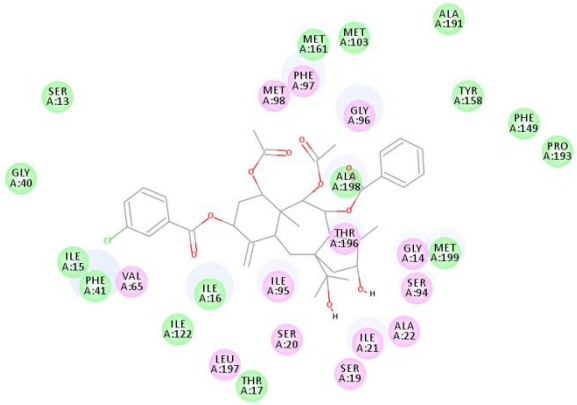


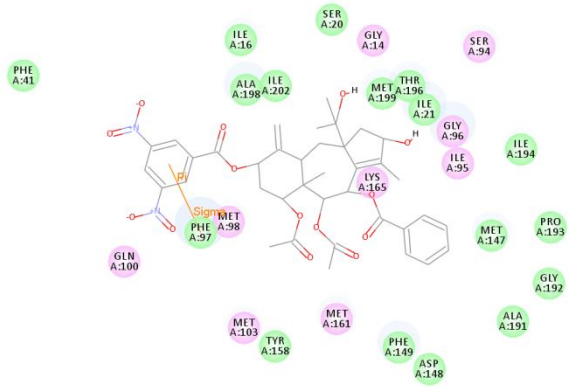
Figure (3): (A) Structural model of enoyl-ACP reductase (PDB ID: 4TRO) with the ligand-binding site (orange sphere). (B-D) Binding site pocket residues with best fit confirmation of compound **7** (green colour), compound **8** (red colour) and Isoniazid (yellow colour)

Table (2): Details of docking based parameters, namely, LibDock score, hydrogen bond, pi interactions, and interactive amino acid residues of candidate compounds in the binding site pocket of target protein inhA (PDB ID:4TRO)

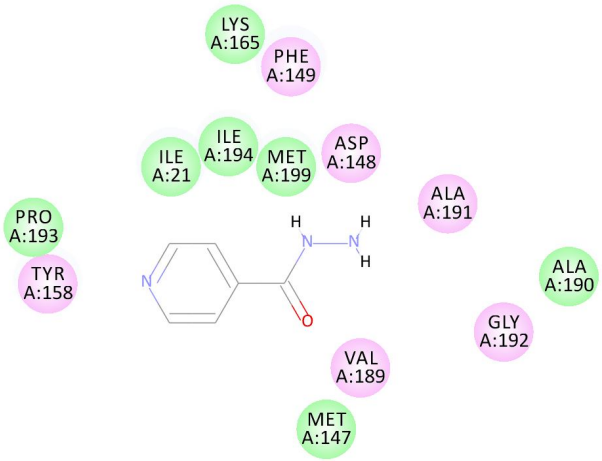
Compound	**LibDock score**	**Binding energy (kcal/mol)**	**H bond**	**-PLP1**	**-PLP2**	**Jain**	**Pi interaction**	**Interactive amino acid residues**
7	152.68	-208.62	SER94 MET98 SER94	98.09	101.53	2.99	None	GLY14, ILE16, THR17, SER20, **ILE21**, ALA22, GLY40, PHE41, VAL65, SER94, ILE95, GLY96, PHE97, MET98, MET103, ILE122, **PHE149, TYR158**, MET161, **ALA191,** THR196, LEU197, ALA198, **MET199**
8	113.55	-175.46	GLN100	84.18	78.91	1.37	PHE97	GLY14, ILE16, SER20, **ILE21,** SER94, ILE95, GLY96, PHE97, MET98, GLN100, MET103, **MET147**, **ASP148**, **PHE149**, **TYR158,** MET161, LYS165, **ALA191**, GLY192, **PRO193**, **ILE194**, THR196, ALA198, **MET199**, ILE202
Control (Isoniazid)	61.63	-81.25	ASP148	47.23	40.3	0.73	PHE149	**ILE21, MET147**, **ASP148**, **PHE149**, **TYR158, ALA191**, **PRO193**, **ILE194**, **MET199**



(A) Compound 7



(B) Compound 8



(C) Control (Isoniazid)

Residue interaction representation

Figure (4): 2-D diagrams illustrating protein-ligand interactions: (A) Compound **7**, (B) Compound **8** and (C) Control (Isoniazid).

3.4.6 Drug target selection for potent derivatives of 7 and 8

There were three possible targets identified namely, inhA (enoyl-ACP reductase), dfrA (dihydrofolate reductase), and katG (catalase-peroxidase) by using STITCH 5.0 program with 0.70 confidence limit and above Figure (5) which indicate stronger association with isoniazid by using the above software and identified 3 possible targets namely inhA, dfrA, and katG . These 3 interactions were related to anti-TB target. The target score given of 0.939, 0.814, and 0.703 respectively. Depending on closeness of score one has to select best appropriate target Table (3).

Table (3): Predicted functional targets of the candidate compound.

S.No.	Target	Target full name	PDB	Amino Acid	Score
1.	inhA	enoyl-ACP reductase	4TRO	269	0.939
2.	dfrA	dihydrofolate reductase	4M2X	159	0.814
3.	katG	Catalase-peroxidase	1SJ2	740	0.703

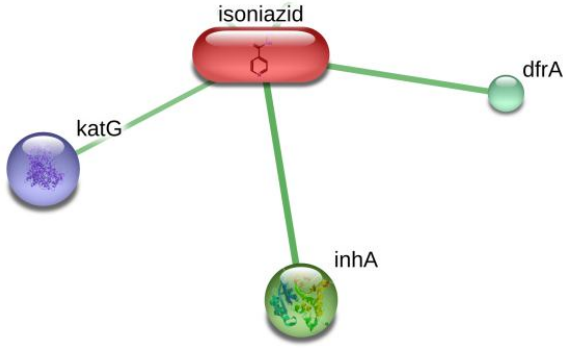


Figure (5): Network representing Isoniazid and protein interactions.

3.4.7 Screening for ADMET risk for esters 7 and 8

For the potent derivative ADME test required to know the feasibility of compound drugness. To know the such risk properties like toxicity, absorption, cytochrome P450 oxidation and mutagenicity were predicted by using ADME predictor ^{TM} (simulation Plus, INC. USA) software. By using above software both the potent compounds and reference drug was recorded ADME risk score. The candidate derivative **7** showed an ADMET Risk score of 10.64, whereas compound **8** shows an ADMET Risk score of 10.41 in comparison to control anti-*Mtb* drug isoniazid, which exhibited a score of 1.42. The risk parameters associated with these derivatives were mainly due to their size, hydrogen bond acceptor, charge, water-solubility, renal clearance issue, lipophilicity, inhibition of testosterone, etc. Table (4). Both the compounds were found to violate the Lipinski rule.

Table (4): Compliance of the candidate compound for ADMET risk assessment

Identifier	Absorption Risk	CYP Risk	MUT Risk	TOX Risk	ADMET Risk	ADMET Code	Rule of 5
Range	**0 – 8**	**0 – 6**	**0 – 4**	**0 – 7**	**0 – 24**		
7	4.92	3.71	0	2	10.64	Size; HBA; ch; Kow; Sw; rat; Xr; 3A4; CL; mi; ti	1
8	5.39	3.01	0	2	10.41	Size; HBA; ch; Kow; Peff; Sw; rat; Xr; 3A4; CL; mi; ti	2
Isoniazid	0	0.42	0	1	1.42	HEPX; 1A2	0

3.4.8 *in silico* pharmacokinetics studies of derivatives 7 and 8

In silico Pharmacokinetic properties were also studied for derivatives **7** and **8** in order to check drugness of above derivatives. To compliance the above test these parameters should be followed; plasma protein binding, blood-brain barrier penetration, aqueous solubility, intestinal absorption and cytochrome P450 2D6 binding were calculated. These parameters should be passed with new chemical entity in order to avoid failure in terms of toxicity of metabolites in active form in the body and unable to cross membrane. Results of this analysis have been provided in the Table (5), together with a biplot as Figure (6).

Table (5): Compliance of compounds to the theoretical parameters of oral bioavailability and drug-likeness properties

Compound	Aqueous Solubility	Blood–brain barrier penetration	Cytochrome P450 2D6 binding	Intestinal absorption	Plasma protein binding	A log P98	PSA
7	yes, low	undefined	Non-inhibitor	poor	highly bounded	5.13	46.55
8	yes, low	undefined	Non-inhibitor	very poor	highly bounded	4.25	32.2
Isoniazid	yes, optimal	low	Non-inhibitor	good	highly bounded	-0.81	67.91

According to the biplot two potent derivatives showed 95% and 99% confidence ellipses for human intestinal absorption (HIA) and the blood-brain barrier penetration models respectively. But the PSA exhibited inverse result with respective to HIA and cell wall permeability. However relationship between PSA and permeability has been well established. Moreover, PSA was estimated as a chemical descriptor for passive molecular transport through membranes. The PSA value higher for derivatives **7** and **8** than the isoniazid. The aq. solubility predictions at 25°C implied to compounds **7** and **8** shown low solubility. Log P value, which is a measure of lipophilicity and is the ratio of the solubility of the compound in octanol compared to its solubility in water, was found to be in the range of the hit active derivatives and follow Lipinski's rule of five, implicating a better oral bioavailability. The excretion process that eliminates the compound from the human body also depends on Log P. The studied compounds were found to be highly bound to carrier proteins in the blood. This binding shows efficiency of the drugs. The drugs which are orally administered must be absorbed by the intestine; here, the predicted result showed that the derivatives **7** and **8** were poorly absorbed by the intestine in comparison to drug isoniazid, which showed a reasonable absorption rate. The derivatives **7** and **8** were found to be non-inhibitors of cytochrome P450 2D6 (CYP2D6), which is known to be one of the crucial metabolic enzyme Table (5).

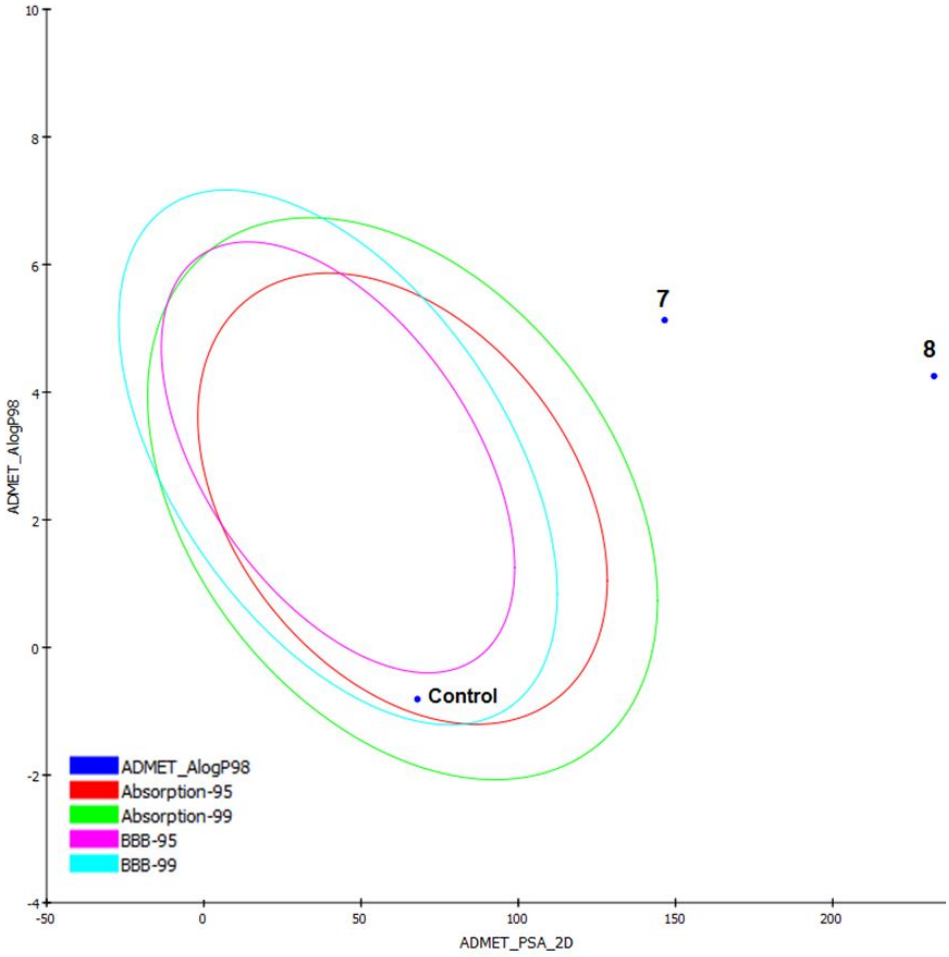


Figure (6): Plot of Polar Surface Area (PSA) versus Log P for candidate compounds showing the 95% and 99% confidence limit ellipses corresponding to the blood-brain barrier and intestinal absorption models.

3.4.9 Prediction of toxic effects of derivatives 7and 8

The derivatives **7** and **8** were subjected to *in silico* toxicity studies. The results showed that the maximum recommended therapeutic dose was above 3.16 mg/kg/day for both the derivatives. The derivative **7** was found to be non-toxic for androgen receptor toxicity, whereas derivative **8** showed toxicity for the same. On the other hand, both derivatives showed toxicity against the estrogen receptor. The derivatives when subjected for skin sensitization studies showed non-sensitizing. Similarly, the derivatives when studied for allergenic respiratory sensitization, none of the derivatives showed sensitizer effect Table (6). The derivatives were found to be non-biodegradable. The bio-concentration factors identified for derivatives **7** and derivatives **8** were 8.01 and 3.38 respectively. The results also showed that there were

no effects on hERG (human Ether-a-go-go-Related Gene) potassium channel inhibition in human due to administration of **7** and **8**. Thus, there may not be adverse cardiac effect. The calculated rapport towards hERG K^+ channel and potential for cardiac toxicity was identified as 5.077 and 4.948 mol/L for **7** and **8** respectively. The results also indicated that there is no drug-induced phospholipidosis (intracellular accumulation of phospholipids). These results suggested that the derivatives **7** and **8** may not be associated with unwanted side effects, such as QT prolongation, myopathy, hepatotoxicity, nephrotoxicity, or pulmonary dysfunction. Feasible reproductive toxicity was also estimated for the derivatives **7** and **8**, and the result showed that both derivatives were toxic. Drug hepatotoxicity, which causes acute and chronic liver disease, results in elevated levels of ALP, GGT, LDH, AST, and ALT. The derivatives **7** and **8** were also tested for the elevation of these enzymes, and the results showed that the level of GGT, LDH, and AST were not elevated by administion of these derivatives. On the other hands, it was found that the level of ALP and ALT were elevated by both the compounds Table (6). An alternate way of animal testing was applied to predict the dose-dependent toxicities such as tumorigenic dose and LD_{50} values. Exhibited results indicated that the LD_{50} for lethal rat acute toxicity was 86.01 and 44.57 mg/kg for derivatives **7** and **8** respectively. The Fathead minnow and lethal toxicity after 96 hours of exposure was calculated, this was 4.11E-05, 0.001 mg/L for **7** and **8** respectively. On the other hand the *Daphnia Magna* (water flea) lethal toxicity after 48 hours of exposure was calculated, which was 0.054 and 0.282 mg/L for **7** and **8**, respectively. The results suggested that 1.96 and 2461.53 mg/kg/day of derivative **7** and 0.65 and 3960.38 mg/kg/day of derivative **8** respectively were required to induce tumorigenesis in the rat and mice Table (6).

Table (6): *In silico* toxicity studies of the candidate compounds-7 and 8.

Identifier	7	8	Isoniazid
Maximum recommended therapeutic dose administered as an oral dose (mg/kg/day)	Above 3.16	Above 3.16	Above 3.16
Estrogen receptor (rats)	Nontoxic	Toxic	Nontoxic
Androgen receptor toxicity	Toxic	Toxic	Nontoxic
Allergenic skin sensitization (mice)	Non-sensitizer	Non-sensitizer	Sensitizer
Allergenic respiratory sensitization in rat	Non-sensitizer	Non-sensitizer	Sensitizer

Fathead minnow lethal toxicity after 96 h of exposure (mg/L)		4.11E-05	0.001	85.22
Tetrahymena pyriformis growth inhibition toxicity (mmol/L)		2.752	3.922	-1.122
***Daphnia magna* (water flea) lethal toxicity after 48 h of exposure (mg/L)**		0.054	0.282	10.346
Bio concentration factor		8.01	3.383	1.565
Biodegradation		No	No	No
Likelihood of the hERG potassium channel inhibition in human		No	No	No
LD_{50} for lethal rat acute toxicity (mg/kg)		86.006	44.57	579.14
Tumorigenic dose rat (mg/kg/day)		1.96	0.65	9.64
Tumorigenic dose mice (mg/kg/day)		2461.53	3960.38	85.4
Triggering the mutagenic chromosomal aberrations		Nontoxic	Nontoxic	Toxic
Causing phospholipidosis		Nontoxic	Nontoxic	Nontoxic
Reproductive/developmental toxicity		Toxic	Toxic	Toxic
Hepatotoxicity	**Levels of ALP enzyme**	Elevated	Elevated	Elevated
	Levels of GGT enzyme	Normal	Normal	Elevated
	Levels of LDH enzyme	Normal	Normal	Elevated
	Levels of AST enzyme	Normal	Normal	Elevated
	Levels of ALT enzyme	Elevated	Elevated	Elevated

From the above, it may be concluded that phenyl esters of brevifoliol having electron withdrawing group at their *meta* position(s) were more active and we must focus our future strategies in this direction to get better candidates.

3.5 Conclusion

In this study as per our desire results provides evidence that the brevifoliol derivatives **7** and **8** possess anti-tubercular activity against the *Mtb* H37Ra. This was further supported by the molecular docking studies, which showed 2 to 2.5 times more docking (LibDock) and binding energy scores for the derivatives **8** and **7** to that of the known drug Isoniazid on the mycobacterium enzyme inhA (enoyl-ACP reductase).

Finally, both the derivatives (**7** and **8**) showed no cytotoxicity towards the healthy liver cell lines CHANG.

3.6 Experimental section

3.6.1 General experimentation

General experiment explanation given in the chapter-1 in section 1.6.1.

3.6.2 Chemistry

3.6.3 Fractionation process

The needles (68 Kg) of *Taxus wallichiana* were collected from Jageswar, district Almora, Uttarakhand, India. The needles were shade dried, powdered (13.0 Kg) and then successively extracted with hexane (20 L x 4), chloroform (20 L x 5) and ethyl acetate (20 L x 5), which afforded Hexane fraction (206.89 g), Chloroform fraction (66.74 g), and Ethyl acetate fraction (82.81 g). The fractionation flow chart given in Chapter-2A 2.6.2. Isolation of brevifoliol Figure (10).

3.6.4 Isolation of brevifoliol

A portion of ethyl acetate fraction (40 g) was subjected to column chromatography over silica gel (600 g, 60-120 mesh). Gradient elution of the column was carried out with chloroform, chloroform- acetone (up to 1-10%), and chloroform-methanol (up to 1-8%). Pure brevifoliol (0.79 g) was obtained in the fractions eluted with chloroform-methanol 6-8%. Further this process was repeated for the rest of the ethyl acetate fraction for the isolation of brevifoliol.

3.7 Biological assays

3.7.1 Determination of MIC

The Minimum Inhibitory Concentration (MICs) of brevifoliol-derivatives and antitubercular drugs INH were determined against Mtb H37Ra by Agar dilution assay [24]. Briefly, brevifoliol-derivatives were dissolved in dimethyl sulfoxide (DMSO) to make stock (5 mg/mL). Serial dilutions from stocks were also made in DMSO. To 1.9 mL MB 7H11 agar medium (in tubes, temp. 45-50ºC, with OADC supplement), 0.1 mL of a compound or DMSO (negative control) was added. The contents were mixed

and allowed to solidify as slants. Three weeks old culture of Mtb H37Ra was harvested from L-J medium and its suspension (1 mg/mL equivalent to 108 bacilli approximately) was made in normal saline (containing 0.05% Tween-80). 10 μL of 1:10 dilution of this suspension (~105 bacilli) was inoculated into each tube and incubated at 37 ºC for 4 weeks. The lowest concentration of a compound up to which there was no visible growth of bacilli was its minimal inhibitory concentration (MIC).

3.7.2 Drug target selection

Generally target selection study can be performed for the active molecules. In this selection procedure the program of STITCH 5.0 was used. This is the program we can explore the known and predicted interaction of proteins and potent compounds. Analysis of target selection for potent molecules along with the control. Target selection can be performed in two ways, one standard drug based and other one is similarity of the compounds [25].

3.7.3 Target preparation

To prepare target protein, the structure of the target protein, i.e., inhA (PDB ID: 4TRO), dfrA (PDB ID: 4M2X) and katG (PDB ID: 1SJ2) as well as the coordinates of the crystal structure of identifying target proteins were collected from the protein data bank (https://www.rcsb.org/). Before docking studies, the target protein was prepared by using the protein preparation protocol of Discovery Studio v3.5 (Accelrys, USA, 2013). Through protein preparation protocol, different tasks were performed such as inserting missing atoms in incomplete residues, modelling missing loop regions, standardizing names of the atoms, deleting alternate conformations, protonating titratable residues and removing water. The force field 'CHARMM' was used for protein preparation. Before further processing, the hydrogen atoms were added [26].

3.7.4 Protein-ligand docking

In silico molecular docking studies and post-docking visualization studies were performed by using the Discovery Studio software for molecular modelling [27]. The docking exercise was completed by a LibDock program of Discovery Studio so that to reveal the bioactive binding site poses of potential inhibitors within the active site of the selected drug targets. This LibDock program uses protein site features referred to as hot spots, and were of two types (polar and a polar). Then this ligand poses were

placed into this polar and a polar receptor interactions site. For energy minimization, the Merck Molecular Force Field (MMFF) was used in the parameterization step. To generate the conformations, a Conformer Algorithm based on Energy Screening and Recursive build-up (CAESAR) method was used [28]. The other docking and scoring parameters kept at their default sets. Further, to identify specific interacting residues of the receptor with bound ligand a 2-D diagram of docking was also performed.

3.7.5 Rule of five and ADMET risk study

All the compounds were checked for violation of Lipinski's rule of five. Later the compounds were screened for ADMET risk by ADMET Predictor ^{TM} (Simulation Plus Inc., USA). The overall ADMET risk was considered to be in the range of 0 – 24, where lower value represents better druggability [29].

3.7.6 Pharmacokinetics parameters

ADMET states the absorption, distribution, metabolism, excretion, and toxicity properties of a molecule within an organism, and was predicted using ADMET descriptors in Discovery Studio. Through this module, six mathematical models (aqueous solubility, blood-brain barrier penetration, cytochrome P450 2D6 inhibition, hepatotoxicity, human intestinal absorption, and plasma protein binding) were used to quantitatively predict properties of a set of rules that stipulate ADMET characteristics of the chemical structure of the molecules [30].

3.7.7 *In silico* toxicity studies

The safety of compounds is crucial for a successful drug. To calculate the toxicity risk assessments, the parameters such as hepatotoxicity, androgen receptor toxicity, neurotoxicity, mutagenicity, and developmental toxicity were calculated along with the effect of compounds on some of the liver-associated enzymes such as gamma-glutamyltransferase (GGT), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) enzymes. This toxicity studies led to define, how the candidate compound behaves in the human body and also helpful to set dose-ranges [31].

3.8 References

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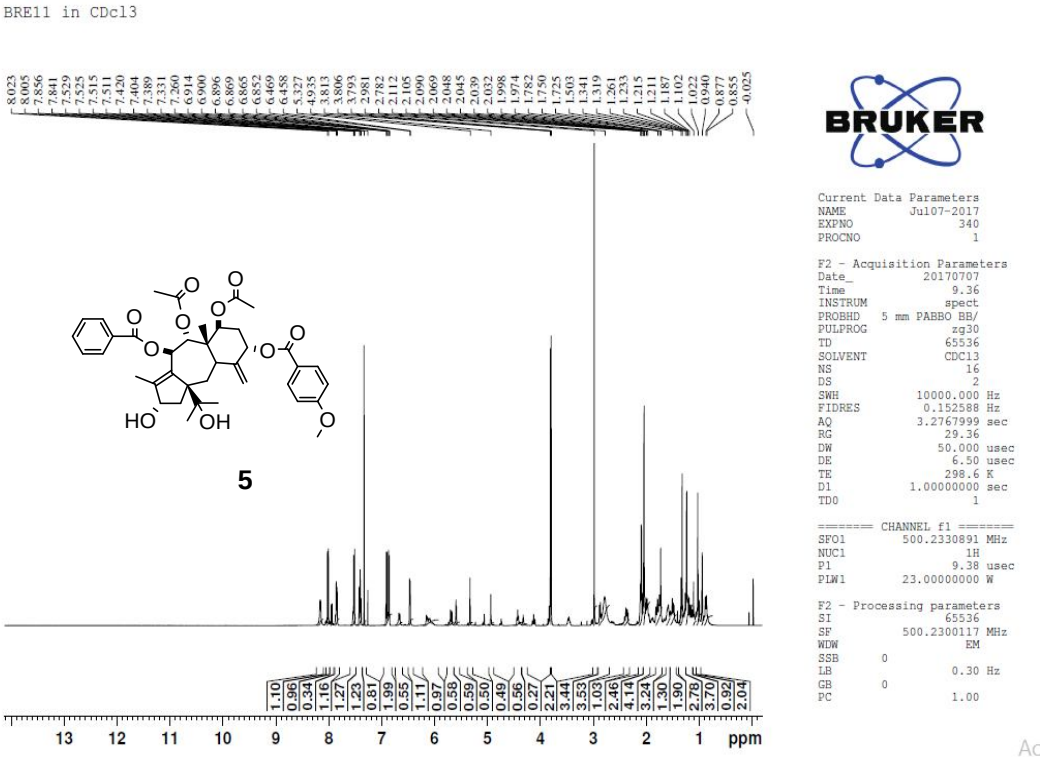
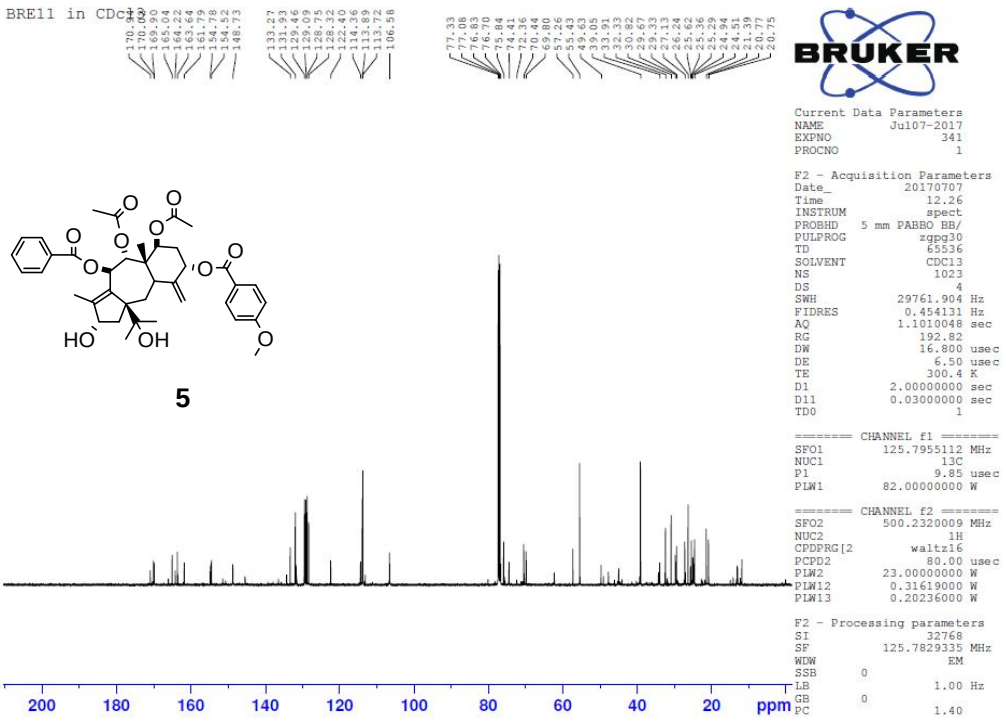
3.9 Spectra of final compounds

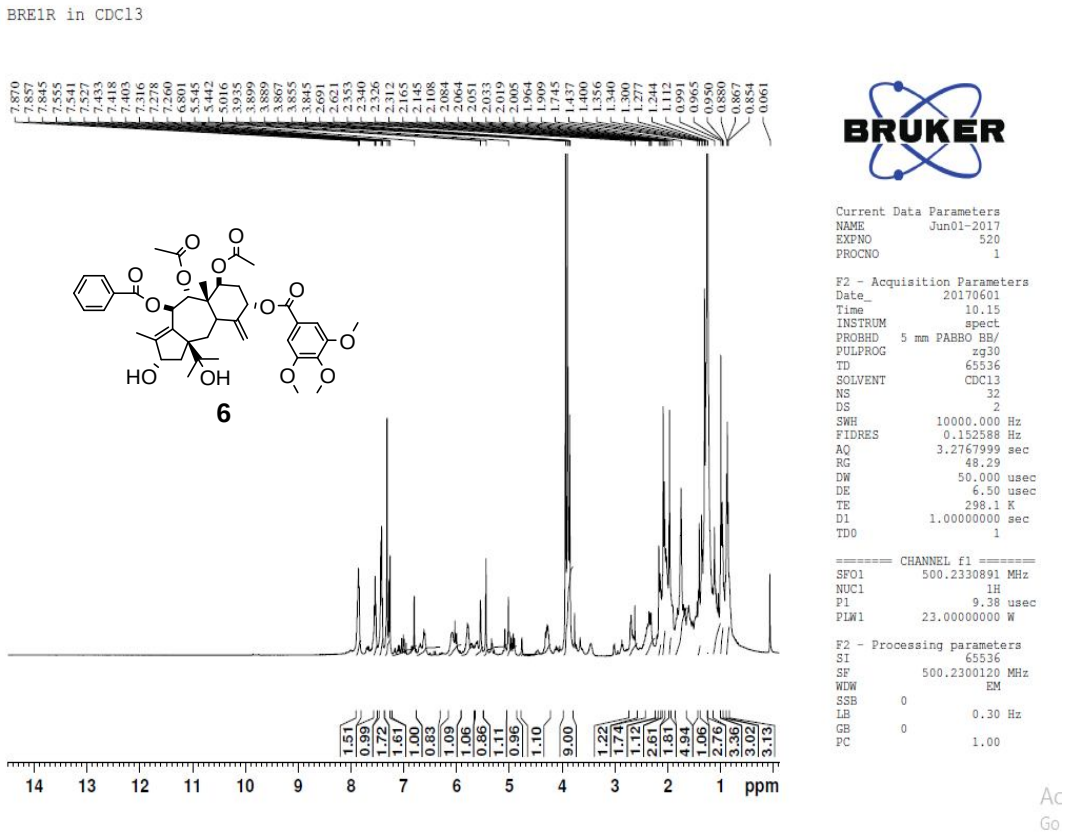
Compound 5: ^{1}H NMR



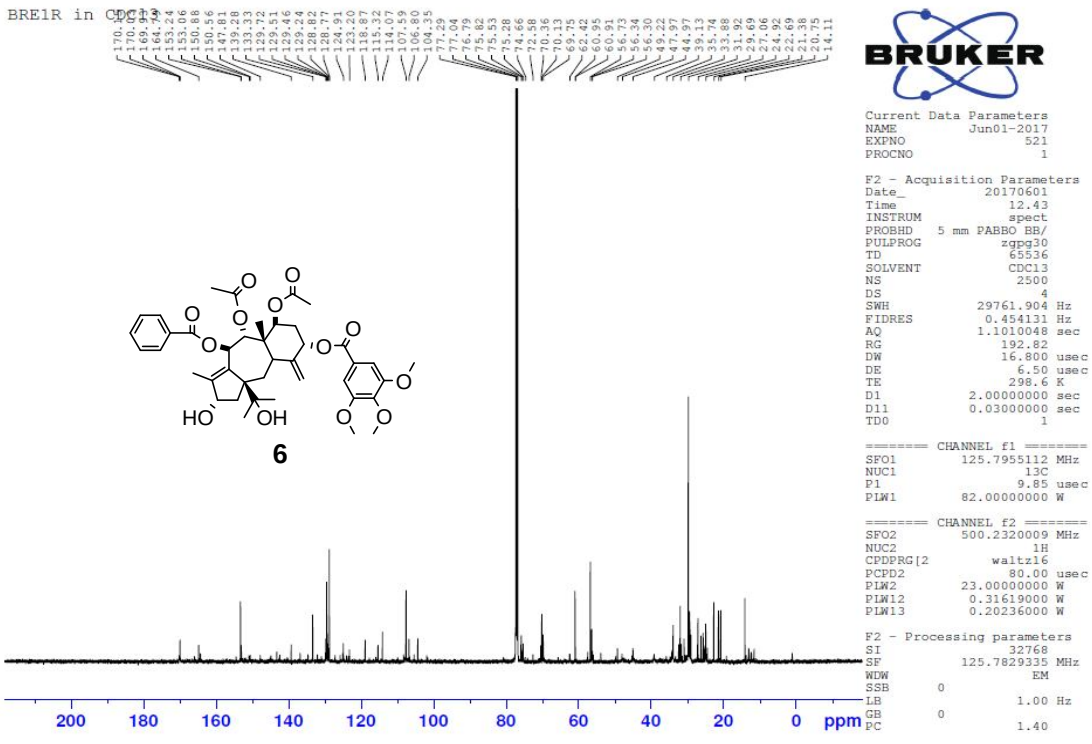
Compound 5: ^{13}C NMR



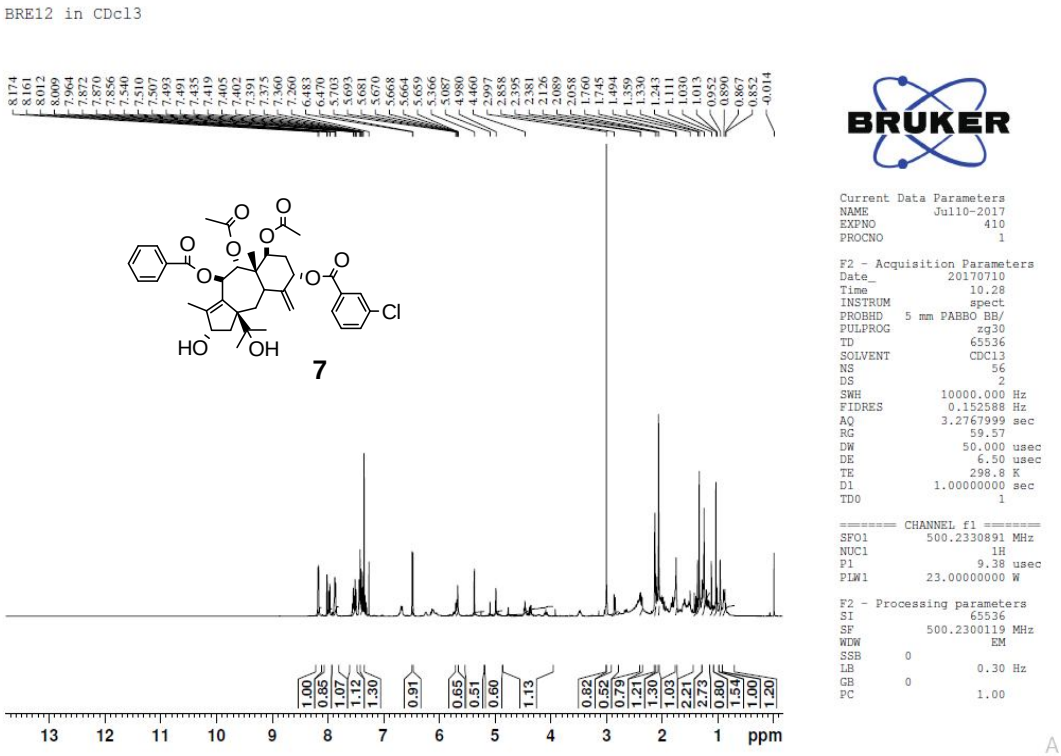
Compound 6: ^{1}H NMR



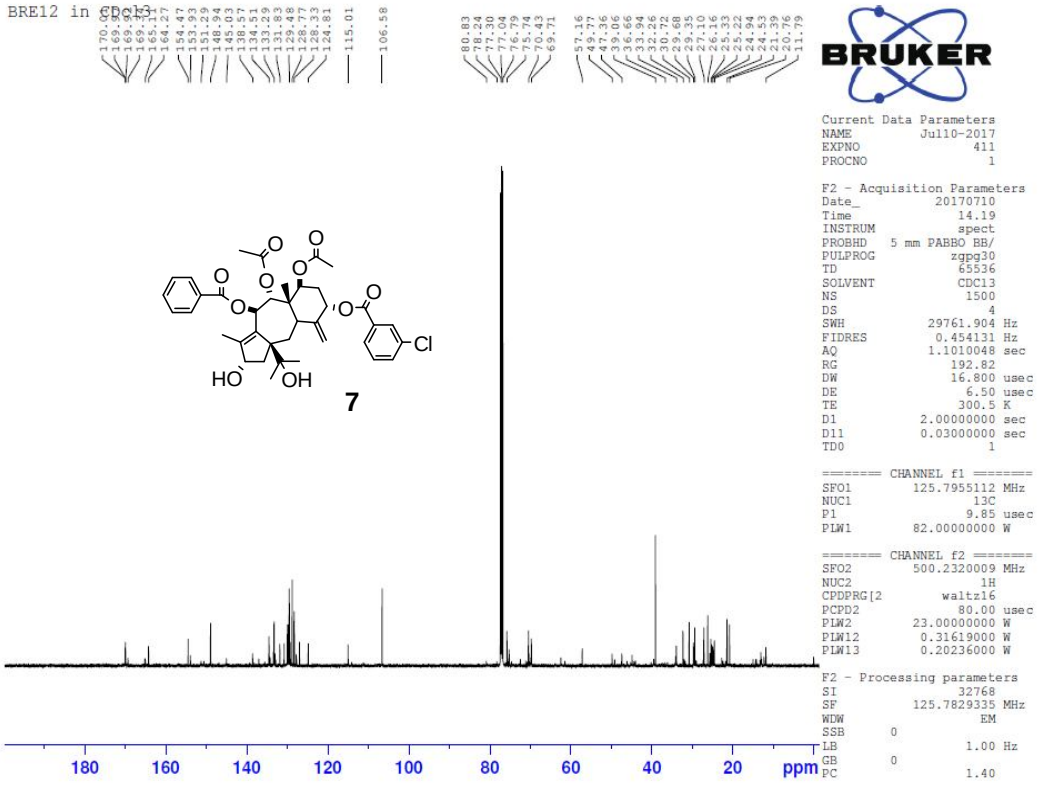
Compound 6: ^{13}C NMR



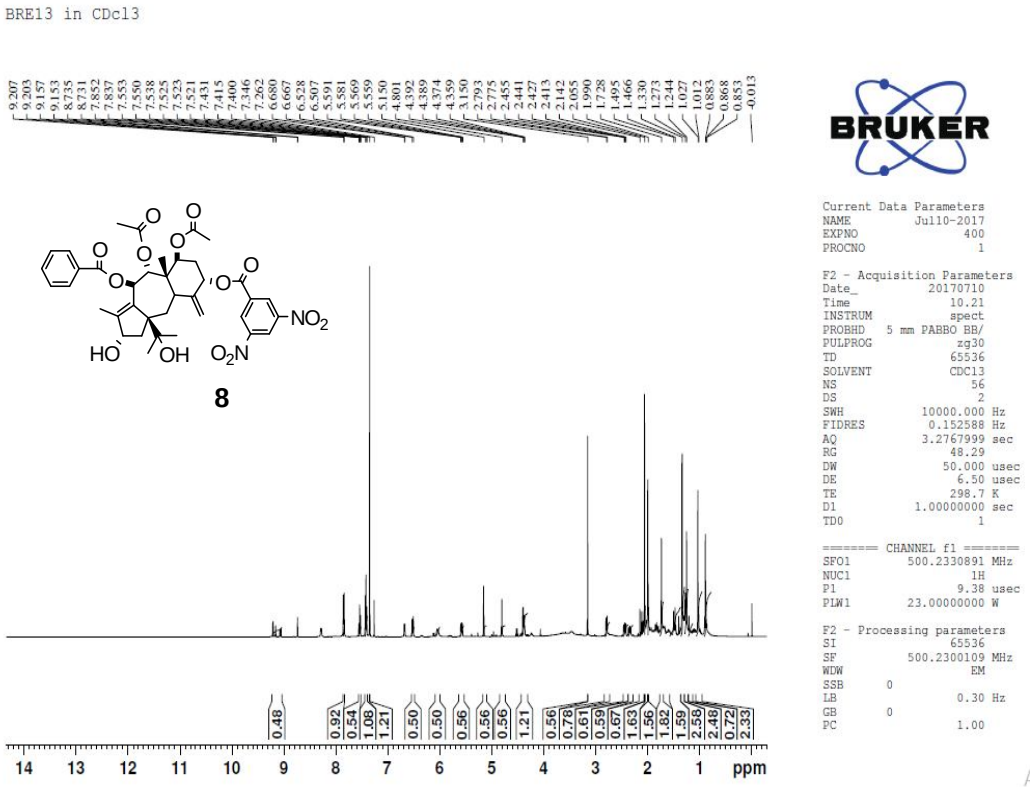
Compound 7: ^{1}H NMR



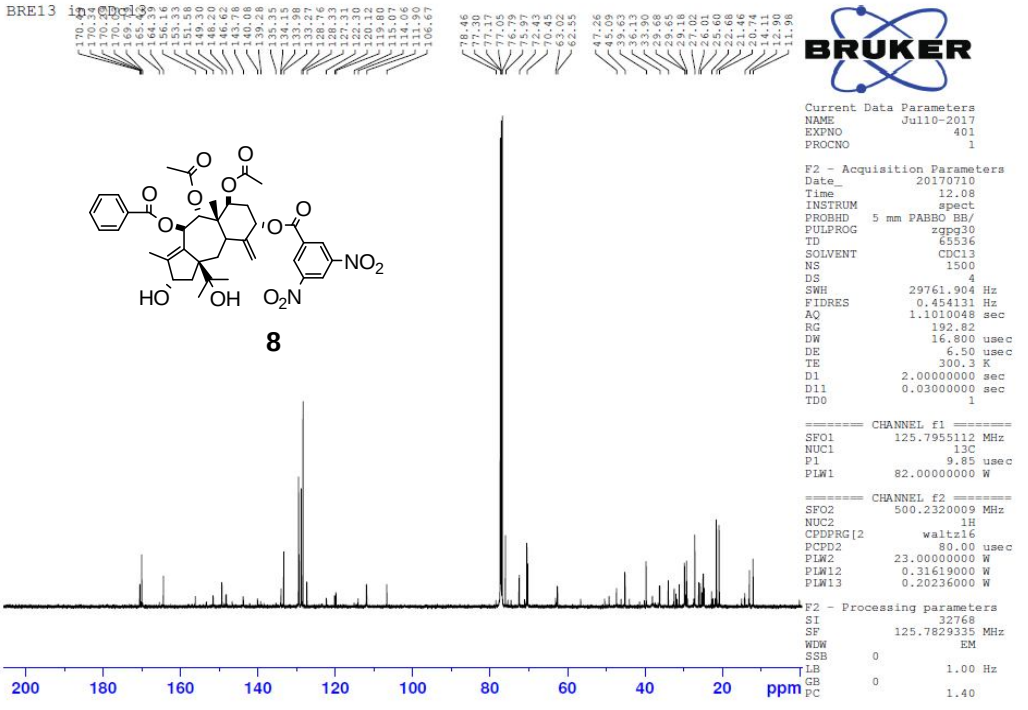
Compound 7: ^{13}C NMR



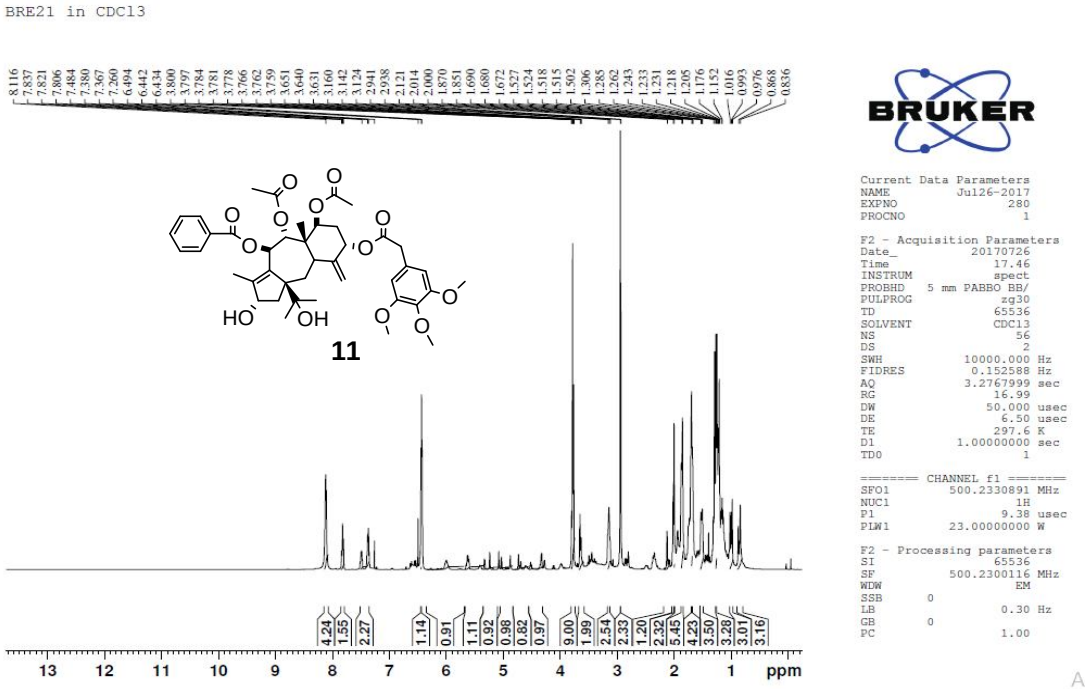
Compound 8: ^{1}H NMR



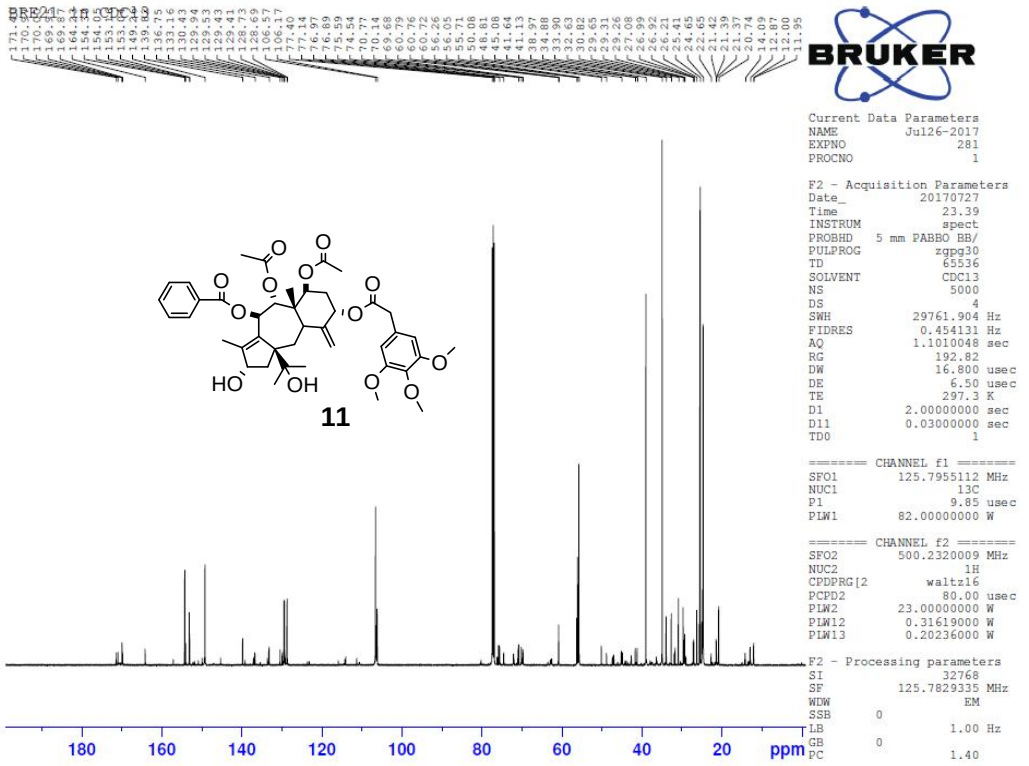
Compound 8: ^{13}C NMR



Compound 11: ^{1}H NMR



Compound 11: ^{13}C NMR



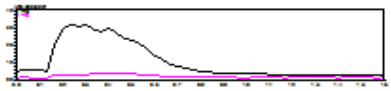
Compound 5: Electrospray mass for $C_{41}H_{38}O_{11}$ (MeOH) : 713 $[M-CH_2CO)+K]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

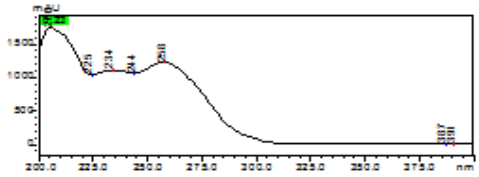
Sample Code : ASN_BRE11
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

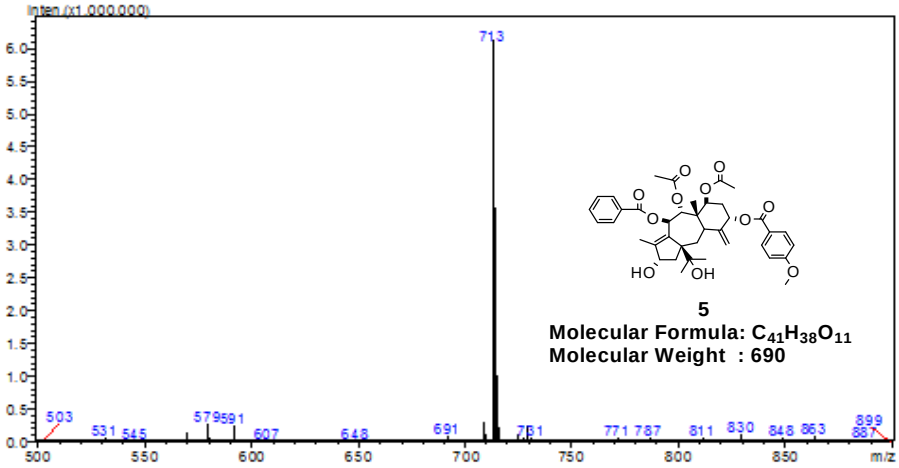
Mass chromatogram



UV-Spectra

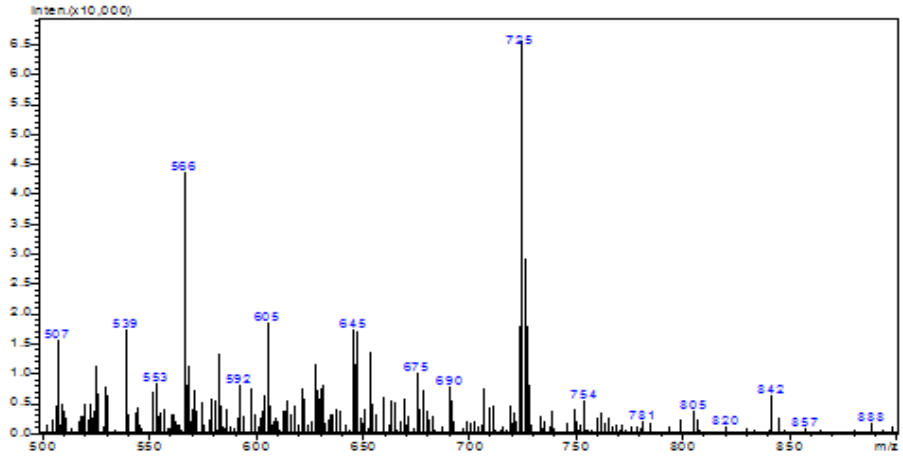


ESI+



5
Molecular Formula: $C_{41}H_{38}O_{11}$
Molecular Weight : 690

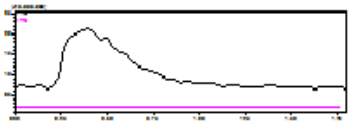
ESI-



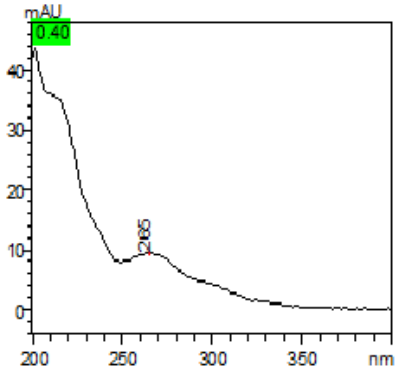
Compound 6: Electrospray mass for $C_{41}H_{50}O_{13}$ (MeOH): 773 $[M+Na]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule
Sample Code : ASN_BRE1R
Name of the Scientist : Dr. A.S.NEGI
Project Code: MLP-02
Mass Range: 500-900

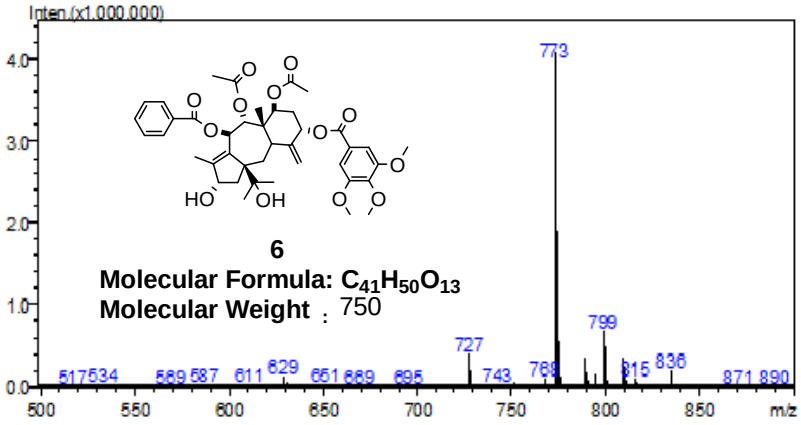
Mass chromatogram



UV-Spectra

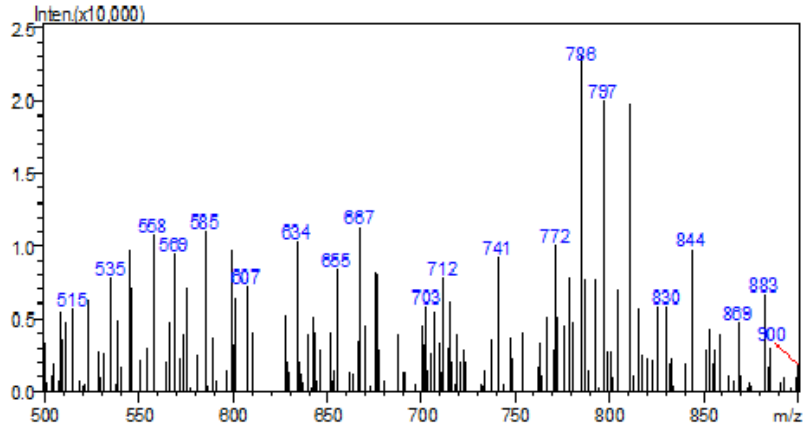


ESI+



6
Molecular Formula: $C_{41}H_{50}O_{13}$
Molecular Weight : 750

ESI-



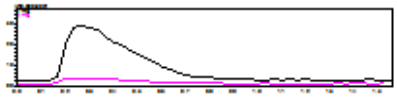
Compound 7: Electrospray mass for $C_{38}H_{43}O_{10}Cl$ (MeOH): 717 $[M+Na]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

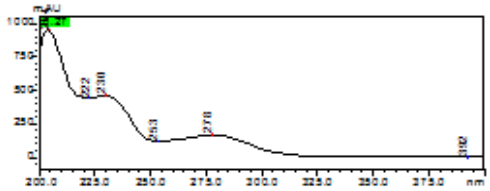
Sample Code : ASN_BRE12
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

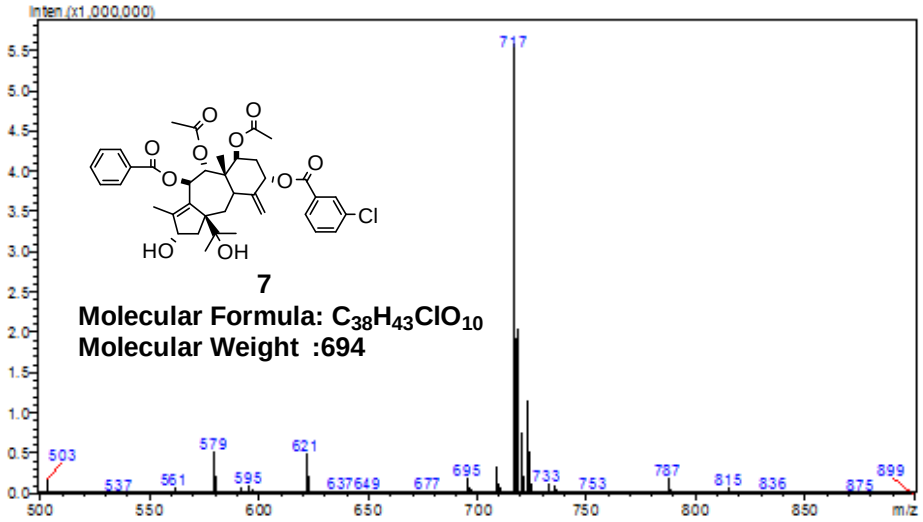
Mass chromatogram



UV-Spectra



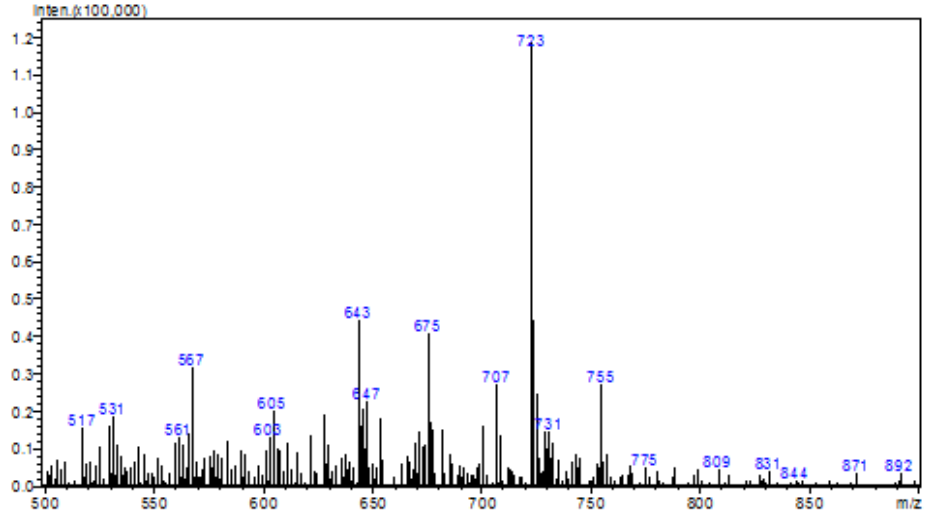
ESI+



7

Molecular Formula: $C_{38}H_{43}ClO_{10}$
Molecular Weight :694

ESI-



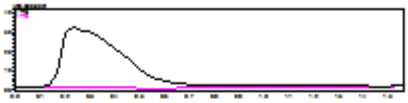
Compound 8: Electrospray mass for $C_{38}H_{42}N_2O_{14}$ (MeOH): 769 $[M+H_3O]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

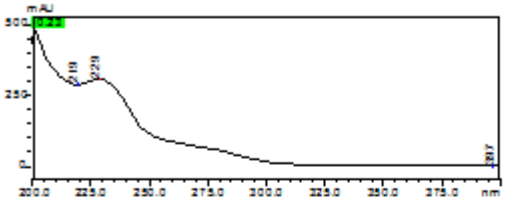
Sample Code : ASN_BRE13
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

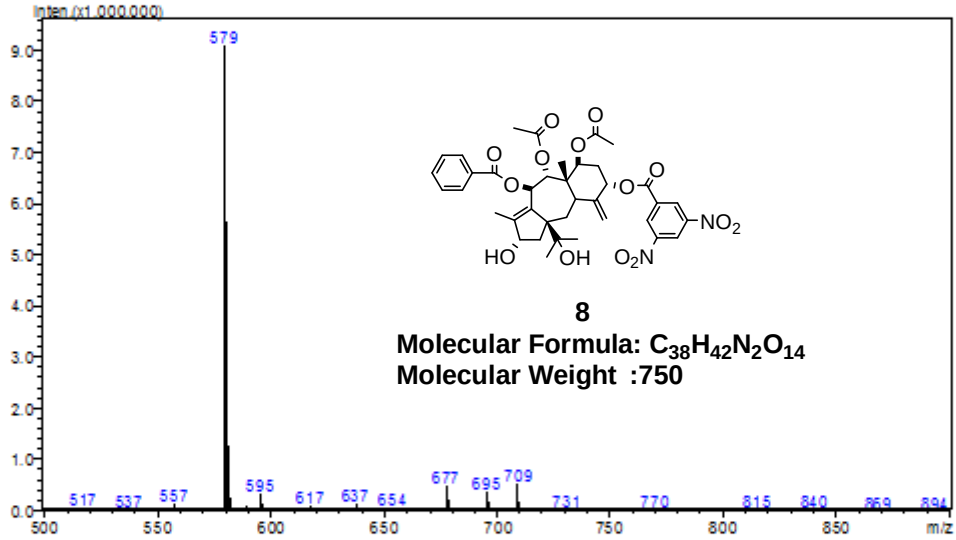
Mass chromatogram



UV-Spectra



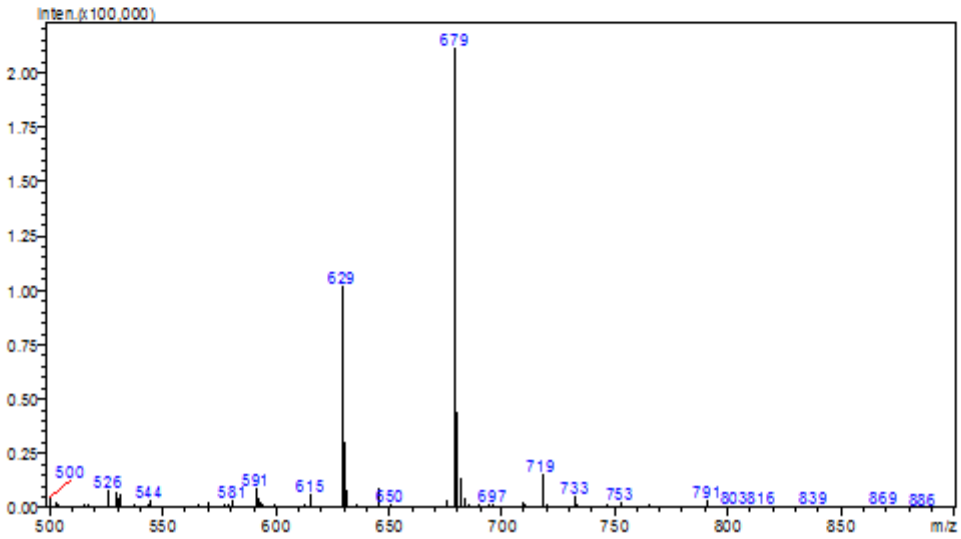
ESI+



8

Molecular Formula: $C_{38}H_{42}N_2O_{14}$
Molecular Weight :750

ESI-



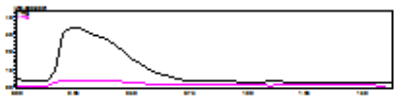
Compound 11: Electrospray mass $C_{42}H_{52}O_{13}$ (MeOH) : 787 $[M+Na]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

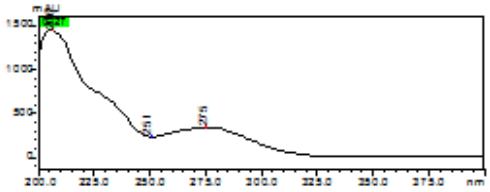
Sample Code : ASN_BRE21
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 500-900

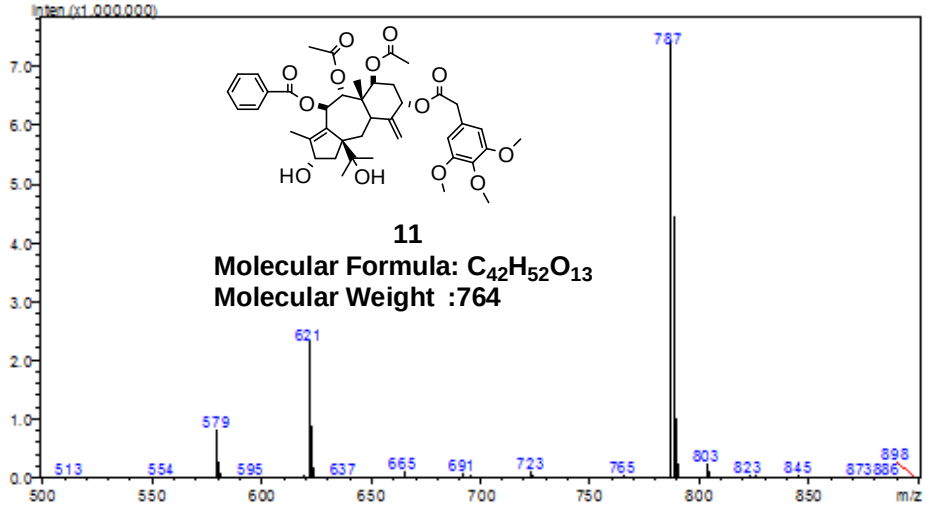
Mass chromatogram



UV-Spectra

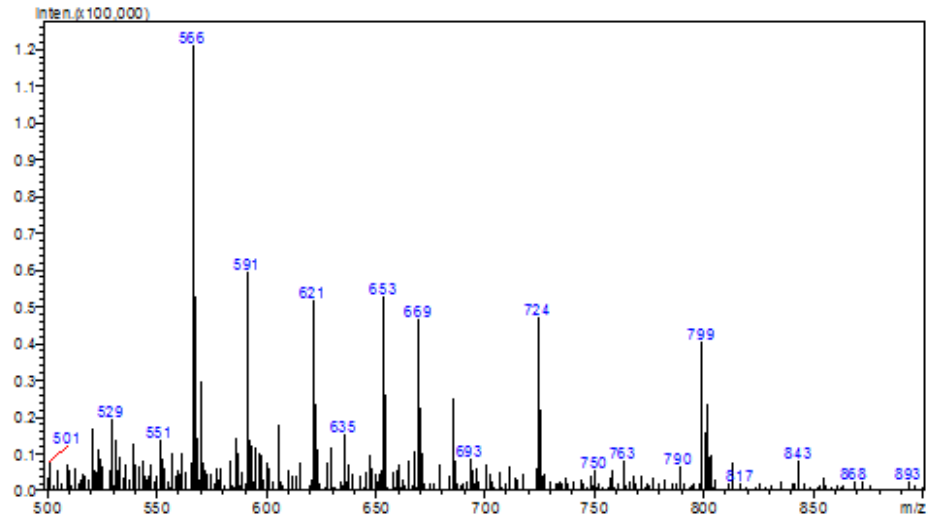


ESI+



11
Molecular Formula: $C_{42}H_{52}O_{13}$
Molecular Weight :764

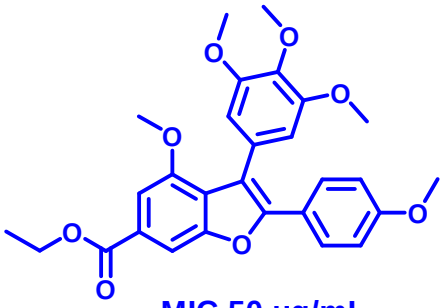
ESI-



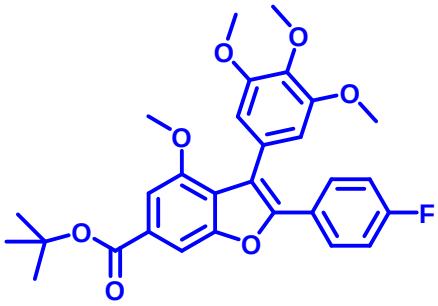
Chapter-3A

2, 3-Diaryl benzofuran as an anti-tubercular agent.

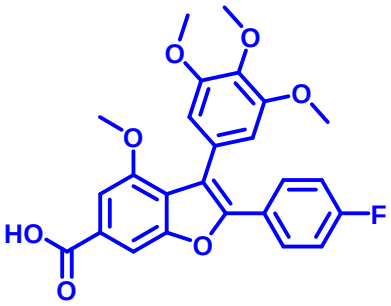
Active derivatives on antituberculosis



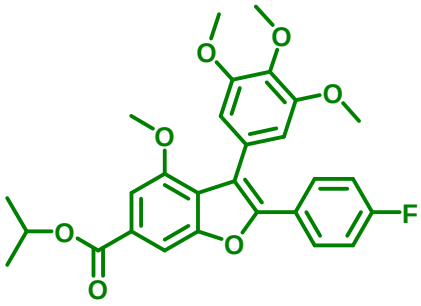
8C MIC 50 µg/mL



11E MIC 50 µg/mL



10 MIC 50 µg/mL



11C MIC 12.5 µg/mL

4.1 Introduction

Tuberculosis (TB) is a bacterial disease caused by the infection of *Mycobacterium tuberculosis* (Mtb) affecting more for male, mainly lungs. Worldwide, in the year 2019, 1.4 million people were died from TB and 3 million people were not diagnosed or not reported officially to national authorities. In 2018 1.5 million people were died from tuberculosis including 0.3 million people also infected with human immunodeficiency virus disease. Almost 66% of world cases were in India, Indonesia, and China, etc. To handle the TB cases through the world (developing countries), it needed at least $ 10.4 billion in the year of 2018. For this further research grants are needed to develop new drugs and development of new tools purpose at least an extra 1.3 billion per year. It has been noticed that the consumption of any generation drugs from long duration that the bacteria has developed to resistant to the drugs. The bacteria resistance developed is divided into mainly three types 1). Multi-Drug-Resistance (MDR), resistance to the first-line drugs isoniazid and rifampicin. 2) Extensive-Drug-Resistance (XDR), resistance to any fluoroquinolones, at least one of second-line injects able drugs in addition to multidrug resistance. 3) Total-Drug-Resistance (TDR), resistance to all first-line and second-line drugs.

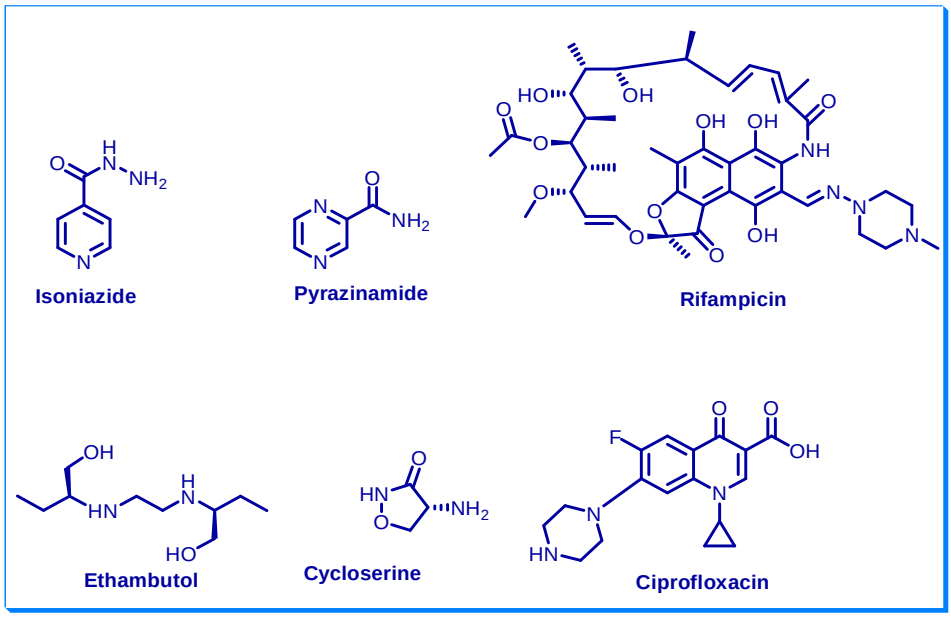


Figure (1): Clinical antitubercular drugs

The total drug resistance term used in only India, Iran and Italy. To handle the situation, two programs were started worldwide such as 'Directly Observed Therapy-Short course' (DOTS) and DOTS-plus programs. But, day by day bacterial resistance is on increasing. To overcome the situation in future, there is an urgent need for combination usage of existing drugs and explore for new antitubercular drugs which can provide better activity with shorten the duration [1-2].

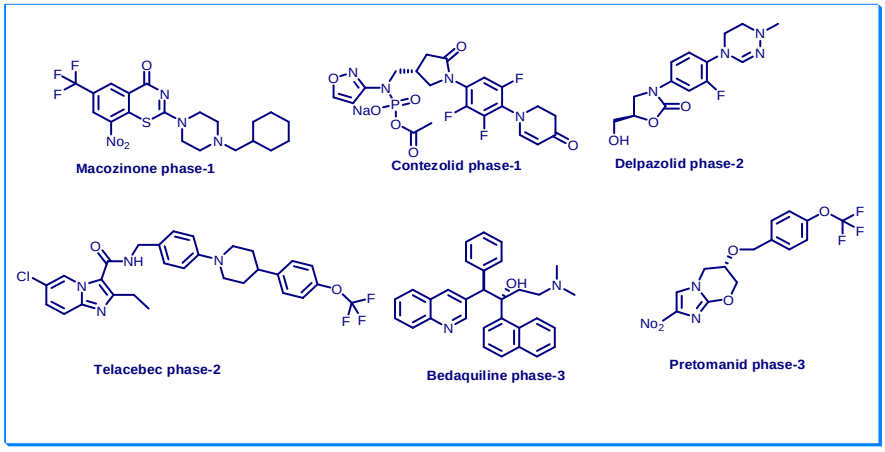


Figure (2): Antitubercular drugs in the pipeline under different phases of clinical trials.

4.1.1 Importance of natural products in tuberculosis

Natural products have complex caged structures and excellent biological activity that to high selectivity. Natural molecule plays a major role in providing unique structural molecule with promising activity, these natural molecules have given an excellent scope to generate the lead molecules. Almost 50% of the food and drug administration approved drugs were either the natural product or natural product derivatives [3, 4, 5]. Natural chemical entities play a major role and give the scope to the chemists to compose their chemical reaction to do total synthesis, semi-synthesis and their derivatization. Most of the natural molecules, which were significantly active, were generally present in plants in very small percentage. Natural molecules are divided into many classes depending on their structures. Here eight classes of compounds were screened on tuberculosis to find out which class of compounds were best suitable to anti-tuberculosis disease. Among them, only flavonoids and terpenoids classes of compounds were more effective against tuberculosis.

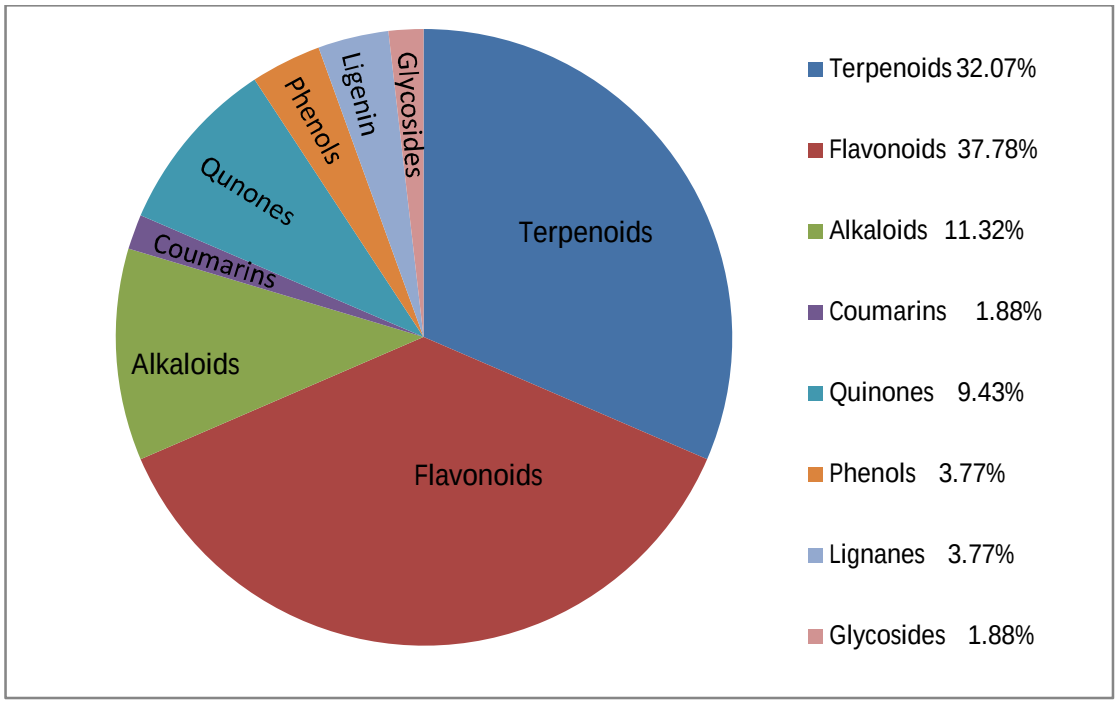


Figure (**3**): Different class of natural molecules and their contribution towards anti-tuberculosis [6].

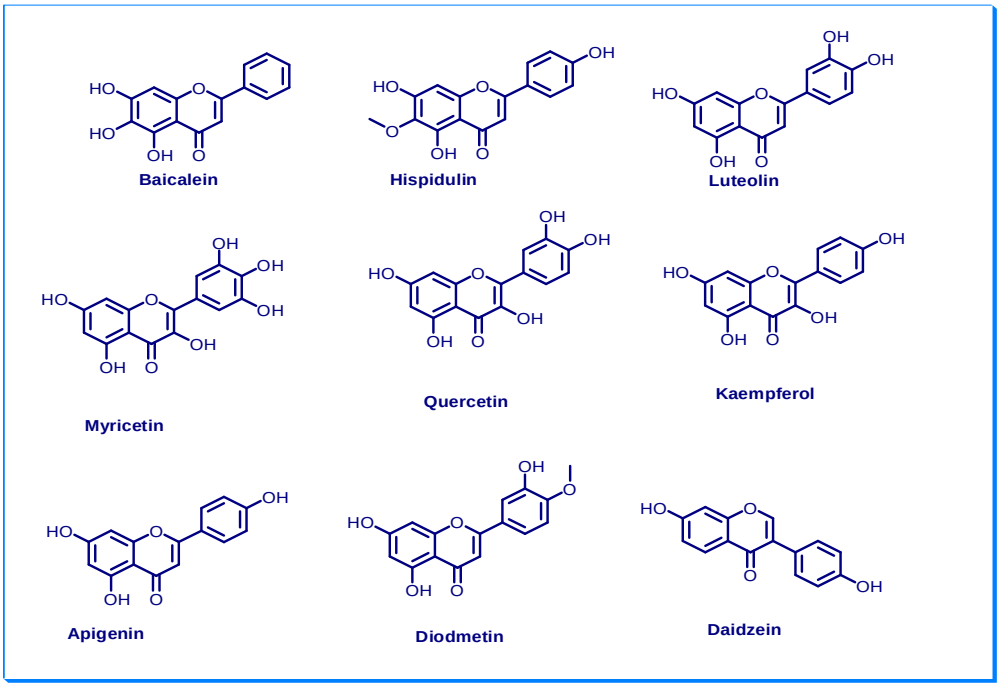


Figure **(**4**).** Structure of some antitubercular flavonoids

4.1.2 Basis of hypothesis

Benzofuran is a versatile pharmacophore, which exhibits significant activity as anticancer [6, 7] anti-tubercular [8, 9] and anti-bacterial agents [10]. There are few benzofuran cores containing the drugs in clinical trials. So benzofuran, as well as flavonoids class of natural new chemical entities, grab the major position in the new drug development. In this regard, we have designed the molecules which contain the core of benzofuran and flavonoids or aromatic rings that can give a few drug candidates towards the anticancer and anti-tubercular activity. Delightfully designed pharmacophore showed activity on anticancer and significant activity on anti-tuberculosis activity.

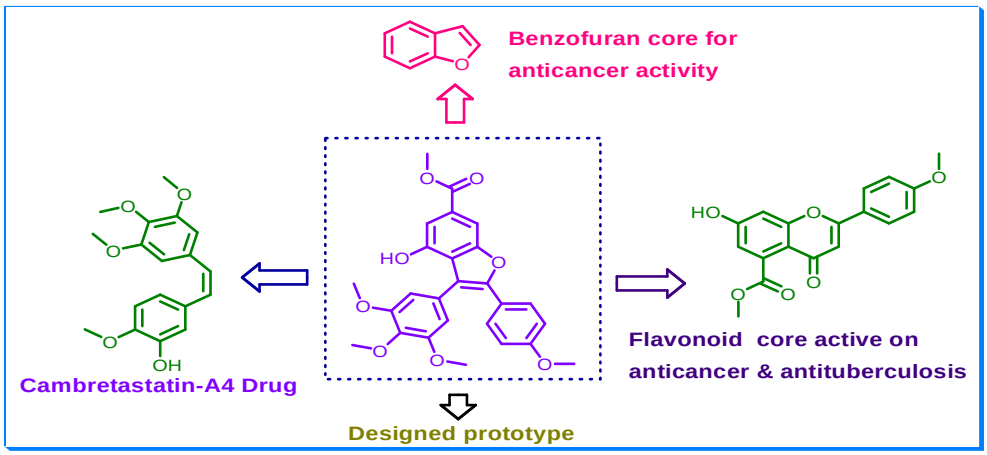


Figure (5): Basis of designing of prototype

Hence, as a part of our drug discovery program for anti-tubercular agents [11-16], design and synthesis of three 2,3-di aryl benzofuran prototypes Figure (6) was carried out and all the derivatives were *in-vitro* evaluated for their antitubercular potential against Mtb H37Ra strain. Further, *in-silico* and cytotoxicity studies were also carried out for the most active derivatives.

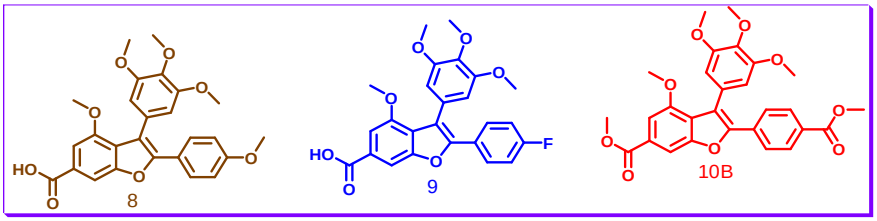


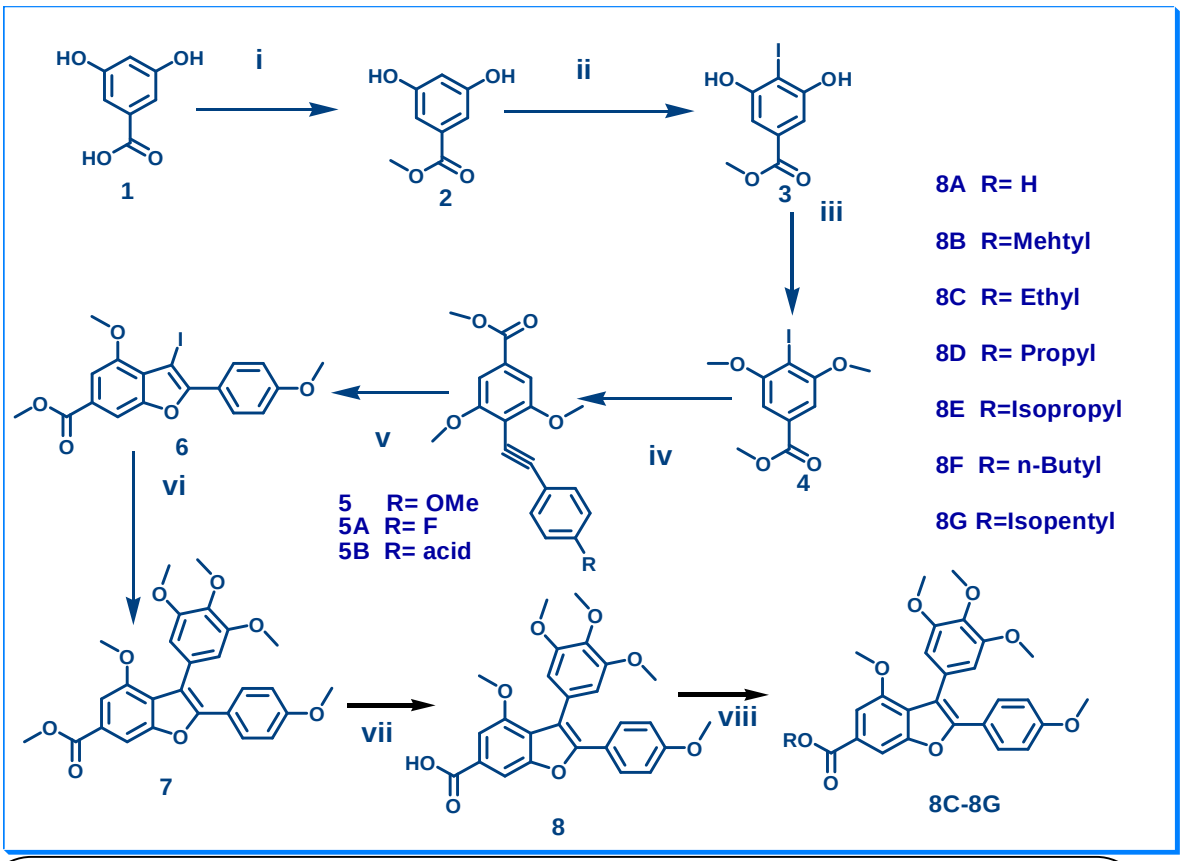
Figure (**6**): Designed prototypes

4.2 Result and discussion

4.2.1 Synthetic strategy

Scheme-1

In scheme-1, we have started 3, 5-dihydroxy benzoic acid **1** as a starting material**.** Compound **1** was methylated with concentrated sulphuric acid in methanol to get **2**. Iodination of compound **2** was done with N-Iodosuccinamide in methanol to obtained **3**. Both phenolic hydroxyls of **3** methylated with methyl iodide in presence of potassium carbonate in N, N-Dimethylformamide to get compound **4**. Iodo derivative of **4** underwent of sonogashira cross-coupling reaction with 4-methoxy phenyl acetylene to afford coupled product **5**.

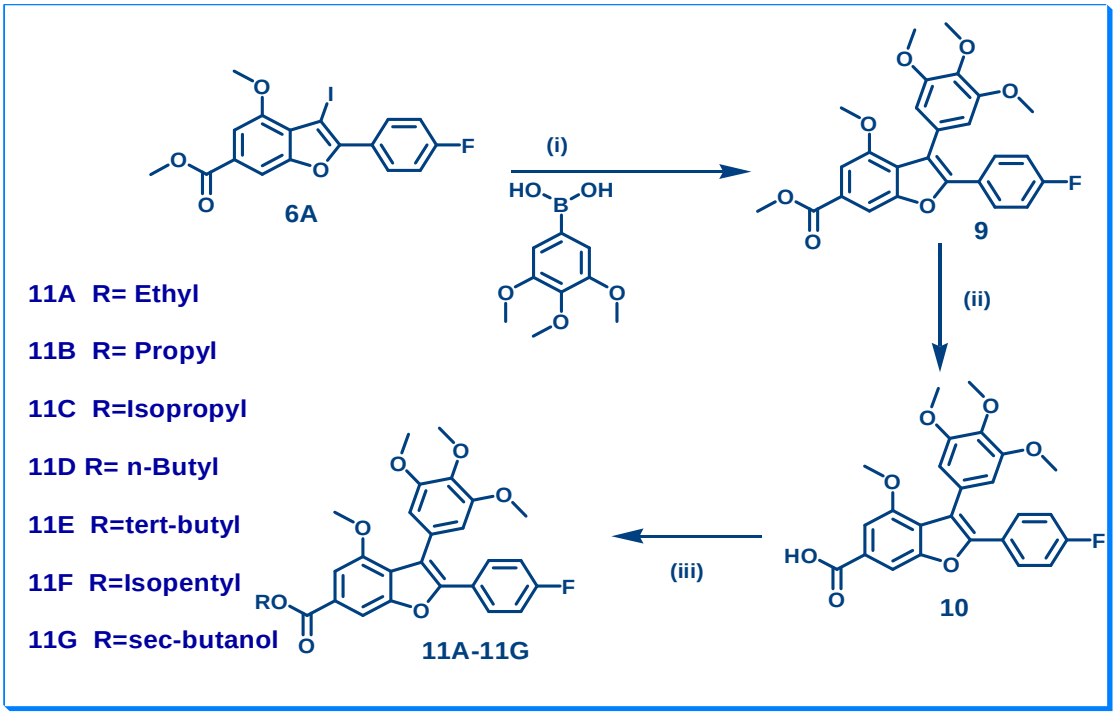


Scheme-1: Reagent and condition: (i) Conc-H_2SO_4, Methanol, temp 80ºC, 4h, 98%; (ii) NIS, Methanol, RT, 16h, 98%: (iii) K_2CO_3, Methyl Iodide, DMF, RT, 15h 98% : (iv) $(Ph_3P)_2PdCl_2$, CuI, Aryl iodide, TEA, 60ºC, 5h, 98%: (v) Iodine, CH_2Cl_2, RT, 23h, 99%: (vi) Trimethoxy phenyl boronic acid, $NaHCO_3$, $(Ph_3P)_2PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 96%: (vii) KOH, MeOH/ H_2O (9:1), 80ºC, 6h, 98%: (viii) ROH, H_2SO_4, 80ºC, 1-2h, 80-90%.

Iodine mediated cyclization of compound **5** was done at room temperature by using molecular iodine as a reagent in DCM to get benzofuran **6**. Compound **6** underwent of suzuki-coupling reaction with 3, 4, 5- trimethoxy phenylboronic acid to get 2, 3-diarylbenzofuran ester derivative **7.** Which was hydrolyzed with 10% KOH in methanol and water (9:1) to get free carboxylic acid **8A**. Five ester derivatives (**8C-8G**) have prepared by using **8A.** All the products were confirmed by spectroscopy data.

Scheme-2

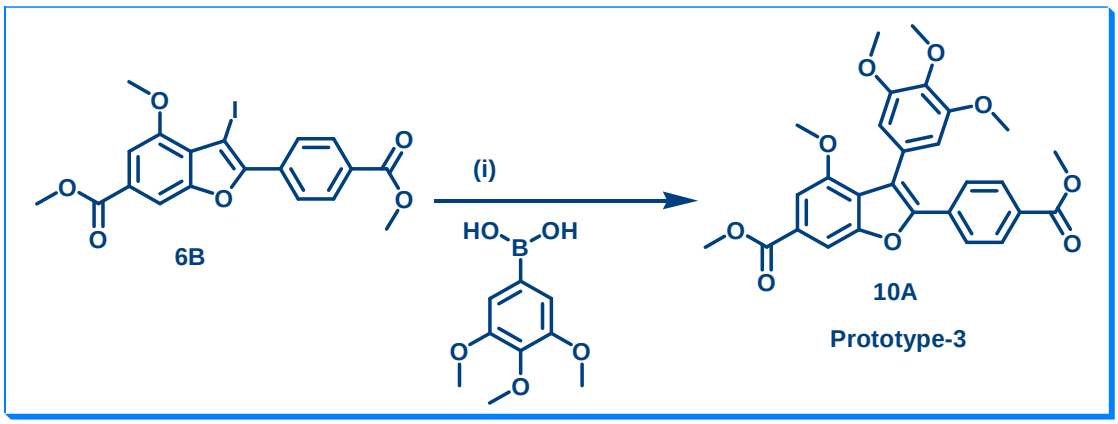
In scheme-2, benzofuran derivative **6** and 3, 4, 5- trimethoxy phenylboronic acid was condensed *via* suzuki cross-coupling reaction to get fluorinated diaryl benzofuran derivatives **9.** Ester group of **9** was saponified with 10% KOH in methanol and water (9:1) to get final pharmacophore **10**. Compound **10** used further derivatization purpose to get derivatives from **11A-11G** and confirmed by spectroscopy.



Scheme-2: Reagent and condition: (i) $NaHCO_3$, $(Ph_3P)_2PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 96%: (ii) KOH,5% in methanol/ water (9:1), 80ºC, 6h, 98%. (iii) Conc-H_2SO_4, methanol, 80ºC, 4h, 98%.

Scheme-3

In scheme-3, benzofuran derivative **6B** underwent suzuki cross-coupling reaction with 3, 4, 5- trimethoxy phenylboronic acid to get compound **10A**.



Scheme-3: Reagent and condition: (i) $NaHCO_3$, $(Ph_3P)_2$ $PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 80%:

4.3 Biological evaluation.

4.3.1 Cytotoxicity evaluation by MTT assay

All the derivatives were evaluated for anticancer activity against a panel of seven human cancer cell lines i.e. A431 (Skin cancer), A498 (Kidney carcinoma), A549 (Lung carcinoma), K-562 (Leukemia), and one normal lung cell line (L-132), NICH-520 (Lung carcinoma), NICH-460 (Lung carcinoma), and PA-1 (Ovarian carcinoma), podophyllotoxin and 5-fluorouracil were used as positive controls [17]. Out of nineteen derivatives seventeen compounds showed anticancer activity IC_{50} <50 µg/mL against the eight human cancer cell lines except compounds 5B and 8D both the compounds were inactive in all these cell lines. Rest all the compounds showed low level of cytotoxicity. So none of the compound was selected for detailed biological study. All the derivatives were also evaluated for their cytotoxicity against the normal lung cell line L-132 by the MTT assay and no toxicity was observed up to 27.4 µg/mL concentration.

Table (1): Cytotoxicity evaluation of brevifoliol derivatives against human cancer cell lines

		A431	A498	A549	K-562	L-132	NCIH-520	NCIH-460	PA-1
	Compound Codes	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
1.	**7**	----	----	----	----	----	----	26.52	29.34
2.	**8A**	----	----	----	----	37.17	42.41	----	----
3.	**8F**	16.81	----	----	----	37.84	28.22	25.22	----
4.	**8D**	----	----	----	----	----	----	----	----
5.	**8G**	39.735	----	----	----	----	----	----	----
6.	**8E**	36.425	----	----	----	----	----	17.44	16.37
7.	**10A**	30.97	----	----	----	----	----	35.30	----
8.	**5A**	34.99	----	----	----	----	----	----	----
9.	**9**	21.645	----	----	----	47.99	----	44.43	----
10	**10**	24.145	----	----	----	----	----	25.60	49.36
11	**11A**	30.11	38.76	----	----	27.40	----	43.18	46.78
12	**11C**	20.975	40.28	----	----	----	38.49	31.80	----
13	**11F**	----	----	----	----	36.38	----	33.73	----
14	**11B**	25.38	----	----	----	40.71	----	44.59	----
15	**11D**	----	40.02	----	----	40.45	21.36	44.01	----
16	**11G**	----	38.43	----	----	30.42	32.85	----	----
17	**11E**	----	21.88	----	----	41.52	28.82	----	43.97

18	**5**	----	----	----	----	----	44.64	----	----
19	**5B**	----	----	----	----	----	----	----	----
20	**Podophyllotoxin**	4.53	28.68	29.61	29.825	33.10	31.13	29.36	11.16
21.	**2-Fluorouracil**	5.305	19.85	31.57	42.965	30.58	38.21	36.02	15.27

IC_{50} >50 µg/mL was considered inactive.

4.3.2 Evaluation of anti-tubercular activity

All the 2, 3-diaryl benzofuran were evaluated for *in vitro*-antitubercular activity, and activity expressed in the form of minimal inhibitory concentration (MICs). Anti-tubercular first line drug INH was used as a standard drug against Mtb H37Ra by Agar dilution assay. The (MIC) is shown in Table (2).

Table (2): Data shown in the column (active at µg/mL) are the MICs of the compounds (active ones marked Bold). Compounds were tested against a virulent strain of Mtb, H37Ra (ATCC 25177).

S. No.	Compound Codes	MIC	Test Concentrations (µg/mL)					
		Active at (µg/mL)	100	50	25	12.5	6.25	3.125
1.	**8A**	Not Active	++	++	++	++	++	++
2.	**8B**	Not Active	++	++	++	++	++	++
3.	**8C**	**50**	--	--	++	++	++	++
4.	**8D**	Not Active	++	++	++	++	++	++
5.	**8E**	Not Active	++	++	++	++	++	++
6.	**8F**	Not Active	++	++	++	++	++	++
7.	**8G**	Not Active	++	++	++	++	++	++
8.	**9**	Not Active	++	++	++	++	++	++
9.	**10**	50	--	--	++	++	++	++
10.	**11A**	Not Active	++	++	++	++	++	++

11.	**11B**	Not Active	++	++	++	++	++	++
12.	**11C**	**12.5**	--	--	--	--	++	++
13.	**11D**	Not Active	++	++	++	++	++	++
14.	**11E**	**50**	--	--	++	++	++	++
15.	**11F**	Not Active	++	++	++	++	++	++
16.	**11G**	Not Active	++	++	++	++	++	++
17.	10A	Not Active	++	++	++	++	++	++
	Std Drug INH	-- (0.025)						
	No drug Control	++++++						

Notes: Growth of Bacilli ++, Growth inhibition –

We have designed the prototypes by keeping the basic benzofuran core and keeping diverse substitution at para position like methoxy and ester at the 2-aryl position only. After synthesizing the final prototypes we kept modified benzofuran core with acid group also.

4.3.3 Molecular docking and mode of action study of derivatives on identified Mtb targets

Mycolic acid is one of the most essential components for Mtb virulence and drug resistance. Till now more than 20 enzymes have been discovered which are involved in Mtb mycolic acid synthesis. These are long chain fatty acids found abundantly in Mtb cell walls which provide high resistance to the bacteria against several antibiotics. Polyketide synthase 13 is one of the essential enzymes involved in advance phase of mycolic acid synthesis in Mtb. Polyketide synthase 13 that exhibits five catalytic domains *viz.*, one acyl transferase domain, two acyl carrier domain, one β-keto acyl-synthase domain and the C-terminal thioester (TE) domain that collectively contribute to the mycolic acid synthesis process. Aggarwal *et al.* (2017) TAM16 is a recently discovered benzofuran class based highly potent PK13 inhibitor named TAM1. TAM1 has found to bind at TE domain of PK13 and block the availability of its catalytic center for substrate. Therefore, here we assessed the docking binding affinity of studied benzofuran derivatives *viz.*, **8C, 10, 11C** and **11E**

against the target PK13. Additionally, three target catalase-peroxidase, dihydrofolate reductase and enoyl-ACP reductase were prioritized for docking binding studies based on the results of target identification tool STITCH Figure (**8**). The results of docking binding affinity of studied derivatives are compiled in Table (3) and Table (4).

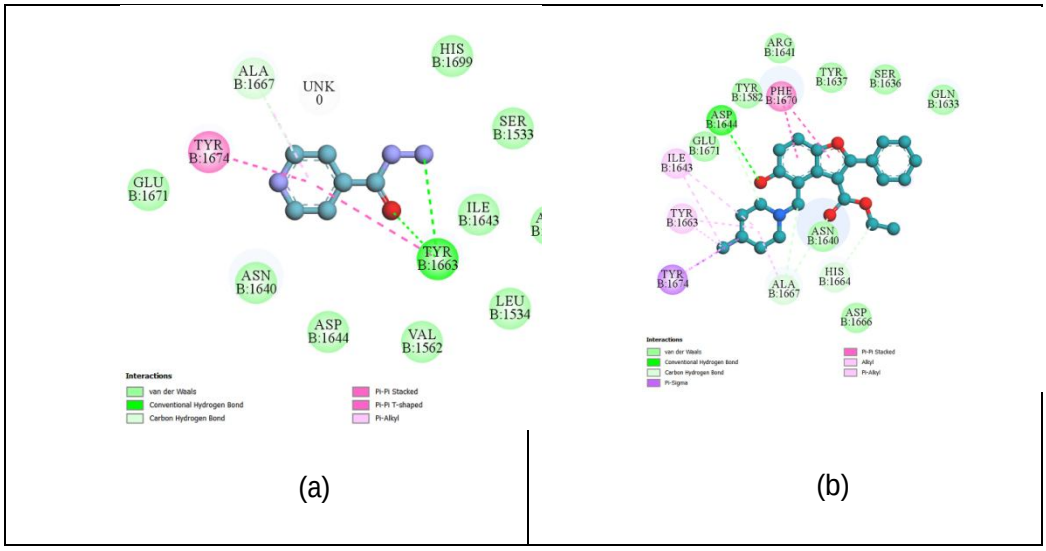
Table (3): Compliance of molecular docking energy kcal/mol results of derivatives 8C, 10, 11C and 11E against Mtb target PK13

Name	**Docking binding energy (kcal/mol)**	**PK13 (PDB: 5V3X) binding pocket residues within 4Å radius**	**Key amino acid and H-bond length (Å)**
ISONIAZID (positive control)	-6.2	SER-1533, LEU-1534, VAL-1562, ASN-1640, ILE-1643, ASP-1644, TYR-1663, ALA-1667, TYR-1674, HIS-1699	TYR-1663 (2.90)
TAM1 (PK13 co-crystallized ligand)	-9.6	TYR-1582, GLN-1633, SER-1636, TYR-1637, ASN-1640, ARG-1642, ILE-1643, ASP-1644, TYR-1663, HIS-1664, ASP-1666, ALA-1667, PHE-1670, GLU-1671, TYR-1674	**ASP-1644 (2.55)**
8C	-8.3	ALA-1477, TRP-1532, SER-1533, TYR-1582, ASN-1640, ILE-1643, ASP-1644, TYR-1663, HIS-1664, ASP-1666, ALA-1667, PHE-1670, GLU-1671, HIS-1699, ILE-1700	**SER-1533 (3.07)**
10	-8.6	ALA-1477, TRP-1532, SER-1533, ASN-1640, ILE-1643, ASP-1644, TYR-1663, HIS-1664, PHE-1670, GLU-1671, HIS-1699, ILE-1700	**SER-1533 (3.06)**
11C	-8.7	ALA-1477, TRP-1579, TYR-1582, VAL-1611, GLN-1633, TYR-1637, ASN-1640, HIS-1664, ASP-1666, ALA-1667, PHE-1670, GLU-1671, TYR-1674, ILE-1700	**GLN-1633 (2.92)**
11E	-8.8	ALA-1477, TRP-1579, TYR-1582, GLN-1633, TYR-1637, ASN-1640, HIS-1664, ASP-1666, ALA-1667, PHE-1670, GLU-1671, TYR-1674, ILE-1700	**GLN-1633 (3.03)**

Analysis of docking results against target PK13 indicate, the studied derivatives viz., **8C**, **10**, **11C** and **11E** showed good binding affinity in comparison to control drug isoniazid. Derivatives **11C** and **11E** showed better binding affinity of -8.7 kcal/mol and -8.8 kcal/mol than derivative **8C** and **10** with binding energy -8.3 kcal/mol and -8.6 kcal/mol respectively. However, the redocking study of TAM1 the known PK13 inhibitor displayed highest binding affinity of -9.6 kcal/mol Table (3). Further, the ligand-receptor interaction analysis revealed all derivatives bind well at hydrophobic pocket of inhibitor TAM1 binding site of PK13. Interaction results displayed derivatives **8C** and **10** form single hydrogen bond with amino acid residue SER-1533 and oxygen atom of trimethoxy phenyl fragment of length 3.07 Å and 3.06 Å respectively.

However they also indicated an unfavorable acceptor-acceptor interaction with amino acid HIS-1699 and trimethoxy phenyl moiety of **8C** and **10** which is highlighted with red color in Figure (7). On the other hand derivatives **11C** and **11E** are making hydrogen bond with carbonyl oxygen present at benzofuran moiety and amino acid residue GLN-1633. While, no unfavorable interaction was observed in the case of **11C** and **11E** derivatives. All derivatives also showed number of non-covalent interactions such as π-π T-shaped, π-π stacked, π-σ, π-alkyl, halogen interaction with aromatic rings of compounds Figure (**7**). Figure (**7**) illustrate aromatic rings of benzofuran moiety of derivatives **8C** and **10** make π-π T-shaped interaction with aromatic ring of PK13 binding site amino acid PHE1670. Whereas derivatives **11C** and **11E** are making π-π stacked based interaction with amino acid PHE-1670. Derivatives **11C** and **11E** make hydrogen bond (green dotted line) of length 2.9 Å and 3.0 Å with amino acid GLN-1633. They also showed halogen-based interaction with fluoride group and binding site amino acid ALA-1667.

The supplemental results of docking study of derivatives **8C**, **10, 11C** and **11E** against other predicted targets viz., KatG, FolA and inhA indicate all derivatives show good binding affinity in comparison to FDA approved drug isoniazid. Analysis of ligand-target interactions showed derivatives make number of interactions with hydrophobic binding sites of targets KatG, FolA and inhA.



(a) (b)

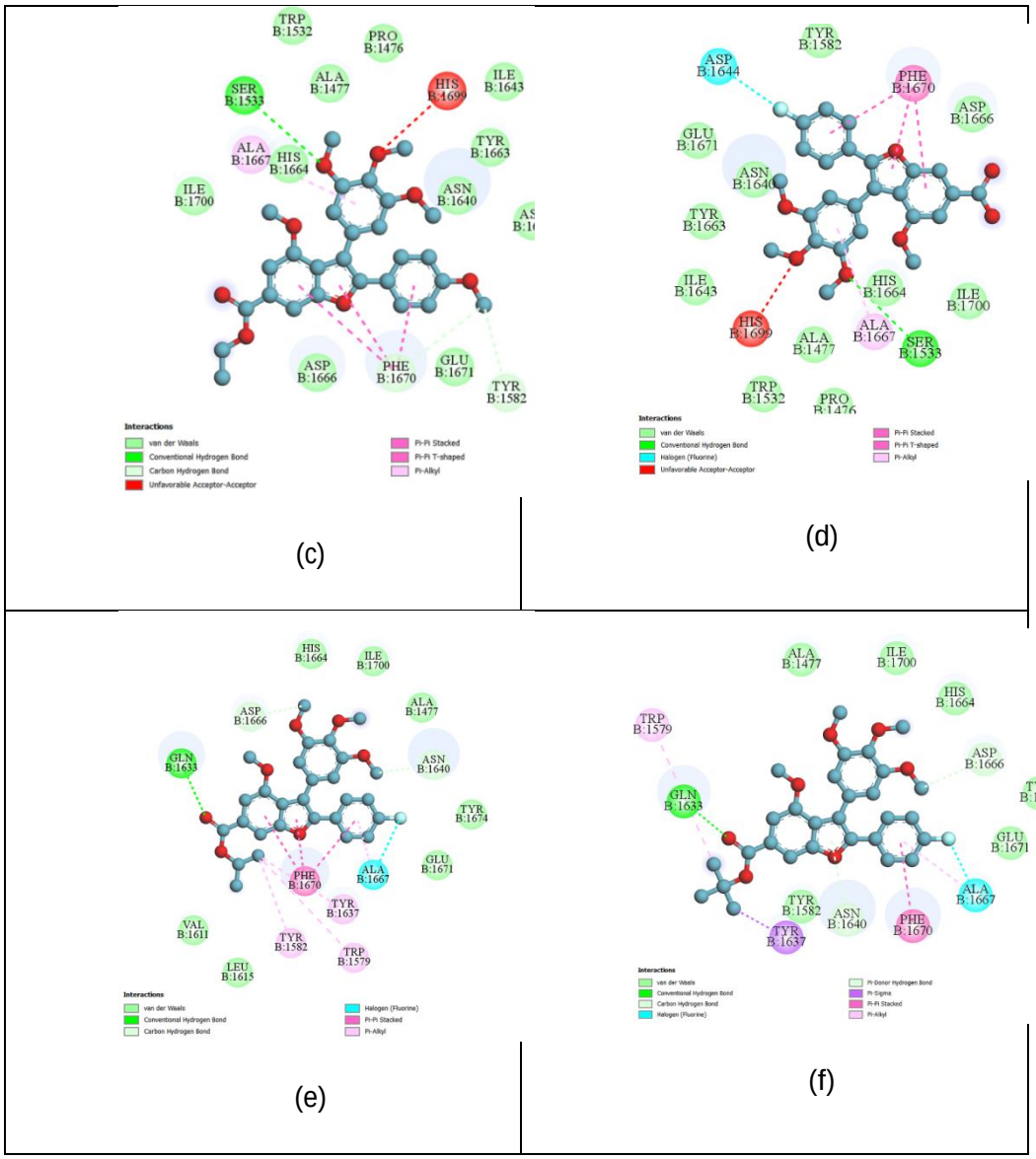


Figure (7): A 2D representation of binding mode of positive control Mtb drug Isoniazid and PK13 co-crystallized benzofuran based inhibitor named TAM1 and studied novel derivatives **8C**, **10**, **11C** and **11E**. (a) propose binding pose of Isoniazid on PK13 binding site with docking binding energy -6.2 kcal/mol, (b) re-docking study of co-crystallized ligand TAM1 on PK13 TE domain (benzofuran based inhibitor), (c) binding pose of derivative **8C** on PK13 binding site with docking binding energy -8.3 kcal/mol, (d) binding pose of derivative **10** on PK13 binding site with docking binding energy -8.6 kcal/mol, (e) binding pose of derivative **11C** on PK13 binding site with docking binding energy -8.7 kcal/mol and (f) binding pose of derivative **11E** on PK13 binding site with docking binding energy -8.8 kcal/mol.

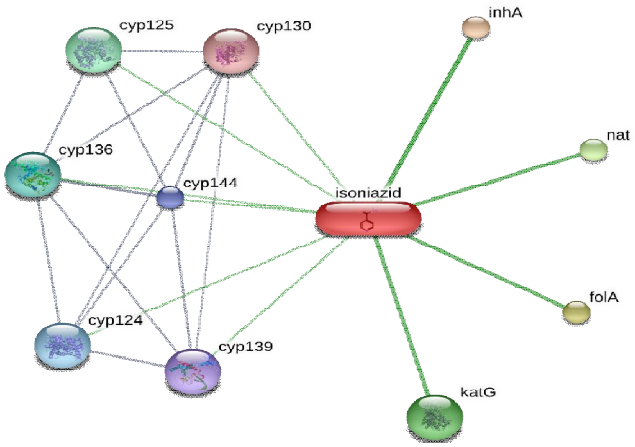


Figure (8): Mtb target prediction for positive control isoniazid through web-based tool STITCH v5.0 (http://stitch.embl.de). inhA, nat folA and katG stands for enoyl-ACP reductase, arylamine V-acetyltransferase, dihydrofolate reductase and catalase peroxidase respectively.

Table (4): Compliance of docking binding energy (kcal/mol) results of derivatives **8C, 10, 11C** and **11E** on predicted Mtb targets catalase peroxidase, dihydrofolate reductase and enoyl-ACP reductase.

Compound Codes	Catalase-peroxidase (PDB: 1SJ2)	Dihydrofolate reductase (PDB: 4M2X)	Enoyl ACP reductase (PDB: 4TRO)
ISONIAZID (positive control)	-6.0	-5.6	-5.9
8C	-7.8	-8.5	-8.0
10	-8.5	-8.5	-8.6
11C	-9.7	-8.5	-8.8
11E	-8.9	-9.0	-8.9

4.3.4 Pharmacokinetic and toxicity assessment

The toxicity predictions results related to female mouse and rat, Ames mutagenicity, indicate active derivative **11C** is non-carcinogenic and non-mutagenic in nature. However, other derivatives *viz.*, **8C**, **11E** and **10** may show hepatotoxicity and developmental toxicity when prolong used. All studied derivatives showed less

oral lethality in rat (LD_{50}) compared to drug isoniazid. A good plasma protein binding capacity of studied derivatives display high biological half-life that influences the biological efficacy of compound. All studied derivatives were predicted to be CYP2D6 non-inhibitor which indicates the compounds are safe for drug-drug based interactions. Derivatives **11C** and **11E** were predicted to be safe for skin and ocular irritancy and sensitization parameters. **8C, 11C** and **11E** derivatives are aerobically degradable means they are non-persistent in nature Table (**4)** and Table (**S1**). Results of Lipinski's rule of five indicate all the studied derivatives violate single rule of RO5 by crossing the cut-off limit of LogP value *i.e.*, 5. Compliance of predicted toxicity parameters and RO5 are given in Table (**5)** and Table (**6)**.

Table (S1): TOPKAT predicted Rat oral LD_{50}, Plasma protein binding (PPB), CYP2D6 inhibition, skin, and ocular irritancy/sensitization and aerobic biodegradability parameters for positive control ISONIAZID and derivatives **8C, 10, 11C** and **11E**.

Name	Rat_Oral_LD_{50} (g/kg_body_weight)	PPB binding	CYP2D6	Skin Irritancy	Skin Sensitization	Ocular Irritancy	Aerobic Biodegradability
INZ	0.50339	False	False	Mild	Strong	Severe	Non-Degradable
8C	2.47624	True	False	Mild	Strong	None	Degradable
10	0.666888	True	False	None	Strong	Mild	Non-Degradable
11C	0.0938587	True	False	None	None	None	Degradable
11E	0.169485	True	False	None	None	None	Degradable

Table (5): TOPKAT predicted toxicity and carcinogenic potency of positive control ISONIAZID and derivatives namely, **8C, 10, 11C** and **11E**

Name	Mouse Female	Mouse Male	Rat Female	Rat Male	Ames mutagenicity	epatotoxicity	DTP
INH	Multi Carcinogen	Multi-Carcinogen	Multi-Carcinogen	Non-Carcinogen	Mutagen	True	Toxic
8C	Non- Carcinogen	Multi-Carcinogen	Single-Carcinogen	Single-Carcinogen	Non-Mutagen	True	Toxic
10	Non- Carcinogen	Non-Carcinogen	Non-Carcinogen	Single-Carcinogen	Non-Mutagen	True	Toxic
11C	Non-Carcinogen	Single-Carcinogen	Non-Carcinogen	Single-Carcinogen	Non-Mutagen	True	Toxic
11E	Non-Carcinogen	Non-Carcinogen	Non-Carcinogen	Single-Carcinogen	Non-Mutagen	True	Toxic

Table (6): Parameter calculation for Lipinski's rule of five (RO5) violations

Name	ALogP98	PSA_2D ($Å^2$)	Molecular weight	No. of H-Acceptor	No. of Donor	No. of violation
INZ	-0.423	67.912	137.139	2	2	0
8C	5.584	83.435	492.517	7	0	1
10	5.231	86.39	452.428	6	1	1
11C	6.183	74.505	494.508	6	0	1
11E	6.388	74.505	508.535	6	0	2

4.3.5 Structure activity relationship

Performed modification at the acid group of benzofuran. When the acid group was free and methylated **8A** and **8B** compounds were inactive, But the ethylated **8C** MIC-50 μg/mL) derivative showed good activity. Similarly we synthesized propylated, isopropylated, n-butylated and isopentylated **8D to 8G** derivatives as inactive. The reason may be that the electron-donating aliphatic chains should be in medium size which can fit with the receptor to exhibit the antitubercular activity. The prototype-2 has a fluorine group at the para position. The derivative of **10** has shown good activity and prompted us to synthesize similar derivatives from **9** to **11G**.when the acid group methylated, ethylated, propylated and n-butylated **9**, **11A**, **11B** and **11D** there was no activity. But the isopropylated **11C** MIC-12.5 μg/mL exhibited very potent activity. Further, we synthesized similar iso group aliphatic chain with increasing in their ethylene group like isopentyl, sec-butyl **11F** and **11G** but found inactive. However tert-butylated ester exhibited significant antitubercular activity **11E** MIC-50 μg/mL. As prototype-3 did not exhibit activity it was not taken for further modifications. The compound **11C** was the best representative of this series.

4.4 Conclusion

In summary, we have synthesized designed molecules of 2, 3-diaryl benzofuran core by the concise method. All the seventeen novel derivatives were screened on antitubercular activity. Out of them four derivatives showed antitubercular activity. Among four derivatives, one derivative was the best from this series that was derivative **11C**. Also, the molecular docking results revealed the active derivative **11C** binds well at inhibitor binding site of Mtb drug target polyketide synthase 13 with binding energy of -8.7 kcal/mol. Amino acid residues GLN-1633 and PHE-1670

playing major role in hydrogen bonding and non-covalent based interactions with PK13. The computational oral bioavailability and toxicity assessment indicate active derivative **11C** may show good pharmacokinetic properties and less toxicity. It did not show any cytotoxicity on **L-132** normal lung cell line.

4.5 Experimental Section.

4.5.1 Reaction protocols

Synthesis of methyl-3, 5-dihydroxybenzoate (2):- To a stirred solution of 3, 5-dihydroxybenzoic acid **(**1 gm, 6.49 mmol), in methanol (10 mL), concentrated sulfuric acid (96% pure in water, 0.5 mL, 9.18 mmol) was added drop wise. The reaction mixture was refluxed for 3h. On completion, solvent was evaporated under reduced pressure. The crude mass was extracted with EtOAc (200mL x 3), washed with water and ethyl acetate layer dried over anhydrous sodium sulfate and distilled on rotary evaporation to get a crude mass, which was purified by column chromatography (Hexane-EtOAc 14-16%) to afford **2** (94%) as a white amorphous solid.

Synthesis of methyl-3, 5-dihydroxy-4-iodobenzoate (3):- In a 100 mL round bottom flask, methyl 3, 5-dihydroxybenzoate (250 mg, 1.49 mmol) was taken in methanol (1 mL) at 0ºC. To this stirred solution N-iodosuccinamide (351.5 mg, 1.57 mmol) was added. Reaction mixture was further stirred at RT for 16 h. Once consumption of starting material completed, then mixture was slowly poured into Ice-cold water (5 mL), transferred into separating funnel then slowly added saturated sodium thiosulfate solution (5 mL). Then waited for 5 min, a white solid compound was formed. The mixture was extracted thrice with EtOAc. The pooled ethyl acetate fraction was dehydrated by using anhydrous Na_2SO_4 and thickened on rotary evaporation to get the crude product **3**. It was as such used in next reaction step.

Synthesis of methyl-4-iodo-3, 5-dimethoxy benzoate (4):- To a 100 mL RB flask, methyl-3, 5-dihydroxy-4-iodobenzoate (230 mg, 0.78 mmol) was taken in DMF (1.5 mL) and K_2CO_3 (386.5 mg, 3.9 mmol) was added to it at RT. It was allowed to stir for 20 min at ambient temperature. To this stirred solution methyl iodide (0.15 mL, 2.35 mmol) was appended and the resulting mixture was stirred at RT for 15 h. On completion, the reaction mixture was uprooted with (50mL x 3 times) EtOAc, pooled

solvent layer was dried over Na_2SO_4 and thickened on rotary evaporation to get the crude product. This residue was refined by column chromatography which yielded white solid compound **4** of 230 mg (81%).

^{1}H NMR (500MHz, $CDCl_3$): δ 3.87 (s, 3H, OCH_3), 3.5 (s, 6H, 2xOCH_3) 7.09 (s, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.51, 56.73, 84.60, 104.36, 131.48, 159.18, 165.85.

General procedure for the synthesis of methyl-3, 5-dimethoxy-4-(2-(4-methoxy phenyl) ethynyl) benzoate (5, 5A and 5B):- In a round bottom flask methyl-4-iodo-3, 5-dimethoxybenzoate (150.0 mg, 0.510 mmol) was taken in triethyl amine (6 mL). To this $PdCl_2$ $(PPh_3)_2$ (45.0 mg, 0.064 mmol), CuI (12.0 mg, 0.063 mmol) and para-methoxy-phenylacetylene (79.0 mg, 0.598 mmol) were added and temperature was raised up to 60ºC for 5h. On completion, the reaction mixture was cooled, uprooted thrice with ethyl acetate, pooled layers dried over sodium sulphate and thickened on rotary evaporation. The residue was refined by using column chromatography with 4% EtOAc/hexane to provide 100 mg, 66% of solid amorphous compound **5, 5A** (65%) and **6B (**45%).

^{1}H NMR (500MHz, $CDCl_3$): δ 3.79 (s, 9H, 3xOCH_3), 3.81 (s, 3H, OCH_3), 7.35 (s, 2H, CH aromatic), 7.36 (d, 2H, CH aromatic), 7.84 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.26, 55.17, 56.17, 84.60, 93.00, 104.68, 113.78, 115.00, 131.79, 133.09, 159.40, 162.60, 166.50.

Methyl-4-(-2-(4-fluorophenyl) ethynyl)-3, 5-dimethoxybenzoate (5A):-

^{1}H NMR (500MHz, $CDCl_3$): δ 3.94 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 7.03 (d, 2H, CH aromatic), 7.43 (d, 2H, CH aromatic), 7.58 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.46, 56.79, 99.30, 104.58, 106.06, 114.08, 115.63, 131.29, 139.31, 161.61, 163.59, 166.58.

4-(2-(4-(methoxycarbonyl(2, 6-dimethoxyphenyl)ethynyl) benzoate (5B):-

^{1}H NMR (500MHz, $CDCl_3$): δ 3.91 (s, 6H, 2xOCH_3), 3.95 (s, 6H, 2xOCH_3), 7.23 (d, 2H, CH aromatic), 7.64 (d, 2H, CH aromatic), 8.01 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.23, 52.38, 56.36, 84.50, 99.42, 104.53, 128.19, 129.47, 129.58, 131.37, 132.47, 161.15, 166.62, 166.62.

General procedure for the synthesis of methyl -3-iodo-4-methoxy-2-(4-methoxyphenyl) benzofuran-6-carboxylate (6, 6A and 6B):- To a solution of alkyne-(**5, 5A and 5B)** (725 mg, 2.22 mmol) in CH_2Cl_2 (60 mL) a solution of iodine (1140 mg, 4.48 mmol) in dichloromethane (15 mL),was added drop wise. Reaction aged for 22 h at ambient temperature. Then diluted reaction mixture with DCM (30 mL) then stirring continued further 10 min at RT. The unreacted amount of molecular iodine was removed by washing with a aqueous $Na_2S_2O_3$ of saturated solution. The organic layer was dried over anhydrous Na_2SO_4 and distilled on rotary evaporation to yield a crude product, which was refined by column chromatography to afford compound **6** (720 mg, 96%), **6A** (65%) and **6B (**40%).

^{1}H NMR (500MHz, $CDCl_3$): δ 3.86 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 7.02 (d, 2H, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.83 (s, 1H, CH aromatic), 8.09 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.35, 53.24, 55.41, 55.82, 105.05, 106.36, 113.88, 122.25, 124.16, 126.96, 129.63, 153.36, 155.38, 160.57, 162.70, 167.03.

Methyl-2-(4-fluorophenyl)-3-iodo-4-methoxybenzofuran-6-carboxylate (6A):-

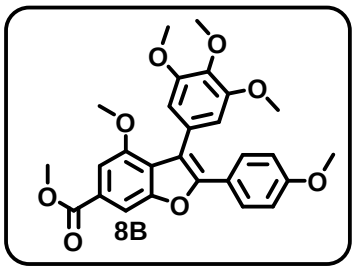
^{1}H NMR (500MHz, $CDCl_3$): δ 3.94 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3) 7.05 (d, 2H, CH aromatic), 7.16 (s, 1H, CH aromatic), 7.42 (d, 2H, CH aromatic), 7.68 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.35, 56.44, 90.23, 104.79, 105.67, 115.82, 116.00, 128.48, 128.57, 128.95, 142.79, 159.57, 161.40, 163.38, 166.02.

Methyl-2-(4- (methoxycarbonyl) phenyl)-3-iodo-4-methoxybenzofuran-6-carboxylate (6B):-

^{1}H NMR (500MHz, $CDCl_3$): δ 3.81 (s, 3H, OCH_3), 3.98 (s, 6H, 2xOCH_3), 7.20 (s, 1H, CH aromatic), 7.46 (s, 1H, CH aromatic), 7.48 (d, 2H, CH aromatic), 8.06 (d, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.27, 52.46, 56.36, 60.43, 104.55, 105.61, 110.54, 127.32, 128.82, 129.77, 129.89, 131.88, 150.96, 156.34, 166.30, 166.58, 166.63.

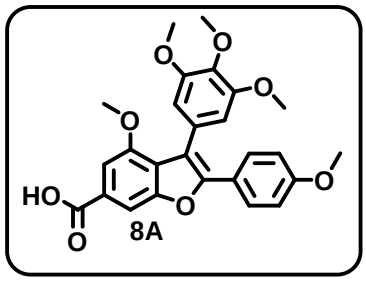


Synthesis of methyl-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl) benzofuran-6-carboxylate (8B or 7):- In a round bottom flask iodobenzofuran-6 (500 mg, 1.14 mmol) in DMF/water (4:1, 20.5 mL), 3,4,5-trimethoxy phenyl boronic acid (350 mg, 1.3 mmol), sodium bicarbonate (133.4 mg, 1.6 mL) and $PdCl_2(PPh_3)_2$ (40 mg, 0.057 mmol) were appended. Reaction mixture was stirred for 10 min at RT, followed by heating at 80ºC for 10h. On completion the reaction mixture was uprooted thrice with EtOAc (200 mL x 3 times), dried over Na_2SO_4 and distilled on rotary evaporation to get a crude mass which was refined by column chromatography with 4% EtOAc/Hexane to obtained 630 mg (89%) of compound **8B** as white solid with mp 149.9ºC.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.84 (s, 9H, 3xOCH_3), 3.94 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3) 6.58 (d, 2H, CH aromatic), 6.97 (d, 2H, J= 8.35 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.83 (s, 1H, CH aromatic), 8.08 (dd, 2H, J= 3.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.34, 53.23, 55.40, 55.81, 56.07, 60.85, 105.05, 105.19, 106.36, 113.88, 122.24, 124.15, 126.97, 129.62, 138.09, 139.30, 153.36, 153.53, 154.41, 155.37, 160.58, 167.01; ESI-MS (MeOH): For $C_{27}H_{26}O_8$, 517 $[M+K]^+$, HRMS (ESI-TOF) m/z [M+H] calculated for $C_{27}H_{26}O_8$, 479.1628 , found 479.1940.

Synthesis of 4-methoxy-3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl) benzofuran-6-carboxylic acid (8A):-



To the RB Flask substrate **7** (300 mg, 0.63 mmol) in methanol/water (9:1, 27.3 mL) was appended KOH (100 mg, 1.785 mmol). The solution was heated for 6 hours at 80ºC. The solvent was concentrated on rotary evaporator to get the crude mass which was primitive drawing out thrice with EtOAc. The combined ethyl acetate layer dried over anhydrous Na_2SO_4, concentrated on rotary evaporation and refined to get the crude which was purified by column chromatography with 4% EtOAc/ Hexane to get 285 mg (97%) of compound **8A** as solid compound with mp 242.5ºC.

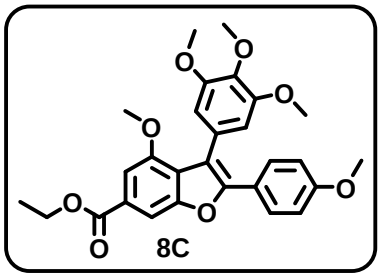
^{1}H NMR (500MHz, $CDCl_3$): δ 3.75 (s, 3H, OCH_3), 3.83 (s, 9H, 3xOCH_3), 3.94 (OCH_3, 3H), 6.59 (d, 2H, CH aromatic), 7.04 (d, 2H, J= 6.5 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 8.00 (d, 2H, CH aromatic), 8.15 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 53.46, 53.94, 58.20, 103.32, 103.77, 112.11, 112.58 119.97, 121.79, 127.44, 129.68, 129.91, 151.07, 152.20, 152.53, 158.42; ESI-MS (MeOH): For $C_{26}H_{24}O_8$, 543 $[M+DMSO+H]^+$**.** HRMS (ESI-TOF) m/z [M+H] calculated for $C_{26}H_{24}O_8$, 465.1471, found 465.1229.

General procedure for the preparation of the substituted esters (8C-8G):-

To a 50 mL round bottom flask contain acid derivative **8A** (30 mg, 0.064 mmol) was taken in ethanol (7 mL) and (0.3 mL, 0.003 mmol) of concentrated sulphuric acid were added. Reaction mixture was heated for a 1-2 hour at 80ºC. The ethanol was concentrated on rotary evaporator to get the crude mass which was extracted thrice with EtOAc. The combined EtOAc was dried over Na_2SO_4, thickened rotary evaporation to get the unrefined mixture which was further refined by column chromatography with 4% EtOAc /hexane to provide 25.5 - 32 mg (80-90%) of all the derivatives.

Ethyl-[4-methoxy-3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl)] benzofuran-6-carboxylate (8C):-



Yield: 80%;

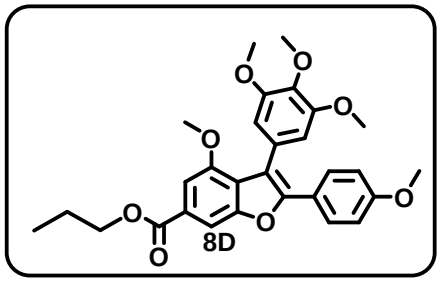
Melting Point: amorphous.

ESI-MS (MeOH): For $C_{28}H_{28}O_{8,}$ 493 $[M+H]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.42 (t, 3H, CH_3), 3.85 (s, 9H, 3xOCH_3), 3.87 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.40 (m, CH_2, 2H), 6.59 (d, 2H, CH aromatic), 7.01 (d, 2H, J= 9.00 Hz, 6.98, J= 3.5 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.78 (s, 1H, CH aromatic), 7.82 (dd, 2H, J= 8.5 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 14.38, 53.23, 55.40, 55.80, 56.08, 61.09, 61.26, 105.06, 105.22, 106.30, 113.88, 122.82, 124.20, 126.73, 129.61, 139.30, 153.35, 154.41, 154.97, 160.32, 167.01.

Propyl-[4-methoxy-3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl)] benzofuran-6-carboxylate (8D):-



Yield: 85%;

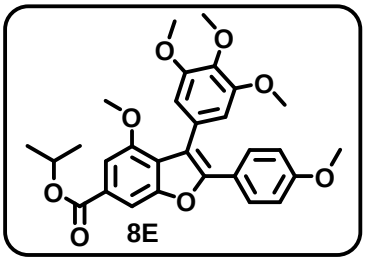
Melting Point: amorphous**.**

ESI-MS (MeOH): For $C_{29}H_{30}O_{8,}$ 507 $[M+H]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ1.41 (t, 2H, CH_3), 1.83 (m, 2H, CH_2), 3.85 (s, 9H, 2xOCH_3), 3.85 (s, 3H, OCH_3), 4.00 ($OCH_{3,}$ 3H), 4.31 (t, 2H, CH_2), 6.98 (d, 2H, CH aromatic), 6.99 (d, 2H, J= 7.0 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.78 (s, 1H, CH aromatic), 7.79 (d, 2H, J= 8.5 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 10.58, 22.20, 52.35, 55.39, 55.78, 56.07, 66.67, 105.08, 105.24, 106.49, 113.88, 122.81, 124.20, 129.60, 139.30, 153.83, 154.41, 154.97, 160.33, 167.06.

Isopropyl-[4-methoxy -3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl)] benzofuran-6-carboxylate (8E): -



Yield: 80%;

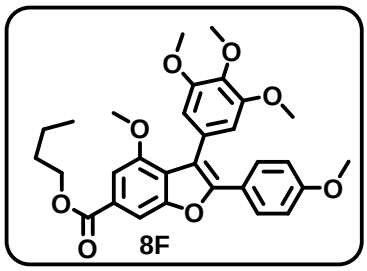
Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{29}H_{30}O_8$, 507 $[M+H]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.39 (s, 6H, 2xCH_3), 3.86 (s, 9H, 3xOCH_3), 3.87 (s, 3H, OCH_3) 3.87 (s, 3H, OCH_3), 5.27 (m, 1H, CH), 6.59 (d, 2H, CH aromatic), 6.99 (dd, 2H, J= 8.0 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.82 (dd, 2H, J= 9.5 Hz, CH aromatic), 8.08 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 22.71, 55.40, 55.81, 56.07, 68.70, 105.05, 105.19, 106.24, 113.88, 115.90, 122.32, 124.07, 126.59, 129.59, 132.23, 139.31, 153.32, 153.53, 154.41, 155.24, 160.54, 166.46.

Butyl-[4-methoxy-3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl)] benzofuran-6-carboxylate (8F):-



Yield: 87%

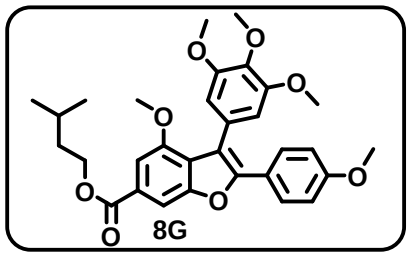
Melting Point: Viscous compound**.**

ESI-MS (MeOH): For $C_{30}H_{32}O_8$, 539 $[M+H+NH_4]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.99 (t, 3H, CH_3), 1.50 (m, 2H, CH_2), 1.77 (m, 2H, CH_2), 3.84 (s, 9H, 3xOCH_3), 3.89 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 4.35 (t, 2H, CH_2), 6.98 (d, 2H, CH aromatic), 7.34 (dd, 2H, J= 7.5 Hz, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.81 (dd, 2H, J= 9.5 Hz, CH aromatic), 8.09 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 13.82, 19.35, 31.94, 52.34, 55.39, 55.78, 56.07, 66.11, 104.34, 106.24, 106.47, 113.87, 115.11, 122.80, 124.17, 126.60, 129.76, 132.07, 139.29, 153.35, 153.53, 154.40, 154.96, 160.32, 167.02.

Isopentyl-[4-methoxy3-(3, 4, 5-trimethoxyphenyl)-2-(4-methoxyphenyl)] benzofuran-6-carboxylate (8G):-



Yield: 90%;

Melting point: Viscous compound.

ESI-MS (MeOH): For $C_{31}H_{34}O_8$, 552 $[M+H]^+$.

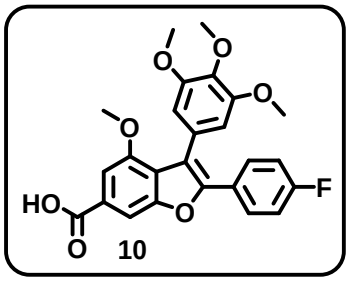
^{1}H NMR (500MHz, $CDCl_3$): δ 1.29 (s, 6H, 2xCH_3), 1.70 (m, 2H, CH_2) 1.83 (m, 1H, CH), 3.86 (s, 9H, 3xOCH_3), 3.88 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.38 (t, 2H, CH_2), 6.99 (d, 2H, CH aromatic), 7.00 (d, 2H, CH aromatic), 7.37 (s, 1H, CH aromatic), 7.79 (d, 2H, J= 8.5 Hz, CH aromatic), 8.10 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 22.71, 25.39, 37.50, 55.40, 53.23, 55.79, 56.07 63.79, 104.34, 106.24, 106.48, 113.88, 114.36, 122.80, 124.19, 126.61, 129.60, 130.27, 139.31, 153.35, 154.96, 155.31, 160.32, 167.05.

General procedure for the preparation of the substituted esters (11A-11G):-

To a solution of acid compound (30 mg, 0.066 mmol) in methanol (5 mL), (0.4 mL, 0.004 mmol) of concentrated sulphuric acid was added. The solution was heated at 80ºC for a 1-4 hour. The solvent was concentrated on rotary evaporation to get the crude residue which was extracted with EtOAc (60mL x 3 times). The combined organic layer was dried over anhydrous sodium sulphate and concentrated on rotary evaporation to get the crude; this crude was subjected to column chromatography with 4% EtOAc/Hexanes to provide 25.8 – 31.2 mg (80-97%) of all the derivatives.

2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl) benzofuran-6-carboxylic acid (10):-



Yield: 91%;

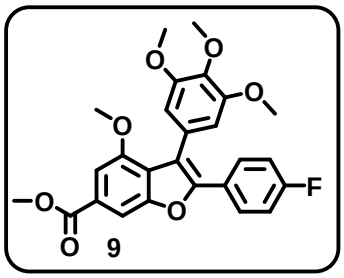
Melting point: 253.0ºC.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{25}H_{21}FO_7$, 453.1305, found 453.1440.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.81 (s, 3H, OCH_3), 3.83 (s, 9H, 3xOCH_3), 6.88 (s, 2H, CH aromatic), 7.07 (d, 2H, J= 7.0 Hz, CH aromatic), 7.29 (d, 2H, J= 2.5 Hz, CH aromatic), 7.44 (bs, 1H, CH aromatic), 7.71 (bs, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.15, 104.23, 104.42, 116.30, 119.32, 123.50, 125.52, 128.56, 132.24, 133.05, 139.28, 151.83, 160.68, 161.08, 163.04, 167.00.

Methyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (9):-



Yield: 93%;

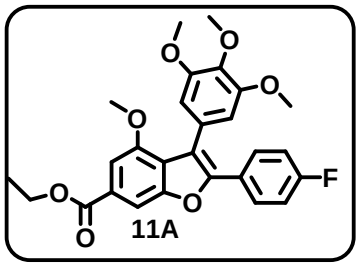
Melting point: Viscous compound.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{26}H_{23}FO_7$, 467.1461, found 467.3140.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.93 (s, 12H, 4xOCH_3), 4.00 (s, 3H, OCH_3), 7.16 (s, 2H, CH aromatic), 7.19 (d, 2H, J= 8.5 Hz, CH aromatic), 7.38 (bs, 1H, CH aromatic), 7.84 (bs, 1H, CH aromatic), 8.12 (m, 2H, J= 3.5 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.34, 54.61, 55.81, 56.35, 105.15, 106.39, 115.51, 115.69 123.92, 125.94, 127.57, 128.55, 130.10, 132.33, 153.59, 154.36, 154.55 162.31, 166.85.

Ethyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11A):-



Yield: 95%;

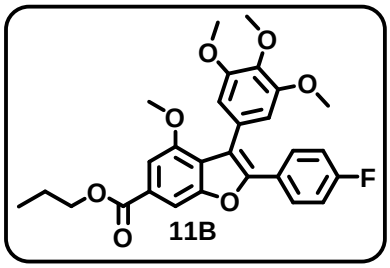
Melting point: 103.5°C.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{27}H_{25}FO_7$, 481.1618, found 481.2600.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.40 (m, CH_3, 3H), 3.96 (s, 12H, 4xOCH_3), 4.38 (m, CH_2, 2H), 7.04 (d, 2H, J= 4.5 Hz, CH aromatic), 7.16 (s, 2H, CH aromatic), 7.56 (bs, 1H, CH aromatic), 7.57 (m, 2H, J= 3.5 Hz, CH aromatic), 7.58 (bs, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 14.10, 54.61, 56.33, 56.35, 61.41, 104.61, 105.15, 115.42, 115.92, 106.39, 123.92, 125.94, 127.57, 128.55, 131.1, 133.60, 153.59 154.55, 154.36, 161.01, 166.09.

Propyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11B):-



Yield: 90%;

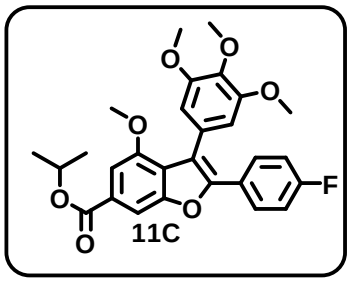
Melting point: Viscous compound.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{28}H_{27}FO_7$, 495.1774, found 495.6310.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.87 (t, 3H, CH_3), 1.79 (m, 2H, CH_2), 3.95 (s, 9H, 3xOCH_3), 4.00 (s, 3H, OCH_3), 4.30 (t, 2H, CH_2), 7.03 (d, 2H, CH aromatic), 7.14 (d, 2H, J= 4.5 Hz, CH aromatic), 7.24 (s, 1H, CH aromatic), 7.57 (d, 2H, J= 5.0 Hz, CH aromatic), 7.87 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 10.50, 22.70, 56.03, 67.04, 104.41, 105.15, 106.00, 114.07, 116.13, 119.59, 126.92, 126.99, 131.42, 139.29, 151.95, 152.81, 156.43 163.58, 166.15.

Isopropyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11C):-



Yield: 91%;

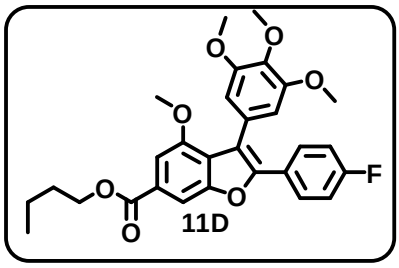
Melting point: Viscous compound.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{28}H_{27}FO_7$, 495.1774, found 495.6310.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.39 (s, 6H, 2xCH_3), 3.96 (s, 12H, 4xOCH_3), 5.27 (m, 1H, CH), 7.03 (d, 2H, CH aromatic), 7.04 (d, 2H, J= 4.5 Hz and 7.05 J= 2.0 Hz, CH aromatic), 7.23 (s, 1H, CH aromatic), 7.57 (d, 2H, J= 3.5 Hz, CH aromatic), 7.58 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 22.71, 55.40, 55.81, 56.07, 68.70, 105.05, 105.19, 106.24, 113.8, 115.43, 122.32, 124.07, 129.59, 132.23, 139.31, 150.31, 153.53 155.24 160.54, 166.46.

n-Butyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11D):-



Yield: 97%;

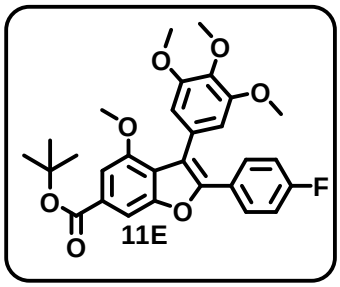
Melting point: Viscous compound.

Electrospray mass for $C_{29}H_{29}FO_7$ (MeOH): 509 $[M+H]^+$

NMR (500MHz, $CDCl_3$): δ 0.87 (t, 3H, CH_3), 1.46 (m, 2H, CH_2), 1.77 (m, 2H, CH_2), 3.95 (s, 9H, 3xOCH_3), 4.00 (s, 3H, OCH_3), 4.34 (t, 2H, CH_2), 7.01 (d, 2H, CH aromatic), 7.03 (d, 2H, J= 3.0 Hz, CH aromatic), 7.24 (s, 1H, CH aromatic), 7.57 (d, 2H, J= 2.5 Hz, CH aromatic), 7.58 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 14.12, 22.70, 33.83, 56.32, 65.35, 104.41, 105.16, 106.53, 114.07, 115.60, 119.60, 126.92, 126.99, 129.68, 130.62, 139.28, 142.77, 151.96, 156.43, 163.58, 166.17.

***tert*-Butyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11E): -**



Yield: 92%;

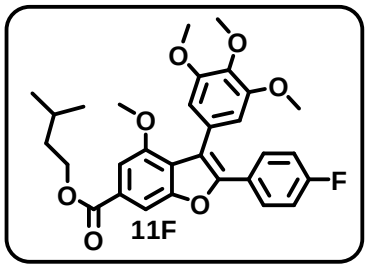
Melting point: 159.5ºC

HRMS (ESI-TOF) m/z [M+Na] calculated for $C_{29}H_{29}FO_7$, 509.1931, found 531.6741.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.20 (s, 9H, 3xCH_3,), 3.91 (s, 12H, 4xOCH_3), 6.97 (d, 2H, CH aromatic), 6.98 (d, 2H, J= 8.5 Hz, CH aromatic), 7.19 (s, 1H, CH aromatic), 7.54 (bs, 1H, CH aromatic), 7.52 (d, 2H, J= 3.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 29.65, 56.24, 80.97, 99.12, 104.49, 105.14, 114.01, 115.54, 119.59, 123.24, 123.77, 131.64, 133.62, 139.22, 152.76, 153.50, 156.34, 163.53, 165.77.

Isopentyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11F):-



Yield: 92%;

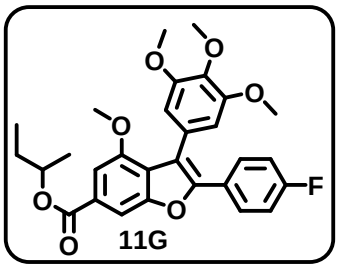
Melting point: Viscous compound;

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{30}H_{31}FO_7$, 423.2087, found 423.3793.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.87 (d, 6H, 2xCH_3), 1.68 (m, 2H, CH_2), 1.76 (m, 1H, CH), 3.95 (s, 9H, 3xOCH_3), 4.00 (s, 3H, OCH_3), 4.24 (t, 2H, CH_2), 7.01 (d, 2H, CH aromatic), 7.05 (d, 2H, CH aromatic), 7.14 (s, 1H, CH aromatic), 7.56 (d, 2H, CH aromatic), 7.70 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 22.37, 24.51, 37.08, 64.06, 55.95, 104.40, 105.16, 115.18, 115.36, 122.09, 122.91, 130.93, 139.34, 143.17, 160.78, 166.11.

***sec*-Butyl-[2-(4-fluorophenyl)-4-methoxy-3-(3, 4, 5-trimethoxyphenyl)] benzofuran-6-carboxylate (11G):-**



Yield: 95%;

Melting point: Viscous compound.

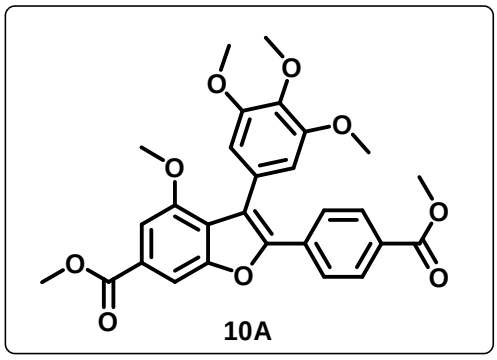
HRMS (ESI-TOF) m/z [M+H] calculated for $C_{29}H_{29}FO_7$, 509.1931, found 509.1667.

^{1}H NMR (500MHz, $CDCl_3$): δ 0.83 (m, 3H, CH_3), 1.43 (m, CH_3, 3H), 1.92 (m, CH_2, 2H), 3.91 (s, 12H, 4xOCH_3), 4.24 (m, CH, 1H), 6.97 (d, 2H, CH aromatic), 7.00 (d, 2H, CH aromatic),7.19 (s, 1H, CH aromatic), 7.54 (bs, 1H, CH aromatic), 7.83 (d, 2H, J= 3.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 8.46, 22.57, 29.58, 56.24, 62.03, 65.32, 99.20, 105.12, 106.03, 113.62, 115.29, 119.48, 123.54, 130.60, 131.10, 133.55, 139.11, 153.09, 156.34, 163.57, 166.24.

Synthesis of methyl-[2-(4-methoxycarbonyl) phenyl)-4-methoxy-3-(3, 4, 5-trimethoxy phenyl)] benzofuran-6-carboxylate (10A):-

In a round bottom flask contain iodobenzofuran **6B** (100 mg, 1.14 mmol) in DMF/water (10:4 mL), 3, 4, 5-trimethoxy phenyl boronic acid (70 mg, 0.33 mmol), sodium bicarbonate (27 mg, 0.32 mL) and $PdCl_2(PPh_3)_2$ (8 mg, 0.012 mmol) were appended. Reaction mixture was stirred for 10 min at RT, followed by heating at 60ºC for 8h. On completion the reaction mixture was uprooted thrice with EtOAc (100 mL x 3 times), dried over Na_2SO_4 and distilled on rotary evaporation to get a crude mass which was refined by column chromatography with 4% EtOAc/Hexane to obtained 87 mg (80%) of compound **10A** as white solid with mp 150.4-160ºC.



10A

^{1}H NMR (500MHz, $CDCl_3$): δ 3.92 (s, 6H, 2xOCH_3), 3.94 (s, 6H, 2xOCH_3), 3.96 (s, 6H, 2xOCH_3), 7.25 (d, 2H, CH aromatic) , 7.64 (d, 2H, J= 8.0 Hz, CH aromatic), 7.65 (s, 1H, CH aromatic), 8.01 (d, 2H, J= 8.0 Hz, CH aromatic), 8.02 (s, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 52.25, 52.34, 52.50, 56.37, 104.56, 105.69, 108.93, 114.08, 115.90, 123.51, 124.06, 129.42, 131.36, 135.18, 139.30, 142.85, 151.89, 161.17, 166.53, 166.67; Electrospray mass for $C_{28}H_{26}O_9$ (MeOH): 507 $[M+H]^+$.

4.6 Biological Assays

4.6.1 *In-vitro* anti-tubercular assay using H37Ra (avirulent strain)

Concisely, benzofuran analogues were dissolved in dimethyl sulfoxide solvent to prepare working stock (5 mg/mL). Serial concentration dilutions from stocks solution were also made in DMSO. To 1.9 mL MB 7H11 agar medium (in tubes, temp. 45-

50ºC, with OADC supplement), 0.1 mL of an analogues or DMSO (negative control) was added. The contents were mixed and kept to solidify aslope. After 21days old culture of Mtb H37Ra was taken from L-J medium and its suspension approximately contain the cells (1 mg/mL equivalent to 108 bacilli approximately) was made in normal saline (containing 0.05% Tween-80). 10 µl of 1:10 dilution of this suspension (~105 bacilli) was inoculated into each tube and incubated at 37ºC for 4 weeks. The lowest concentration of a compound up to which there was no visible growth of bacilli was its minimum inhibitory concentration [18].

4.7 *In silico* studies

4.7.1 Identification of anti-Mtb targets, target preparation and molecular docking

The probable anti-Mtb targets were searched through web-based target identification tool STITCH v5.0 (http://stitch.embl.de) and reported literatures. Stitch tool develop score-based interaction network between standard known compound and identified targets reported biological activities. The 3D crystallographic structures of identified targets *viz.*, polyketide synthase 13 (PDB: 5V3X), catalase peroxidase (PDB: 1SJ2), dihydrofolate reductase (PDB: 4M2X) and enoyl-ACP reductase (PDB: 4TRO) were retrieved through protein databank (PDB) (http://www.pdb.org). The protein and ligand structures were prepared by using Autodock Vina (MGL, La Jolla, USA). Initially Autodock Vina prepared extended PDBQT file for receptor and ligands. The method included addition of polar hydrogen's, atomic partial charges and AD4 atom type conversion, providing information about torsional degree of freedom. Afterward the receptor was implanted in 3D grid system with probe atoms at each grid point. The electrostatic and desolvation potentials of each atom type (C, O, N and H) with every grid point was calculated. Further, ligand conformation energy was calculated through grids. The receptor-ligand interaction results were analyzed through Discovery Studio visualize.

4.7.2 Computational assessment for oral bioavailability and potential toxicity

The two major reasons identified for the new drug candidate failure are the poor bioavailability and high toxicity. Hence, the oral bioavailability of the studied compound was assessed through Lipinski's rule of five (RO5). While the toxicity parameters of the studied derivative was assessed though the standard computational

assessment tool TOPKAT (DS v3.5). TOPKAT utilizes its patented optimal predictive space (OPS) to calculate different toxicity parameters for query set component (Enslein, 1988). The oral bioavailability, blood-brain-barrier penetration capacity and intestinal absorption were assessed though DS ADME plots. This included the calculated parameters such as, plasma protein binding, CYP2D6 metabolism, hepatotoxicity, mouse and rat male/female carcinogenicity, developmental toxicity, skin and ocular irritancy etc.

4.8 References

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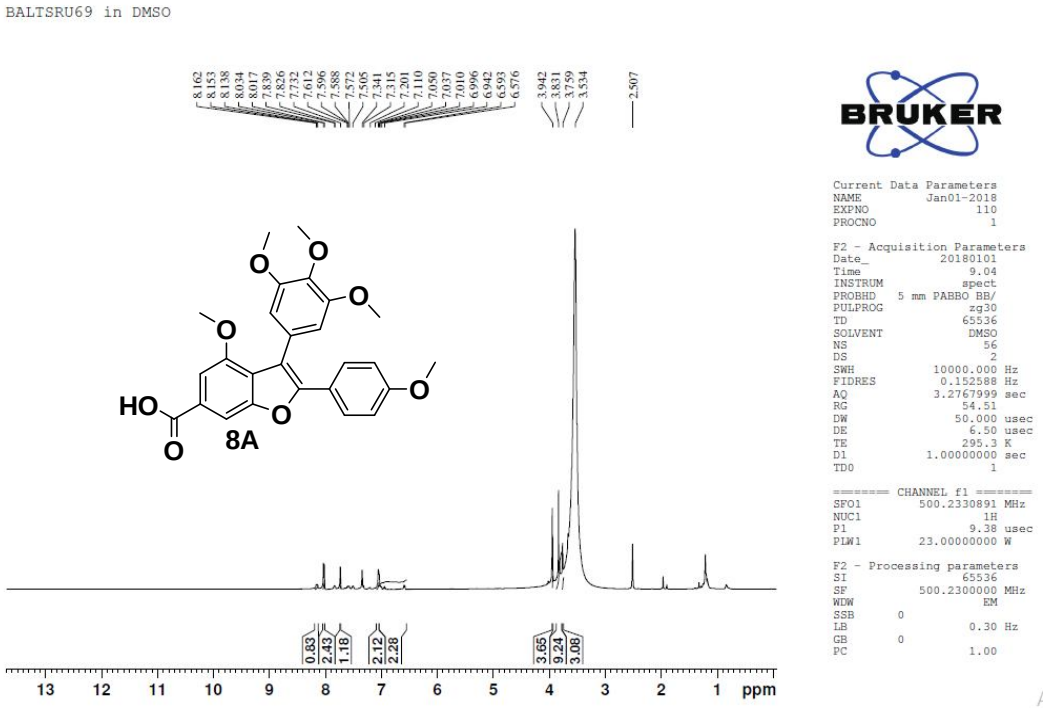
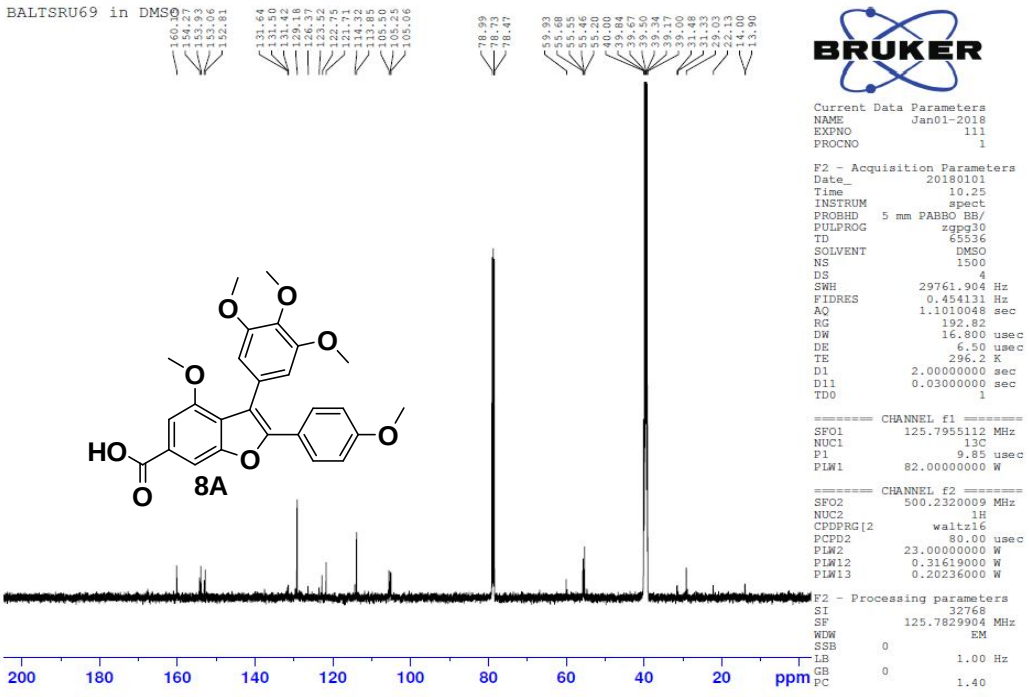
4.9 Spectra of final compounds

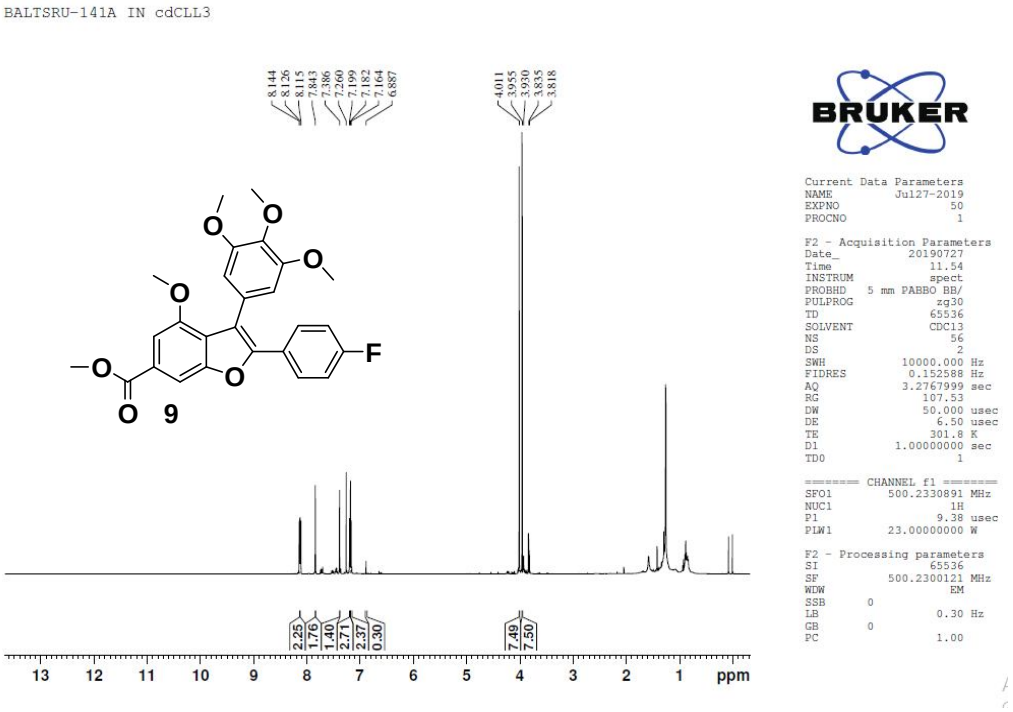
Compound 8A: ^{1}H NMR



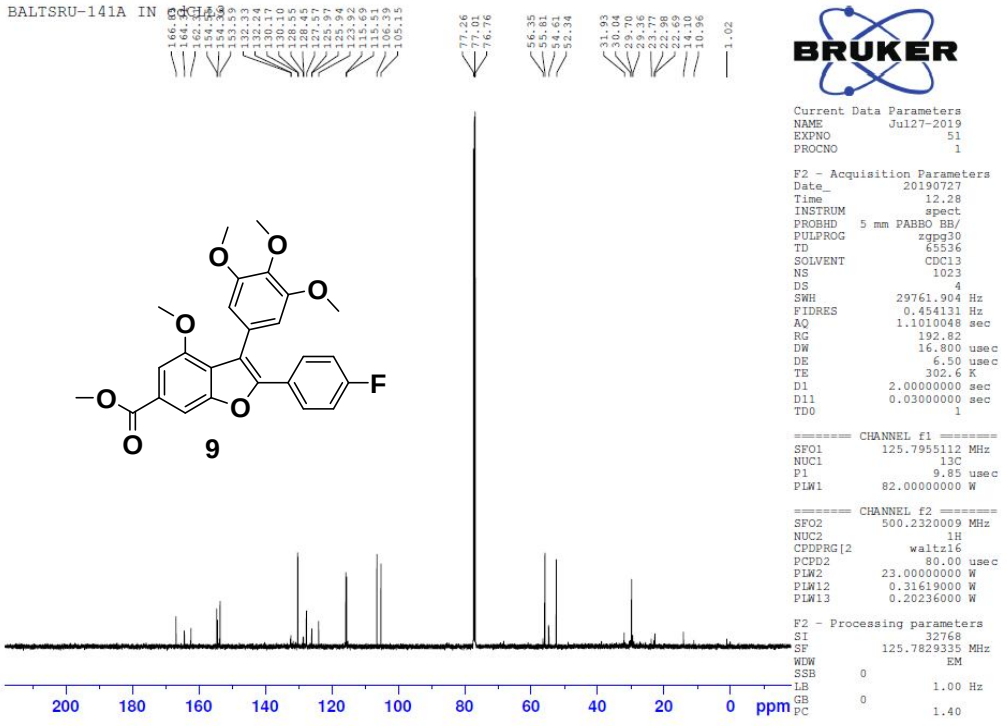
Compound 8A: ^{13}C NMR



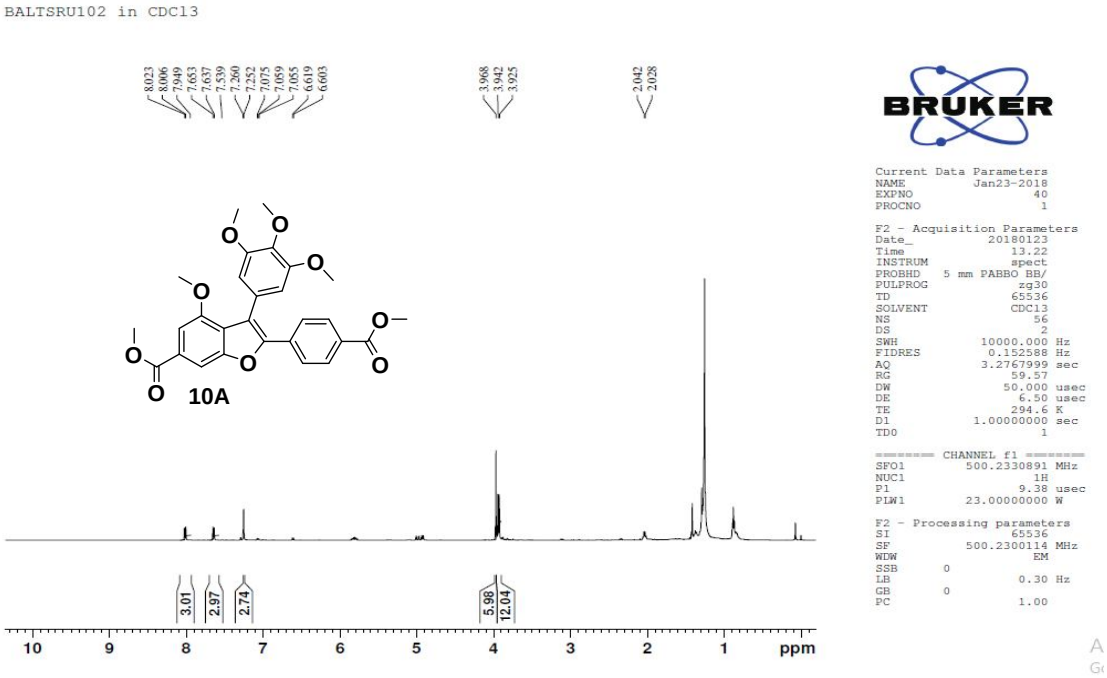
Compound 9: ^{1}H NMR



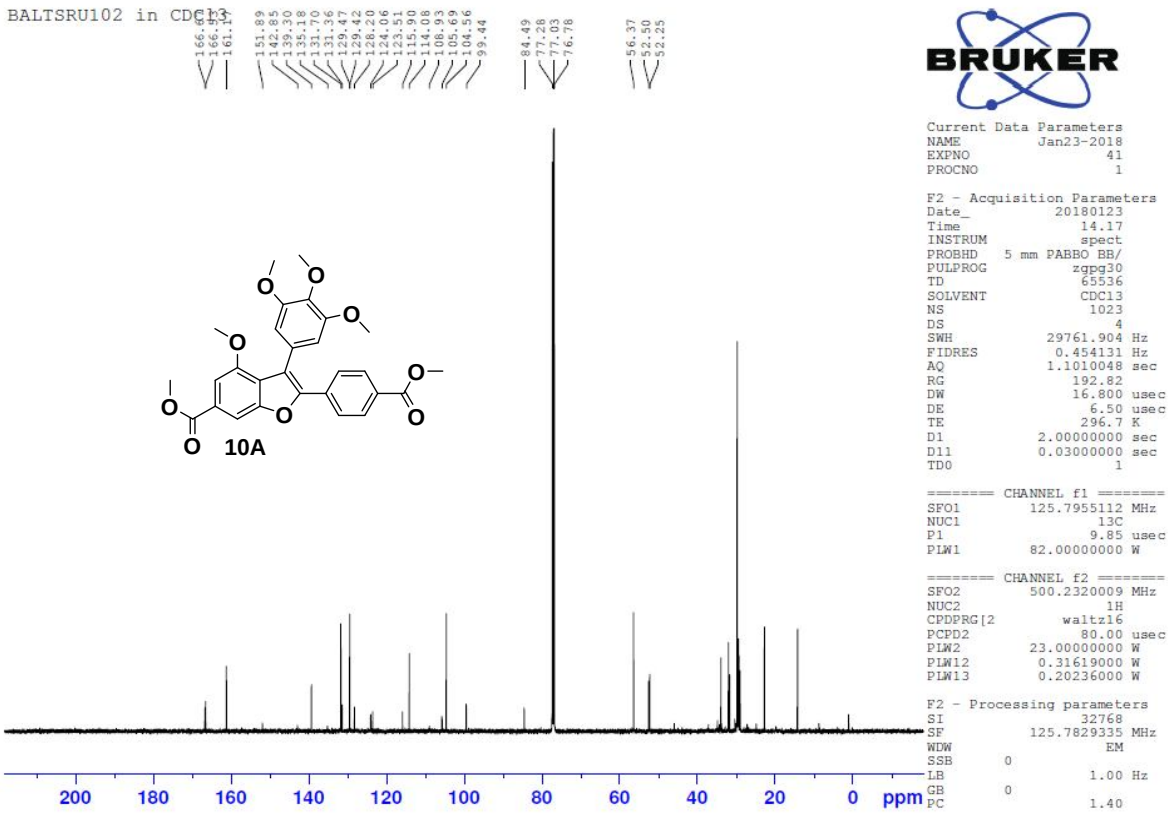
Compound 9: ^{13}C NMR



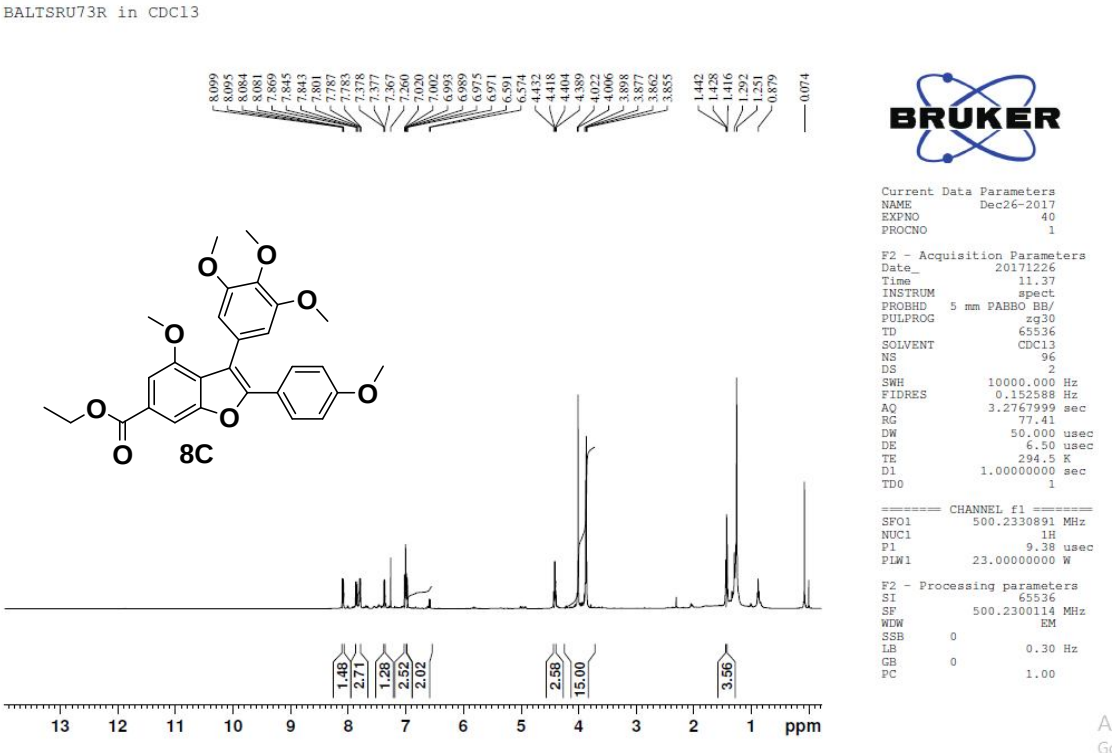
Compound 10A: ^{1}H NMR



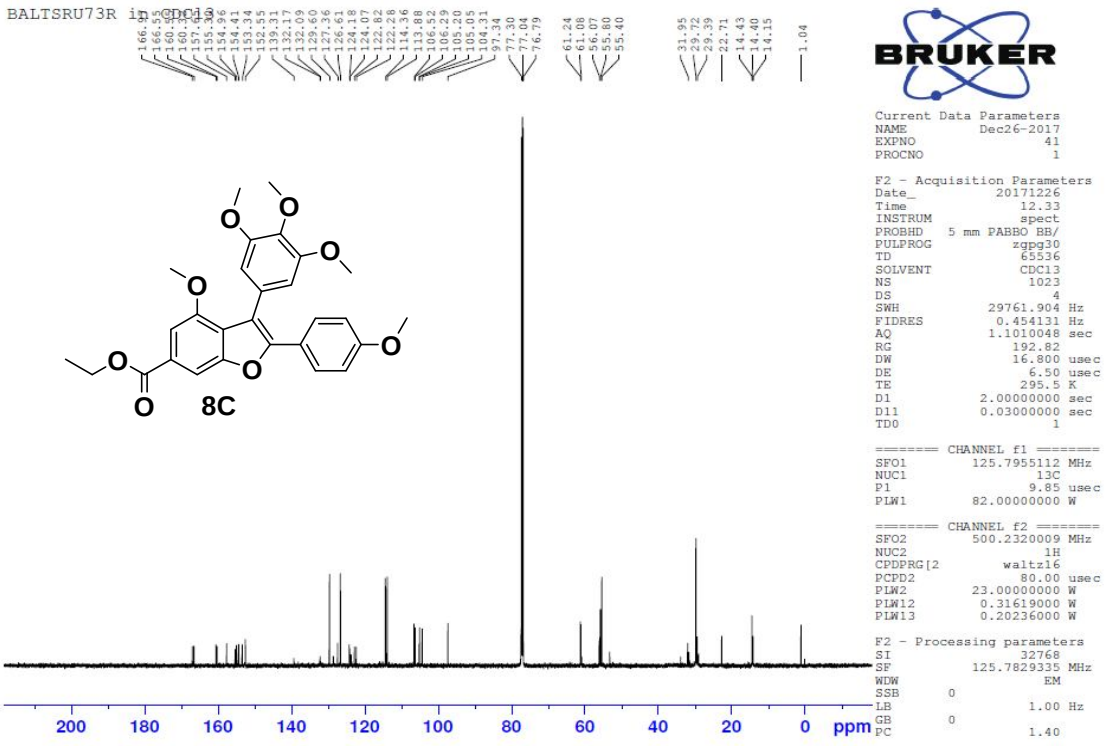
Compound 10A: ^{13}C NMR



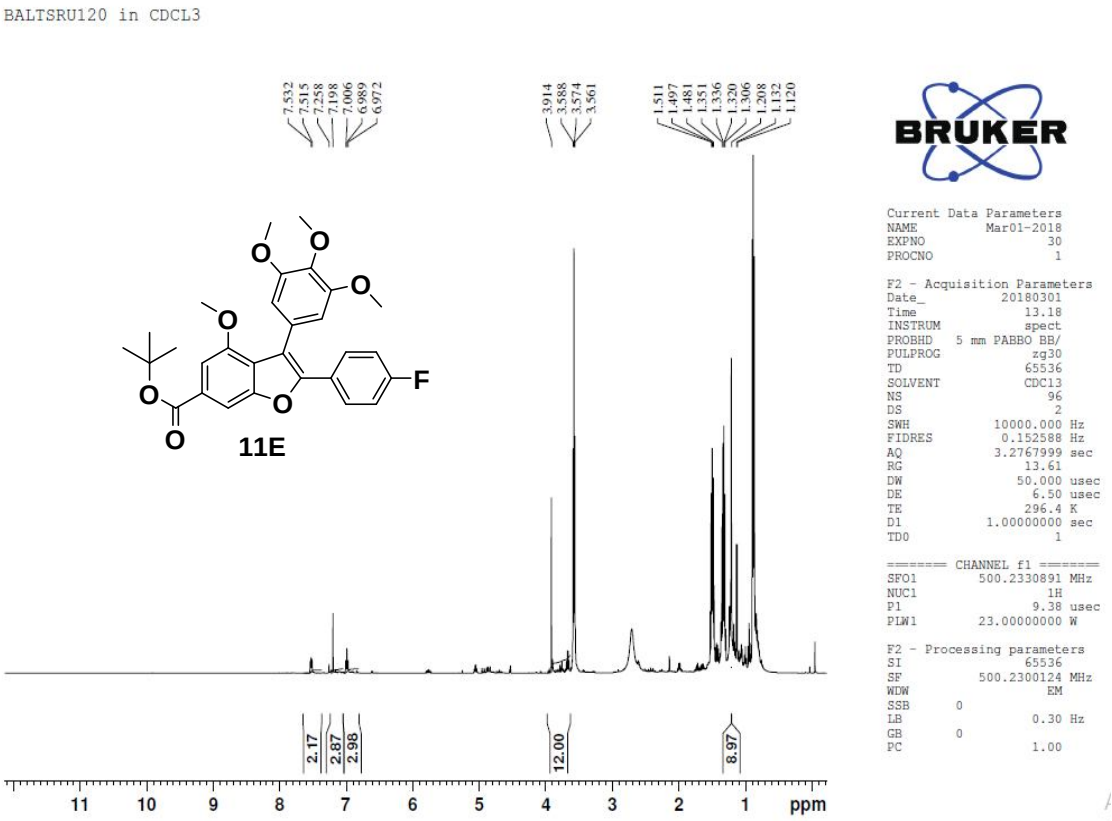
Compound 8C: ^{1}H NMR



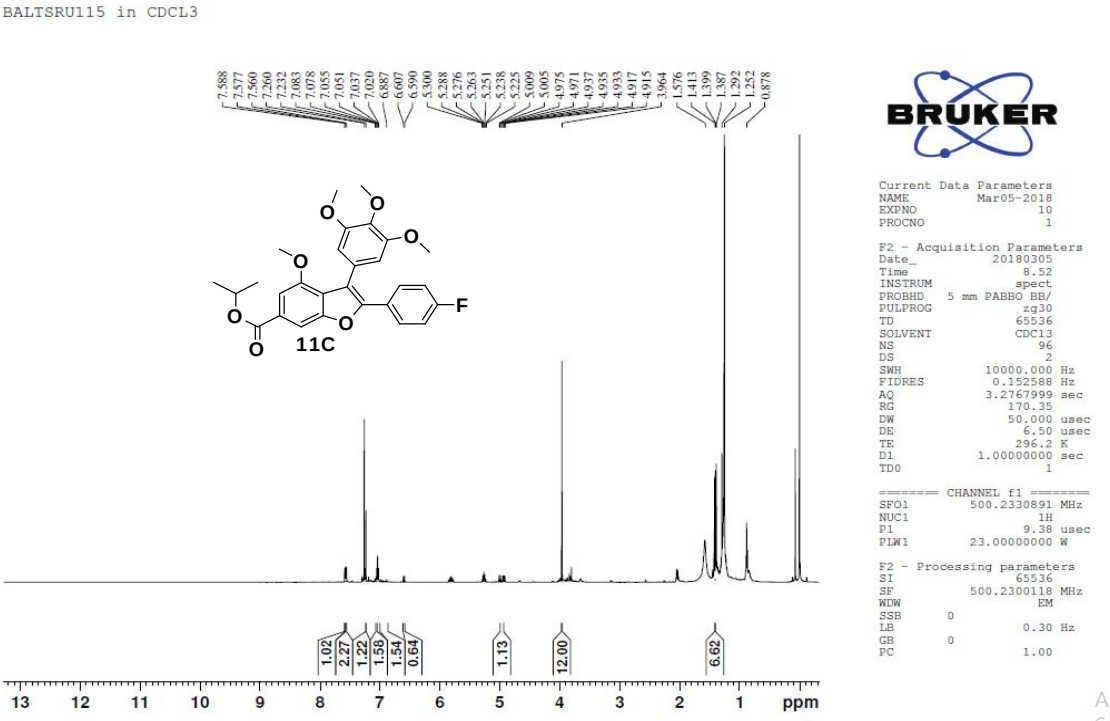
Compound 8C: ^{13}C NMR



Compound 11E: ^{1}H NMR



Compound 11C: ^{1}H NMR



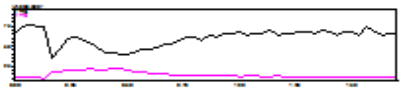
Compound 8A: ESI-MS (MeOH): For $C_{26}H_{24}O_8$, 543 $[M+DMSO+H]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

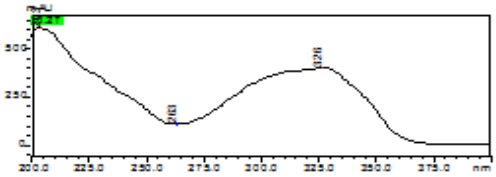
Sample Code : ASN_BALTS69
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 400-700

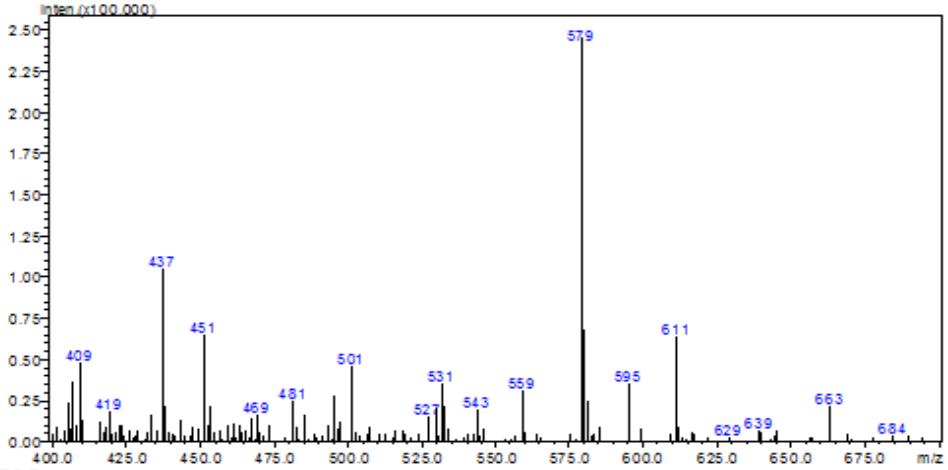
Mass chromatogram



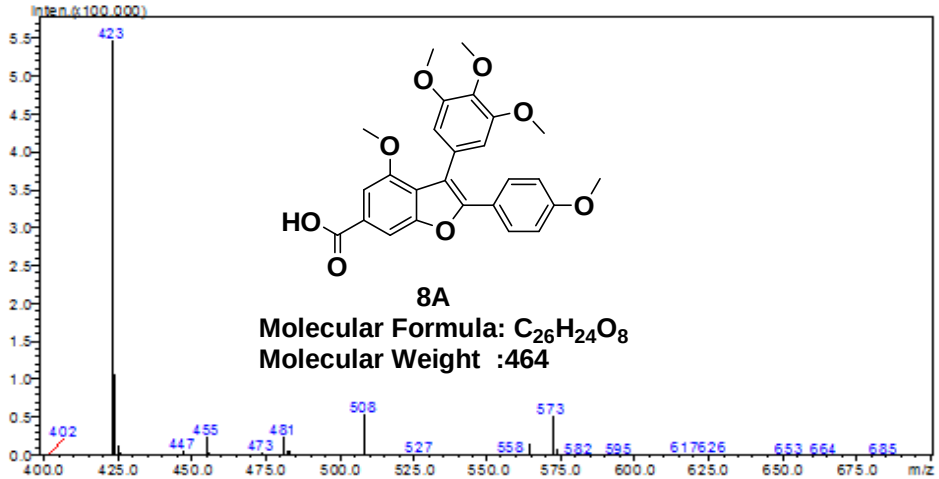
UV-Spectra



ESI+



ESI-



8A

Molecular Formula: $C_{26}H_{24}O_8$

Molecular Weight :464

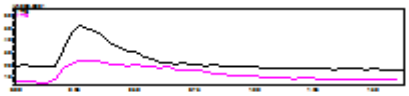
Compound 10A: ESI-MS (MeOH): For $C_{28}H_{26}O_9$, 507 $[M+H]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

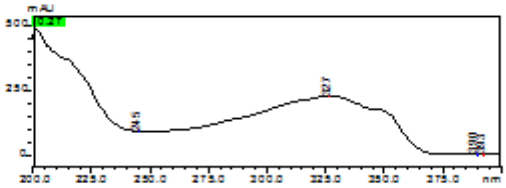
Sample Code : ASN_BALTSRU102
Solubility : MeOH
Name of the Scientist : Dr. A.S.NEGI

Project Code: MLP-02
Mass Range: 400-600

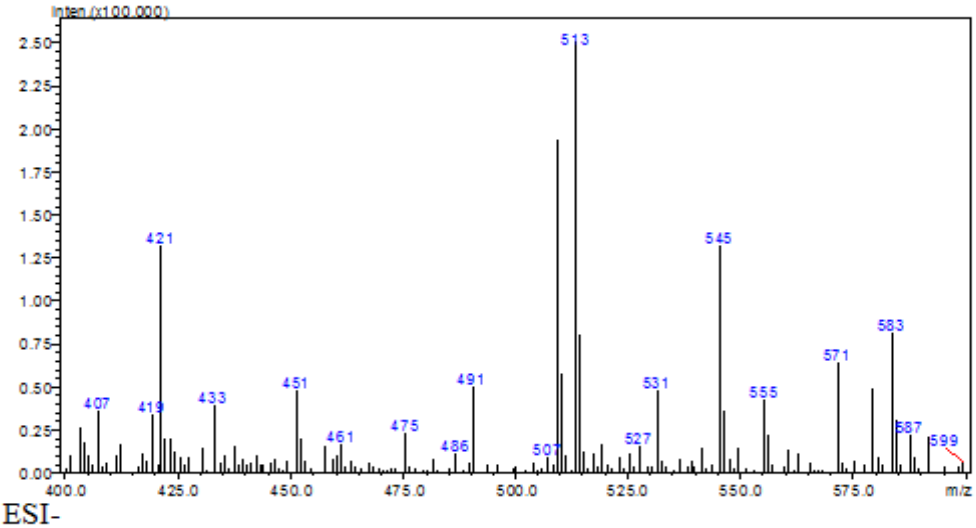
Mass chromatogram



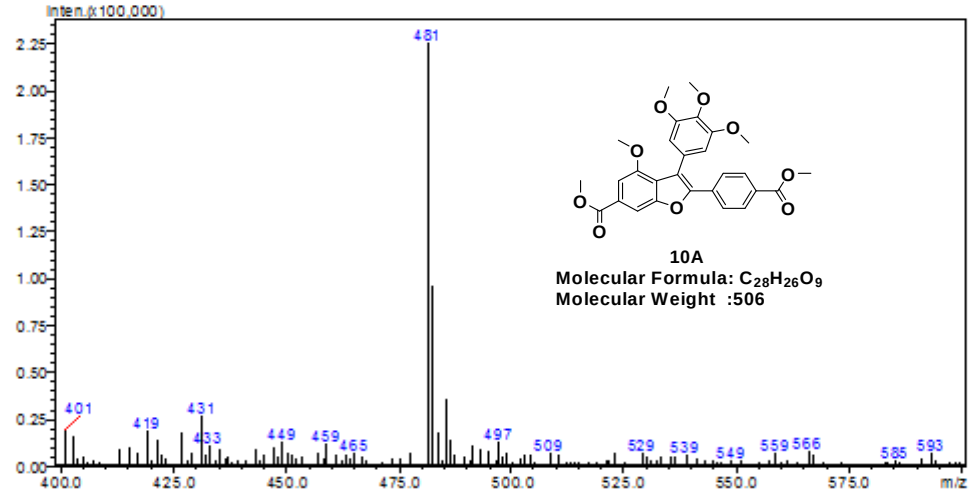
UV-Spectra



ESI+



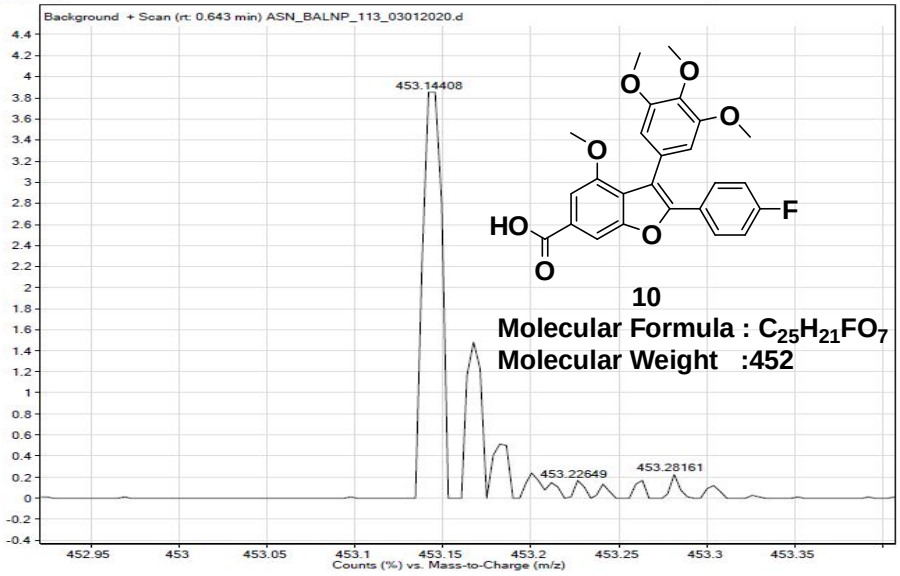
ESI-



10A
Molecular Formula: $C_{28}H_{26}O_9$
Molecular Weight :506

Compound 10: HRMS (ESI-TOF) m/z [M+H] calcd for $C_{25}H_{21}FO_7$, 453.1305, found 453.1440

Sample Name	ASN_BALNP_113	**Position**	P2-B5	**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.1	**InjPosition**	
Sample Type	Blank	**IRM Calibration Status**	Success	**Data Filename**	ASN_BALNP_113_03012020.d
ACQ Method	MeOH_EO_MMS_19012019.m	**Comment**		**Acquired Time**	1/3/2020 1:20:40 PM



10

Molecular Formula : $C_{25}H_{21}FO_7$

Molecular Weight :452

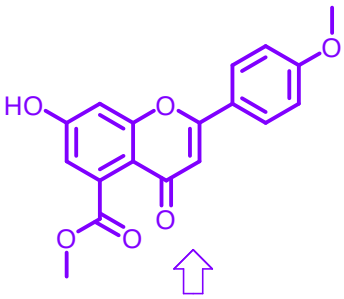
Chapter-3B

Total synthesis of natural product rugosaflavonoid-B.

NB4 IC_{50} 2.2 µM

SHSY5Y IC_{50} 2.5 µM

MCF-7 IC_{50} 2.3 µM



Friedel craft acetylation

Crossed aldol condensation

Iodocyclization

Total synthesis

Rugosaflavonoid-B

Rosa rugosa

5.1 Introduction

Phytomolecules have great importance in our day to day life. We are taking natural products as medicine, supplements, aroma, in the pure form as well as in the form of herbal. Natural molecules may be from any source like medicinal, aromatic plants, marine, microbes etc. For example taxol is isolated from the *Taxus brevifolia* it has many semi synthetic derivatives like taxotere-docetaxel and cabazitaxol.Vincristine (oncovin) and vinblastine (velban) are isolated from the *Catharanthus roseus* navelbine (vinorelbine) is a semi-synthetic derivatives of vinca alkaloids . Podophyllotoxin isolated from the *Podophyllum peltatum*, tenifoside-VM-26, etoposide-vepesid its semi-synthetic derivatives. Camptothecin-camptosar isolated from the plant *Camptotheca acuminate*, it is also has two derivative, topotecan-hycamtin; Irinotecan was derivative of camptothecin. Combretastain A-4 isolated from the *Cambretum caffrum* plant.

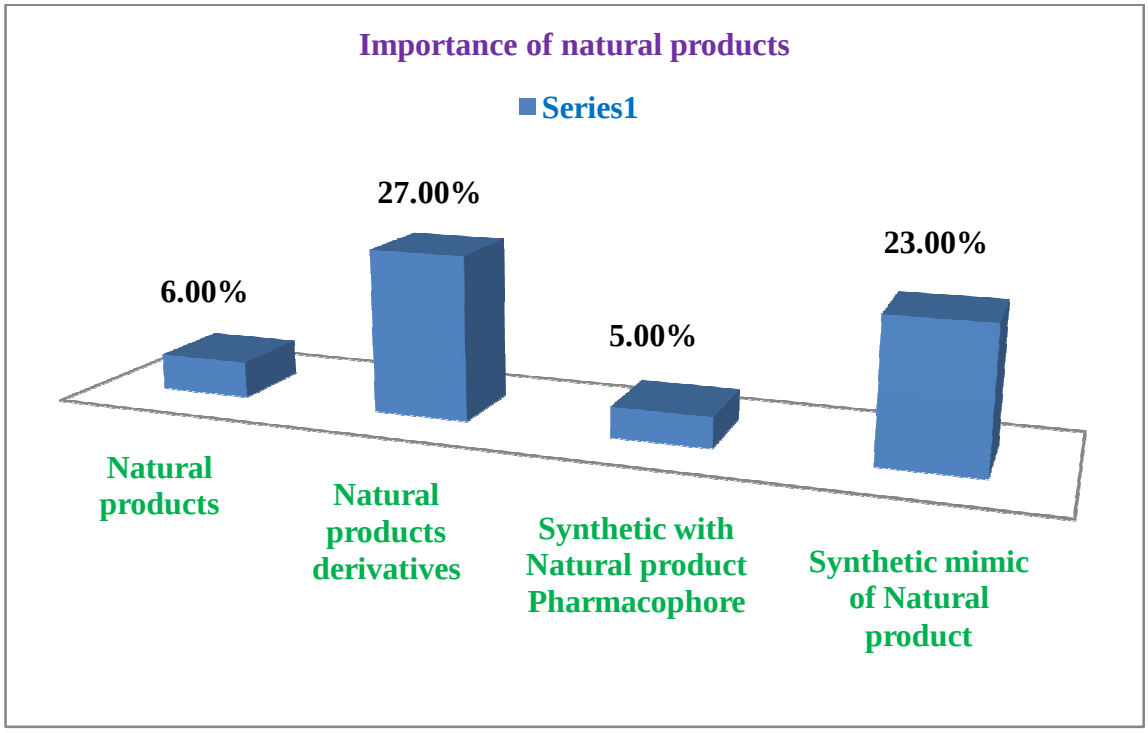


Figure (1): Importance of natural products in market

Natural products and their derivatives effectively served as medicines in last 50 years. Almost 61% of the FDA approved and pre-NDA candidates are natural product or their derivatives [1]. Details are given in the Figure (1). Almost 225 drugs in the testing procedure in the preclinical, clinical and preregistration by FDA. Sources are presented in the Figure (2). From 1940 to 2014 only 140 natural products has been

approved by the FDA. According to recent literature almost 617 molecules labeled as active anticancer molecules and 2892 natural molecules are labeled as inactive molecules [2].

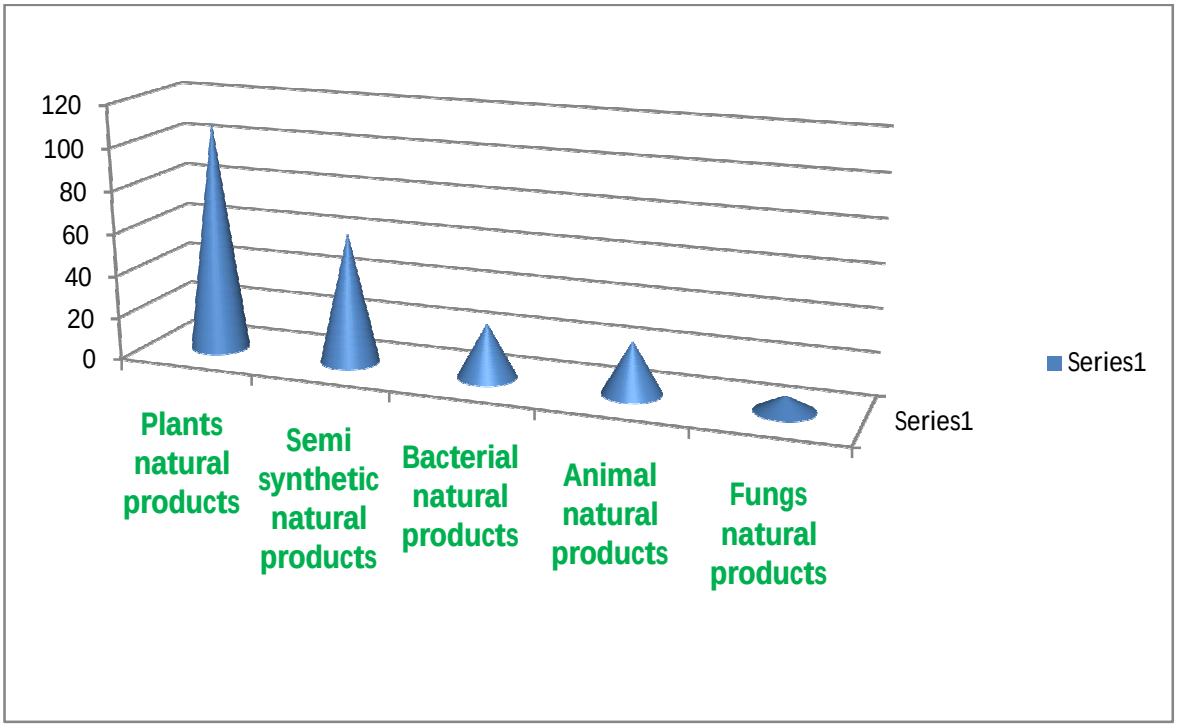
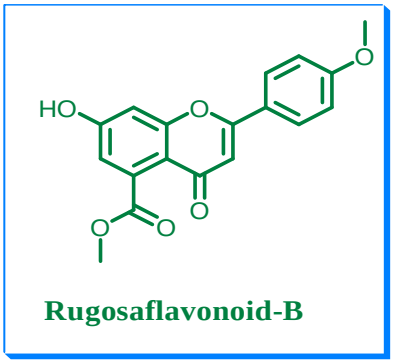


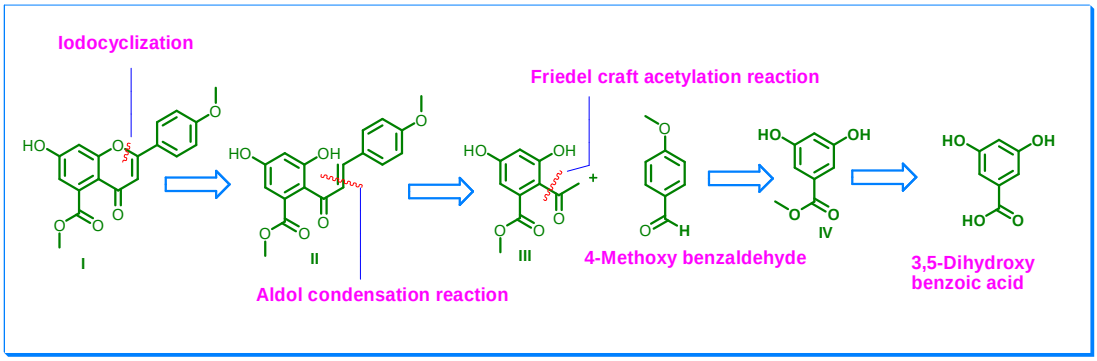
Figure (2): Different sources of natural products

By keeping the importance of natural products we have selected the rugosa flavonoid for the total synthesis. Rugosaflavonoid-B isolated from the common ornamental flower plant *Rosa rugosa* (Rosaceae) which is distributed in the temperate regions of eastern Asia and widely cultivated in Yunnam province [1]. *Rosa rugosa* plant petals and buds are used in the food, incense in china. Its medicinal uses also there like diarrhea, stomachache and gynecological problems. Xue-Mei Gao group isolated rugosaflavonoid-B from this plant and reported its anticancer activity on five human cancer cell lines. We have selected it for total synthesis and its derivatization to generate more lead molecules to see its activity improvement [3-7].

5.2 Retro synthetic approach to rugosaflavonoid-B



Scheme-1



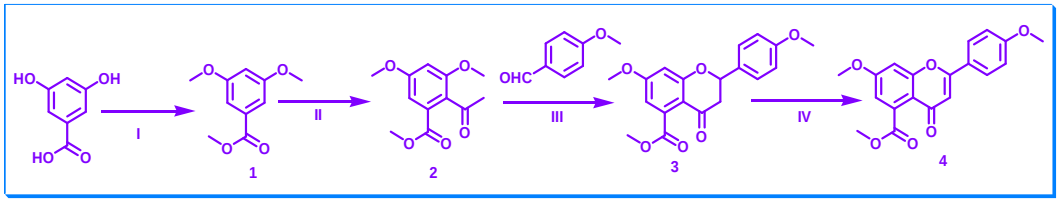
Retro-synthetic approach for the rugosaflavonoid-B presented in the scheme-1.The targeted molecule synthesis can be started with the readily available molecules is 3,5-dimethoxy benzoic acid esterification followed by protection of one hydroxyl group can be provide synthon-IV . Then the synthesis of synthon-III can be done through friedel craft acetylation or boron trifluride etherate. By using synthon-III and 4-methoxy benzaldehyde to prepare synthon-II envisioned *via* aldol condensation reaction to get final target-I.

5.3 Synthetic strategy

In scheme-2, we started with 3, 5-dihydroxy benzoic acid as starting material, methylation done by using dimethoxy sulphate to protect the both hydroxyl and the carboxylic acid group to get compound **1**. Further compound **1** used in friedel-craft

acylation reaction with aluminum chloride in carbon disulfide afforded an acetylated product **2**. Finally compound **2** reacted with anisaldehyde in presence of the molecular iodine and pyrrolidine in DMSO at refluxed condition to get the compound **3**. Further final step and its derivatization is progress in our lab.

Scheme -2



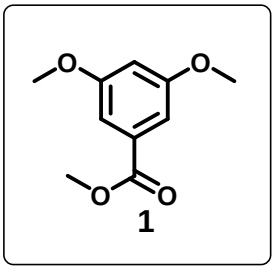
Scheme-2: Reagents and conditions: I). 3, 5-Dihydroxybenzoic acid (930 mg, 6.03 mmol), dry Acetone, Dimethyl sulphate (500 mg, 3.96 mmol), K_2CO_3 (550 mg, 3.98 mmol), 80^0C, 8 h, 93%. (II). Compound **1**, Acetyl chloride (1 gm, 5.10 mmol), CS_2 (2 mL, 26.31 mmol), $AlCl_3$ (2 gm, 15.0 mmol), 46%. (III) Compound **2** (50 mg, 0.212 mmol), Anisaldehyde (4 mg, 0.29 mmol), I_2 (5 mg, 0.0197 mmol), Pyrrolidine (6 mg, 0.0845 mmol), Dimethyl sulphate (5 mL), 8 h reflux, 35%. (IV) Compound **3** (200 mg, 0.583 mmol), Iodine (8 mg, 0.0316 mmol), DMSO (5 mL).

5.4 Experimental section

Synthesis of methyl-3, 5-dimethoxybenzoate (1):- Dry 250 mL round bottom flask taken and added 3, 5-dihydroxy benzoic acid (930 mg, 6.03 mmol), dry acetone (30 mL), potassium carbonate (550 mg, 3.98 mmol), dimethyl sulphate (500 mg, 3.96 mmol), were added at RT. Then heated the reaction mixture up to 80ºC, stirring continued for 8 h. After completion of the reaction, the reaction mixture extracted with the EtOAc 3 times, and then combined organic layer were dried over sodium sulphate and concentrated on rotary evaporator to get the compound-**1** in 93% yield.

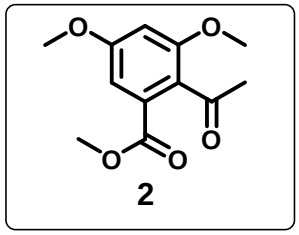
^{1}H NMR (500MHz, $CDCl_3$): δ 3.82 (s, 6H, OCH_3), 3.88 (s, 3H, OCH_3), 6.56 (bs, 1H, CH aromatic), 7.09 (d, 2H, CH aromatic),

^{13}C NMR (125 MHz, $CDCl_3$): δ 52.10, 55.43, 105.51, 107.03, 131.95, 160.62, 107.03, 171.05.



Synthesis of 2-acetyl-3, 5-dimethoxybenzoate (2):- To the stirring solution of the compound-**1** (1 gm, 5.10 mmol) in acetyl chloride (2 gm, 25.0 mmol), to this mixture added carbon disulfide (2 mL, 26.31 mmol) at 0-2ºC, then $AlCl_3$ (2 gm, 15.0 mmol) added at vigorously stirring and stirring continued for 15 min then reaction progress monitored by TLC. The reaction finished within the 1.5 h then the reaction mixture quenched with ice continuous stirring and extracted with ethyl acetate and combined organic layer are dried over sodium sulphate. The solvent concentrated on rotary evaporator the yielded crude subject for the column chromatography to get the compound-**2** in 46% yield.

^{1}H NMR (500MHz, $CDCl_3$): δ 2.52 (s, 3H, CH_3), 3.82 (s, 6H, OCH_3), 3.88 (s, 3H, OCH_3), 6.62 (d, 1H, CH aromatic).

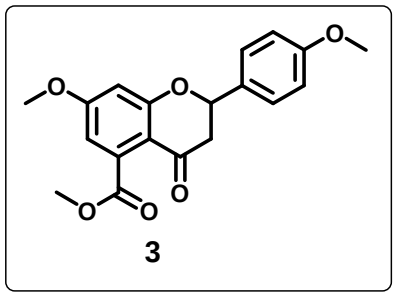


2

^{13}C NMR ($CDCl_3$, 125 MHz): δ 52.49, 56.00, 102.88, 105.23, 126.83, 129.61, 160.81, 166.56, 203.35.

Synthesis of methyl -3, 4-dihydro-7-methoxy-2-(4-methoxyphenyl-4-oxo-2H-chromene-5-carboxylate (3):- 100 mL round bottom flask taken and 2-acetyl-3, 5-dimethoxybenzoate **2** (50 mg, 0.212 mmol) in DMSO (5 mL), mixed with 4-methoxy benzaldehyde (4 mg, 0.29 mmol), molecular iodine (5 mg, 0.0197 mmol) and pyrrolidine (6 mg, 0.0845 mmol), were added and reaction temperature was increased up to reflux for 7h. After completion of the reaction, the reaction mixture cooled to the RT and quenched with water, extracted thrice with the EtOAc, then combined organic layer were dried over sodium sulphate and concentrated on rotary evaporator to get the compound **3** (19 mg) 35% in yield.

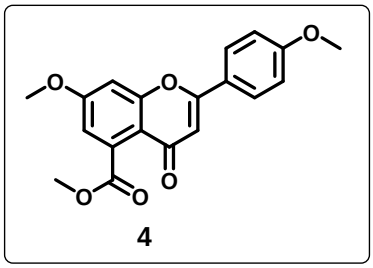
^{1}H NMR (500MHz, $CDCl_3$): δ 3.36-3.44 (d, 2H, CH_2), 3.86 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 5.92 (s, 1H, CH), 6.68 (s, 1H, CH aromatic), 6.81 (d, 2H, CH aromatic), 6.94 (s, 1H, CH aromatic), 7.14 (d, 2H, CH aromatic).



3

^{13}C NMR (125 MHz, $CDCl_3$): δ 41.78, 56.17, 56.28, 99.22, 105.24, 105.34, 105.87, 130.00, 156.03, 168.80, 195.82; HRMS (ESI-TOF) m/z [M+Na] calculated for $C_{19}H_{18}O_6$, 342.1137, found 365.1062.

Synthesis of methyl -7-methoxy-2-(4-methoxyphenyl)-4-oxo-4H-chromene-5-carboxylate (4):- In a 100 mL round bottom flask taken and mixture of compound **3** (200 mg, 0.583 mmol), and molecular iodine (8 mg, 0.0316 mmol), were added in to DMSO (5 mL), then refluxed for 1 h. Reaction progress monitored by TLC. After completion of the reaction, the reaction mixture cooled to the RT and quenched with water, extracted with the EtOAc 3 times, then combined organic layer were dried over sodium sulphate and concentrated on rotary evaporator to get the mixture of compound **4** (approx 3 mg) 42 % in yield.



4

References:

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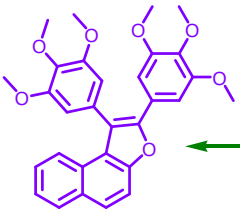
Chapter-4

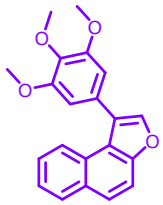
Design, synthesis of 2, 3-diaryl naphthofuran and its derivatives as an antibreast cancer agent.

Potent derivatives on antibreast cancer



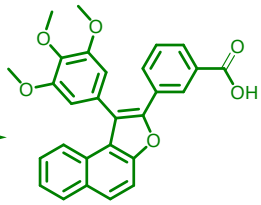
7C MCF-7 15.54±0.1111 µM

MDA-MB-231 16.19±0.083 µM



4 MCF-7 17.56±0.2486 µM

MDA-MB-231 8.154±0.092 µM



7D MCF-7 15.13±0.1515 µM

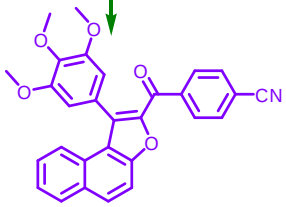
MDA-MB-231 10.04±0.067 µM

Selectivity index= >4.98

Microtubule destabilizer

Bioavailability score: 0.56

Safety/toxicity: safe & well tolerable up to 1000 mg/kg



8E MCF-7 18.05±0.1368 µM

MDA-MB-231 9.921±0.063 µM

6.1 Introduction

Cancer is a group of disease which spreads over one to one another organ. If it is not detected early controlling the disease may not possible even though average life time about 5 years after detection of the disease. Its exact causes remain elusive. Till now there was no such drug to cure completely the cancer. According to WHO breast cancer was one of the main cancers in female which was responsible for 42170 deaths in female and in male 520 deaths in the year 2020. According to the cancer Facts and Figures-2020 [1] the cancer generally develops in above 55 years old persons and breast cancer affecting both genders relative percentage less in male. Cancer prevalence increased one who consumes alcohol and tobacco. There were many other reasons like family background, genetic effect and physical activity increasing the cancer occurrence. American cancer society made few recommendations to early detection of the cancer to avoid the risk. 1). Mammography's test should be taken in the age group of 40-44 years for ladies. 2) Mammography test should be conducted to the females every year between the age group 45-54 years.

Breast cancer is the most common cancer diagnosed in women. It is a multifactorial disease with diverse morphology and complications [2]. Early, accurate and affordable diagnosis is one of major problems associated with the disease [3]. Generally, breast cancer is defined with three biomarkers estrogen receptor (ER), progesterone receptor (PR) and human oncogene HER2 basis. Hormone dependent breast cancer is the most abundant type breast cancer which covers about 65% of total breast cancer cases. Estrogen antagonists like tamoxifen and raloxifen and aromatase inhibitors like anastrozole, exemestane and fulvestrant as estrogen receptor down-regulator are used to tackle such type of breast cancer [4]. On an average the hormone responsive breast cancer can be managed more than 15-20 years. On the other hand, triple negative breast cancer (ER-ve, PR –ve, and HER-2 –ve) is very aggressive type cancer which occurs in about 15-20% of all breast cancer cases. Triple negative breast cancer (TNBC) is fatal and considered difficult to treat. Various types of cytotoxic drugs are used tackle this type of breast cancer with meager success only. Some of the notable drugs are paclitaxol and docetaxel (Antimitotic), doxorubicin (Topoisomerase-II inhibitor), cisplatin (DNA intercalation), 5-fluorouracil (Antimetabolite), abemaciclib (CDK inhibitors), Olaparib (PARP inhibitors), and Gefitinib (EGFR inhibitor) etc, Figure (1). However, TNBC is very difficult to treat

due to lack of prognostic biomarkers [5]. There is no systematic and accurate treatment available for this type of breast cancer cases.

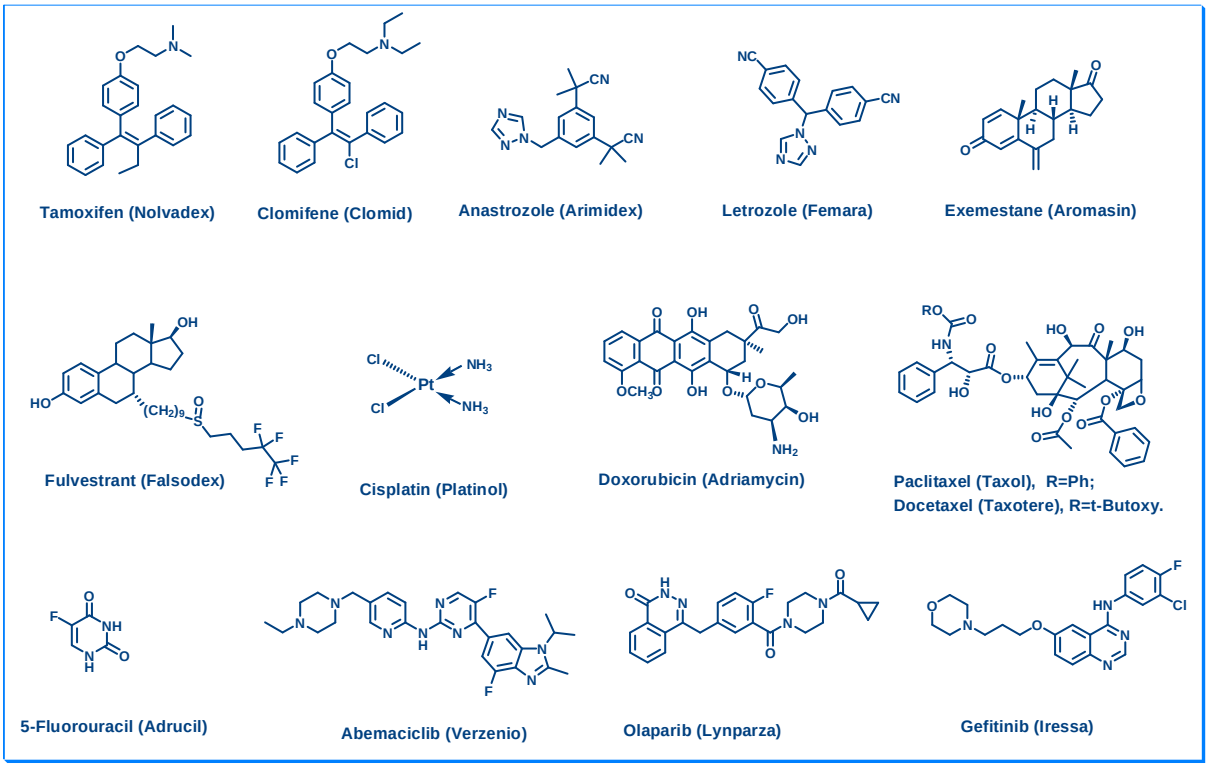


Figure (1): Some of the notable drugs for the breast cancer

6.2 Naphthofuran core as anticancer agents

Naturally occurring naphthofuran core containing molecules exhibiting several biological activities were antibacterial [6] anticancer [7] antioxidant, antifungal [8] and dual inhibitors of alzheimer's disease [9]. Furomollugin was isolated from the *Rubia cordifolia* [10] and Rubioncolin A and Rubioncolin B were isolated from the *rubia oncotricha* as well as *Rubia cordifolia.* In traditional medicine, *Rubia cordifolia* were using to treat the various diseases like cough, joint inflammation, bladder and kidney stones, uterine hemorrhage and uteritis [11, 12]. Radermachol compound was isolated from the plant of *Radernacgera xylocarpa* in India and it has been used for the treatment of nervine, calmative and other diseases [13, 14].

More than 30 drugs which contain naphthofuran moieties have been approved by Food and Drugs Administration (FDA) [15]. On the basis of furomollugin derivatives were synthesized and it was significantly inhibited tumor necrosis factor (TNF) alpha-induced inflammatory responses in HT-29 human colon epithelial cells [16].

Compound-8 was identified as an efficient lifespan altering agent of eukaryotic cells [17]. Then compound balsaminone A was used to treat antipruritic activity.

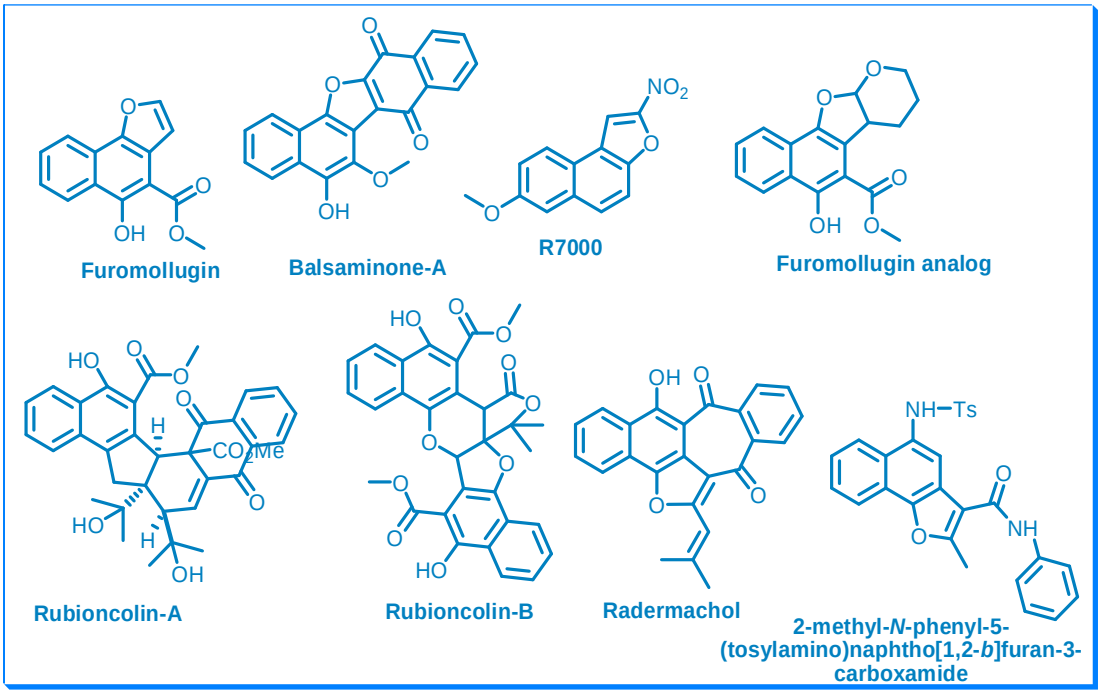


Figure (2): Naphthofuran core contains natural and synthetic naphtho [1, 2-b] furans compounds.

The broad range of biological activity of naphthofuran has attracted too many chemists to do total synthesis and their derivatization to generate a library of compounds in order to get hit molecules. Then we have developed four-step efficient methods which can provide high yield through brominating, etherification, cyclization and heck- cross-coupling reaction which provide various derivatives.

6.3 Basis of hypothesis

Generally, nature was the best source for the new class chemical entities and plants were the finest chemist in this world. Nowadays most of the drugs in the market were from medicinal plants. For example *Taxus wallichiana* (Taxol), *Catharanthus roseus* (vincristine and vinblastine), *Artemisia annua* (artemisinin), *Berberis aristata* (berberine), *podophyllum* (podophyllotoxin) and *Camptotheca acuminata* (camptothecin), topotecan was derivative of camptothecin. Colchicine isolated from the plant of *Colchicum autumnal*.

Most of the molecules which were active on cancer having trimethoxy phenyl system on aryl ring which was mandatory for the activity this has been revealed from their structure-activity relationship. Here some examples were presented below.

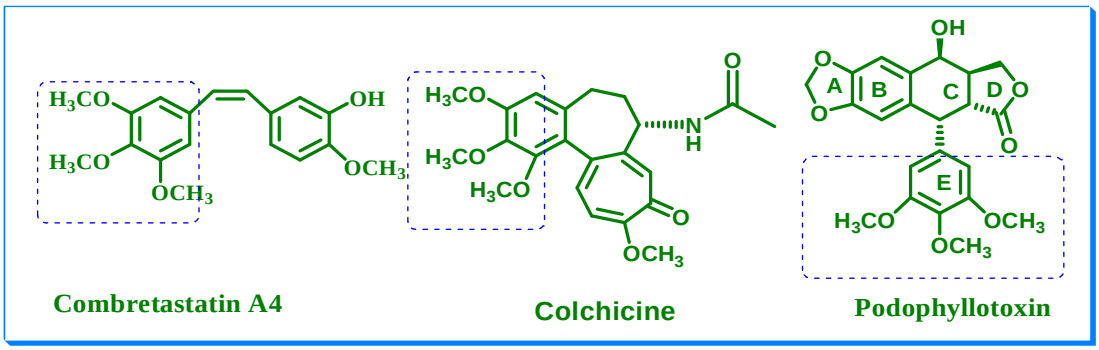


Figure (3): Natural molecules contain a trimethoxy system.

Endogenous estrogens were mainly affected growth, differentiation, and function of female reproductive organs. Estradiol also plays a major role in maintaining bone density as well as protection against osteoporosis. All the above effects were mediated through its binding to estrogen receptors which were over expressed in hormone-dependent breast cancer.

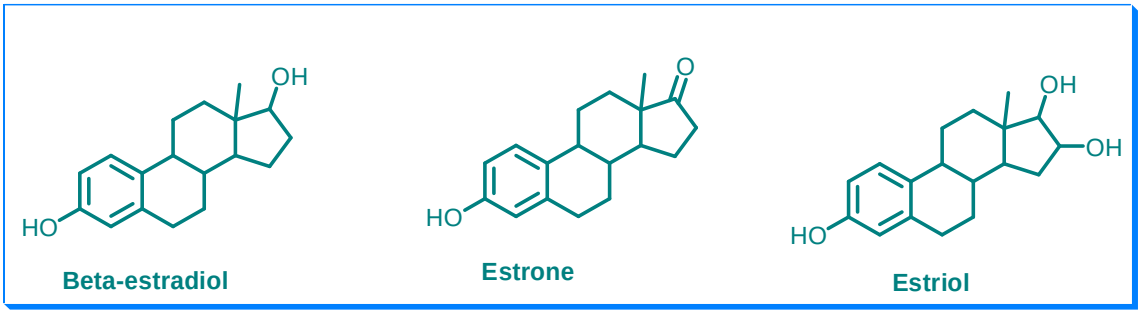


Figure (4): Beta-estradiol, estrone and estriol as a carrier.

Fragment based drug discovery (FBDD) is a systematic and relatively a new approach to design pharmacophore in drug discovery [18]. This approach identifies a small fragment (or motif) in a pharmacophore to have quality interactions with the biological target [19, 20] Nowadays, low molecular weight “fragment hits” are being validated to bind to a target protein which has become an effective starting point for drug discovery [21]. It has become so popular that pharmaceutical industries have adopted this approach and yielded some drug candidates [22-24]. In a similar approach, we identified a small fragment i.e. 3, 4, 5-trimethoxyphenyl as an

antitubulin motif [25, 26]. This fragment is also present in some of the naturally occurring antitubulin like colchicine, podophyllotoxin, combretastatin A4 etc [27, 28]. Based on this preliminary information, we designed prototype on naphthofuran core.

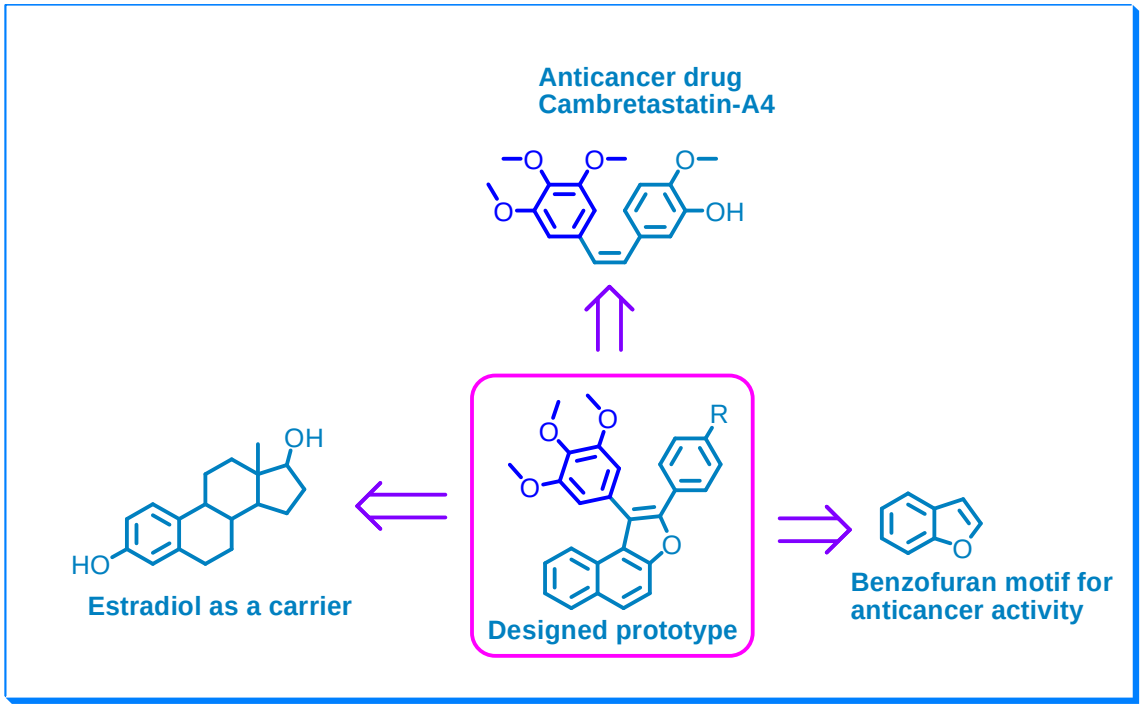


Figure (5): Designed prototype

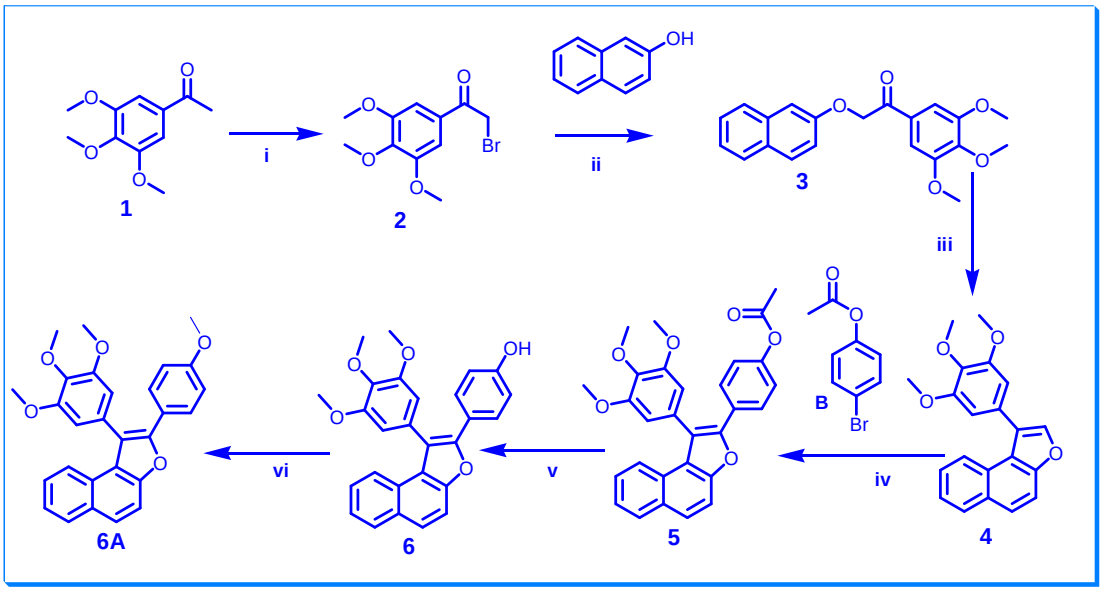
6.4 Results and discussion

6.4.1 Synthetic strategy

Scheme-1

In scheme-1, we have started with 3, 4, 5- trimethoxy acetophenone **1** as a starting material it was brominated with Br_2 in diethyl ether at room temperature to get compound **2** in 88% yield**.** Then the compound **2** with 2-naphthol esterfication has done in DMF with potassium carbonate at room temperature to get compound **3** in 95% yield. The cyclization of compound **3** with trifluoroacetic acid as a solvent cum reagent at room temperature to get compound **4** in 90% yields**.** Compound **4** and compound B were condensed together in the presence of heck cross-coupling reaction at 80ºC temperature to get compound **5** in 92% yield and compound **5** used in deprotection of phenolic group in presence of potassium hydroxide 5% in methanol

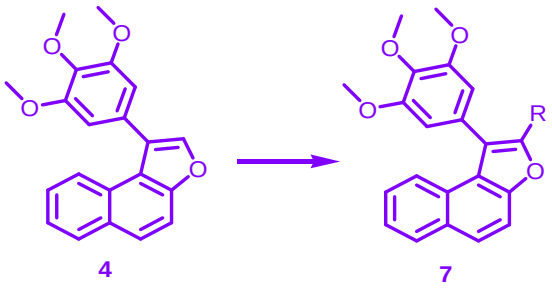
and water at room temperature to get the final pharmacophore **6** in 98% yield. By using final pharmacophore **6** we prepared one derivative of **6A** in 90% yield.



Scheme-1: Reagent and condition: **(i)** Br_2, Diethyl ether, RT, 2h, 88% **(**ii**)** 2-Naphthol, K_2CO_3, DMF, RT, 4h, 95% **(**iii**)** Trifluroacetic acid, RT, 5h, 90 % **(**iv**)** Para-acetoxy bromobenzene, DMA, $Pd(OAc)_2$, 80ºC, 4h, 92 %. **(**v**)** KOH, Methanol / H_2O (9:1), 80ºC, 30 min, 98%. (vi) Dimethyl sulfate, K_2CO_3, Acetone, 80ºC, 1h, 90%.

Scheme-2

In scheme-2, 1-(3, 4, 5-trimethoxy phenyl) naphtho [2, 1-b] furan **4** was taken as a starting substrate, by using the heck cross-coupling reaction in presence of different aryl benzoyl substrate (halogenated), N, N-Dimethylacetamide, palladium acetate and potassium acetate were added to the reaction at RT. Then temperature was raised upto at 80ºC for 4-6 hours, to get the final compound **7**. Further we have prepared seven derivatives from **7A-7G** by using pharmacophore **7**. Their yields were in the range of 85-90%. All the derivatives are confirmed by NMR spectroscopy and mass spectrometry data.



7A. R= p-Flurobenzene

7B. R= 3,5-Dimethoxy benzene

7C. R= 3,4,5-Trimethoxy benzene

7D. R= m-Benzoic acid

7E. R= Methyl-3-benzoate

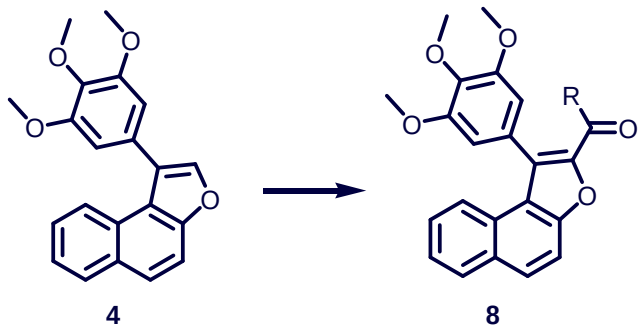
7F. R= Ethyl-3-benzoate

7G. R= Furan-2-carbaldehyde

Scheme-2: Reagent and condition:- Aryl halides, DMA, $Pd(OAc)_2$, KOAc, 80ºC, 4h, 86-90 %.

Scheme-3

1-(3, 4, 5-trimethoxy phenyl) naphtho [2, 1-b] furan **4** was taken as a starting substrate in the scheme-3. By using the heck cross-coupling reaction in presence of different aryl benzoyl halide substrate, N, N-Dimethylacetamide, palladium acetate and potassium acetate were added to the reaction at RT. Then temperature increased up to at 80ºC for 4-6 hours, to get the final compound **8**. Further, we have prepared five derivatives from **8A-8E** by using pharmacophore **8**. Their yields are in the range of 80-90%. All the derivatives are confirmed by NMR and mass data.



8A. R= 3-Nitrophenyl

8B. R= 3,4,5-Trimethoxy phenyl

8C. R= 2-Nitrophenyl

8D. R= 4-Chlorophenyl

8E. R= 4-Cyanophenyl

Scheme-3: Reagent and condition: Benzoyl halide, DMA, $Pd(OAc)_2$, Potassium acetate, 80ºC, 4h, 85-89%.

6.5 Biological evaluation

6.5.1 Cytotoxicity evaluation by sulphorhodamine B (SRB) assay

Sixteen new derivatives were prepared and evaluated for their anti-cancer activity evaluation. All the derivatives were tested on two human cancer cell lines MCF-7 and MDA-MB-231 by Sulphorhodamine assay. Out of sixteen molecules four molecules were active against MCF-7 and MDA-MB-231 cells i.e. IC_{50}<20 µM Table (1). Two molecules **7D** (IC_{50}=10.04 µM) and **8E** (IC_{50}=9.92 µM) showed significant anticancer activity. Further, all these compounds were also evaluated against normal cell line HEK-293T to assess the toxicity of the compounds. Compounds did not show any toxicity up to 50 µM concentration.

Table (1): Cytotoxicity evaluation of 2, 3-diaryl naphthofuran derivatives against human breast cancer cell lines

S. no.	Compound codes	Cell lines IC_{50} (µM)			
		MCF-7	MDA-MB-231	Hek-293T	Selectivity index (SI)[#]
1	5	>20	>20	>50	
2	6	>20	>20	>50	
3	6A	>20	>20	>50	
4	7A	>20	>20	>50	
5	7B	>20	>20	>50	
6	7C	15.54±0.1111	16.19±0.083	>50	>3.09
7	**7D**	**15.13±0.1515**	**10.04±0.067**	>50	>4.98
8	7E	>20	>20	>50	
9	7F	>20	>20	>50	
10	7G	>20	>20	>50	

11	8A	>20	12.00±0.028	>50	>4.17
12	8B	>20	>20	>50	
13	8C	>20	>20	>50	
14	8D	>20	>20	>50	
15	8E	18.05±0.1368	**9.921±0.063**	>50	>5.04
16	4	17.56±0.2486	**8.154±0.092**	>50	>6.13
17	Doxorubicin	2.585±0.1213	4.127±0.0739	>20	>4.84
18	Tamoxifen	10.74±0.2730	9.501±0.1163	>20	>2.11

6.5.2. Tubulin polymerization inhibition

Tubulin kinetics provides conversion of α/β tubulin monomer/dimer units (tubulins) to polymer unit (microtubules). The rate of polymerization is either stabilized or destabilized by antimitotic agents. The lead compound **7D** destabilized the polymerization process which is evident from the kinetic curve.

In Figure (6) the curves of paclitaxel (PAC) are above the control curves (GTB and DMSO) showing stabilization effect. While podophyllotoxin (PDT) curves are below the control curves showing destabilization effect on microtubules. Compound **7D** curves also exist below the control curve, clearly indicating destabilization effect but less than PDT. The naphthofuran derivative exhibited tubulin polymerization inhibition. Modulation of tubulin-microtubules dynamics is one of the most effective targets for cancer chemotherapeutics [29]

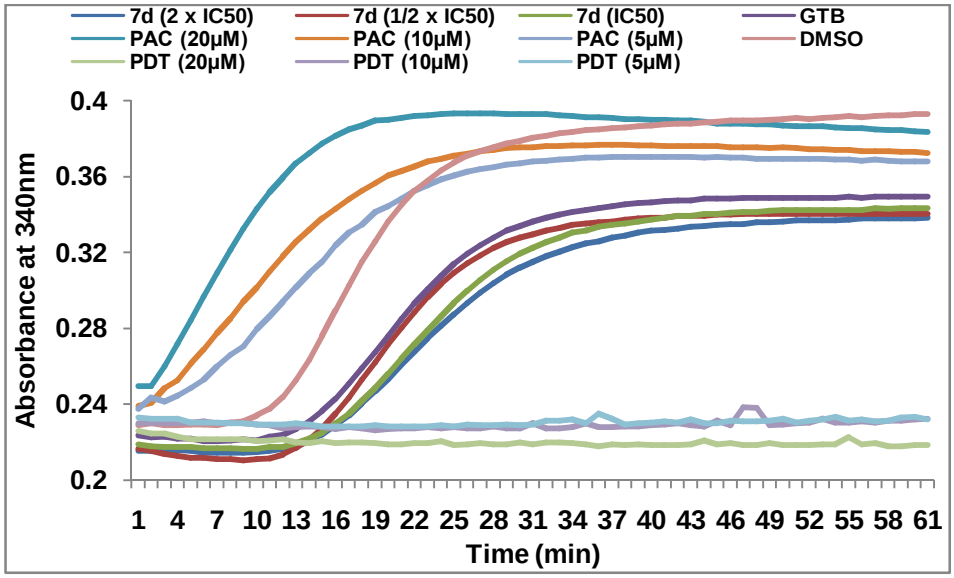


Figure (6): Tubulin polymerization kinetic curve GTB and DMSO are control curves, PAC is paclitaxol (Stabilizer), PDT (destabilizer), and diarylnaphthofuran derivative **7D** at half IC_{50}, IC_{50} and double IC_{50}.

6.5.3. Molecular docking studies

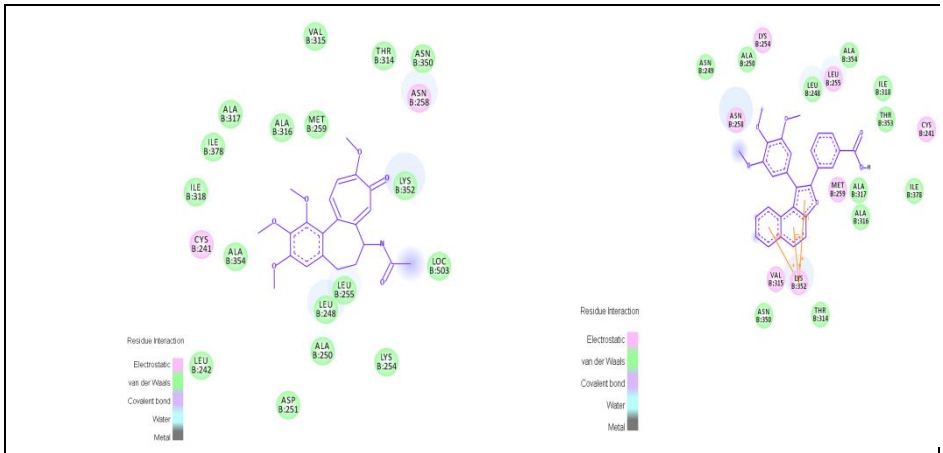
Naphthofuran derivative **7D** interactions were assessed with target protein i.e β-tubulin. Further, ADME properties were also predicted to see their bioavailability.

6.5.3.1 Interaction with β-tubulin

The naphthofuran lead compound **7D** was evaluated for affinity with β-tubulin. In molecular docking studies, the compound **7D** occupied same binding pocket of colchicine Table (2). The binding energy was quite comparable to colchicine. There were sixteen common residual amino acids i.e. CYS241, LEU248, ALA250, LEU255, ASN258, MET259, THR314, VAL315, ALA316, ILE318, ASN350, LYS352, LYS254, ALA317, ILE378 and ALA354 clearly indicating same binding pocket of β-tubulin occupied by all the three ligands. Comparing with colchicine for docking energy, the ligand **7D** showed additional binding *via* π-π stacking through phenyl rings (as shown in docked view Figure (7) which might have provided stronger affinity with the residual amino acids as compared to colchicine (no such interaction found)

Table (2): Docking energy and residual amino acids within 4Å of colchicine (positive control) and naphthofuran derivative **7D** with the protein target β-tubulin (PDB: 4O2B).

S. No.	Compounds	Docking Energy kcal/mol	Interacting Amino Acids within region of 4Å
1.	Colchicine	-8.5	**CYS241**, LEU242, **LEU248**, **ALA250**, ASP251, **LYS254**, **LEU255**, **ASN258**, **MET259**, **THR314, VAL315**, **ALA316**, **ALA317**, **ILE318**, **ASN350**, **LYS352**, **ALA354**, **ILE378.**
2.	**7D**	-8.8	**CYS241**, **LEU248**, ASN249, **ALA250, LYS254**, **LEU255**, **ASN258**, **MET259**, **THR314, VAL315, ALA316**, **ALA317**, **ILE318**, **ASN350**, **LYS352**, THR353, **ALA354**, **ILE378**.



Colchicine	**7D**

Figure (7): Docked view of naphthofuran derivative **7D** at active site showing crucial interactions with amino acids. Colour coded interactions: Pink, electrostatic; green, Vander Walls; purple, covalent bond; sky-blue, water and gray, metal.

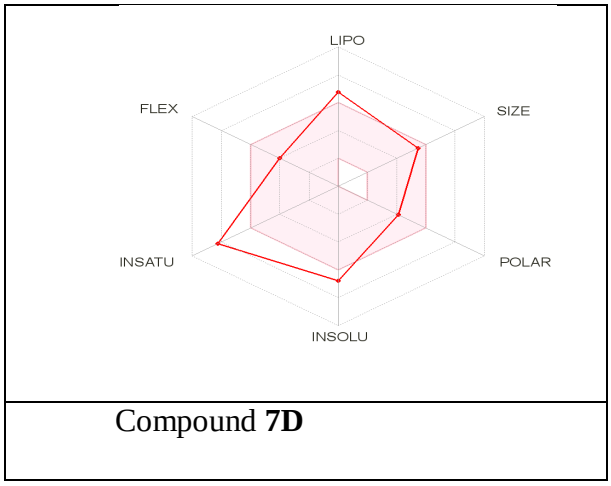
6.5.3.2 *In-silico* prediction of ADME properties

Physicochemical properties of a drug candidate give an idea about the druggability of the molecule. To be an effective drug candidate, the molecule must reach to its biological target in the body in sufficient concentration, and stay there for sometimes in a bioactive form to induce desired biological response. Pharmacokinetic properties of the naphthofuran derivative **7D** were determined using online Swiss ADME software program [30]. Physicochemical parameters, water solubility, lipophilicity, pharmacokinetics, drug likeness and medicinal chemistry aspects were calculated. The compound showed good druggability. Most of the required parameters were within the desired limits Table (3). Compound **7D** did not possess PAINS (Pan-assay interference compounds) structure. However, the lead compounds have violation within acceptable limit in Lipinski's rule of five [31]. Overall, with these physiochemical parameters the compound **7D** possess low oral bioavailability and hence moderate druggability. Further, Figure (8) exhibits bioavailability radar of compound **7D** slightly deviate at some places which is due to high level of unsaturation (Low C_{Sp3} ratio). The bioavailability radar enables a first glance at the drug-likeness of a molecule. The compound **7D** were possessed moderate bioavailability scores limits.

Table (3): Various druggability parameters of naphthofuran derivative- **7D**.

Parameter	**7D**	Acceptable range	Parameter	**7D**	Acceptable range
Physicochemical properties			Lipophilicity		
Molecular formulae	C28H22O6	---	Log $P_{o/w}$	5.18	≤5
M. Wt.	454.47	≤500	Pharmacokinetics		
Rotable bonds	06	≤10	GI absorption	Low	---
H-bond acceptors	6	≤10	BBB permeability	No	---
H-bond donors	1	≤5	P-gp substrate	Yes	---
Molar Refractivity	131.03	40-130	CYP1A2 inhibition	No	---
C_{Sp3} hybridisation fraction	0.11	Not less than 0.25	CYP2C9/19 inhibition	Yes	---

TPSA	78.13Å^2	20 Å^2 to 130 Å^2	Drug likeness		
Water solubility			Lipinski rule	no violation	Up to 1 violation
Water solubility	0.08 µg/mL		Bioavailability score	0.56 good	moderate
Solubility class	Low	acceptable	Medicinal chemistry		
Log S	-7.73	>-6	PAINS (False bioactivity)	No	---
			Synthetic accessibility	3.96	1-10 Easiest-most difficult



Compound **7D**

Figure (8): Two dimensional bioavailability radars for the naphthofuran derivative **7D**

(Six physicochemical properties are taken into account: lipophilicity, size, polarity, solubility, flexibility and saturation. The pink area represents the optimal range for each properties (lipophilicity: XLOGP3 between − 0.7 and + 5.0, size: MW between 150 and 500 g/mol, polarity: TPSA between 20 and 130 A^2, solubility: log S not higher than 6, saturation: fraction of carbons in the sp3 hybridization not less than 0.25, and flexibility: no more than 9 rotable bonds. In this example, the compound **7D** possess moderate bioavailability but low water solubility)

6.6 Safety studies

6.6.1 *In-vivo* acute oral toxicity of 7D in *Swiss albino* mice

In-vivo acute oral toxicity experiment for the lead compound **7D** was carried out in *Swiss albino* mice at four different doses 5, 50, 300 and 1000 mg/kg once orally. No significant changes were observed in all the parameters studied like morbidity, mortality, observational parameters, body weight, serum biochemical parameters, organ weight and most of the haematological parameters up to the dose level of 1000 mg/kg body weight.

Table (4): Effect of naphthofuran derivative **7D** as single acute oral dose at 5, 50, 300, 1000 mg/kg on body weight haematological and serum biochemical parameters in *Swiss albino* mice (Mean±SE; n=6).

Parameters		Dose of compound **7D** at mg/kg body weight as a single oral dose				
		Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (gm)		27.68±0.88	26.85±1.48	30.33±1.37	27.58±1.13	29.82±0.88
Haematological profile	Haemoglobin (gm/dL)	12.66±0.623	12.03±0.42	13.26±0.568	11.90±0.52	11.52±0.66
	RBC (million/mm3)	7.37±0.23	6.99±0.19	7.03±0.35	7.75±0.32	7.56±0.26
	WBC (1000*/mm3)	4.69±0.64	4.50±0.24	5.60±0.48	4.11±0.42	4.93±0.28
Liver Function Test	ALP (U/L)	170.63±15.68	152.43±5.45	151.32±15.92	172.15±15.62	176.17±8.92
	SGOT (U/L)	25.54±1.45	31.24±3.14	27.08±2.06	26.59±2.85	24.02±1.72
	SGPT (U/L)	11.19±0.58	16.22±2.67	16.12±1.07	13.77±2.03	13.57±1.05
	Albumin (g/dL)	2.82±0.156	2.77±0.16	3.01±0.136	3.06±0.11	2.97±0.13
	Bilirubin (mg/dL)	0.19±0.008	0.18±0.011	0.182±0.012	0.167±0.011	0.162±0.007
	Serum Protein mg/mL)	6.44±0.09	6.09±0.08	6.48±0.30	6.15±0.09	6.005±0.25
Lipid profile	Triglycerides (mg/dL)	162.15±4.72	184.24±6.00	192.11±5.79	174.72±14.55	187.40±8.51

	Cholesterol (mg/dL)	149.83±9.82	166.42±5.52	166.72±10.03	162.25±3.64	181.63±7.41
Kidney function test	Creatinine (mg/dL)	0.33±0.03	0.36±0.09	0.31±0.05	0.33±0.02	0.35±.02

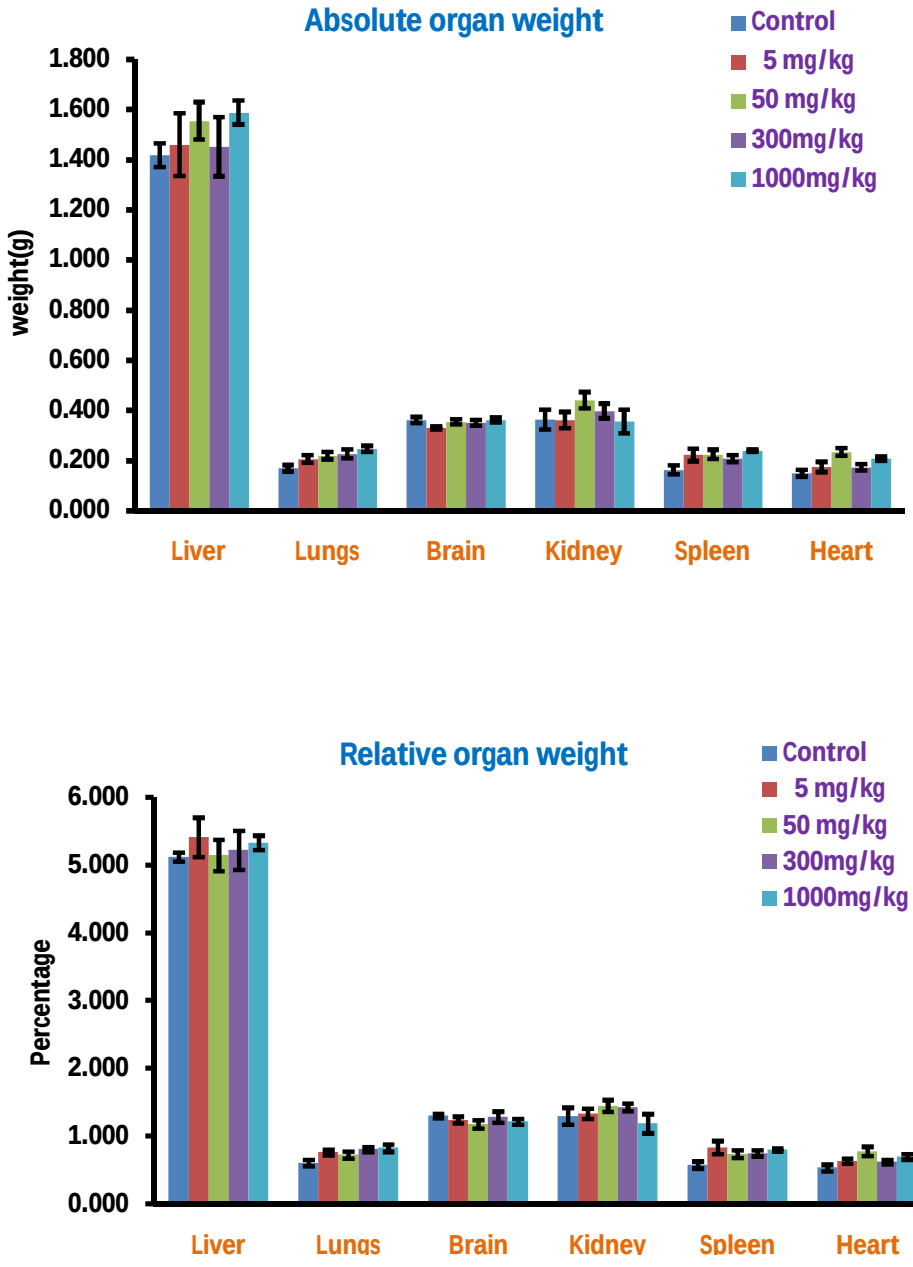


Figure (9): Effect of compound-**7D** as single acute oral dose at 5, 50, 300 mg/kg, and 1000 mg/kg body weight on absolute and relative organ weight in *Swiss albino* mice (Mean±SE; n=6).

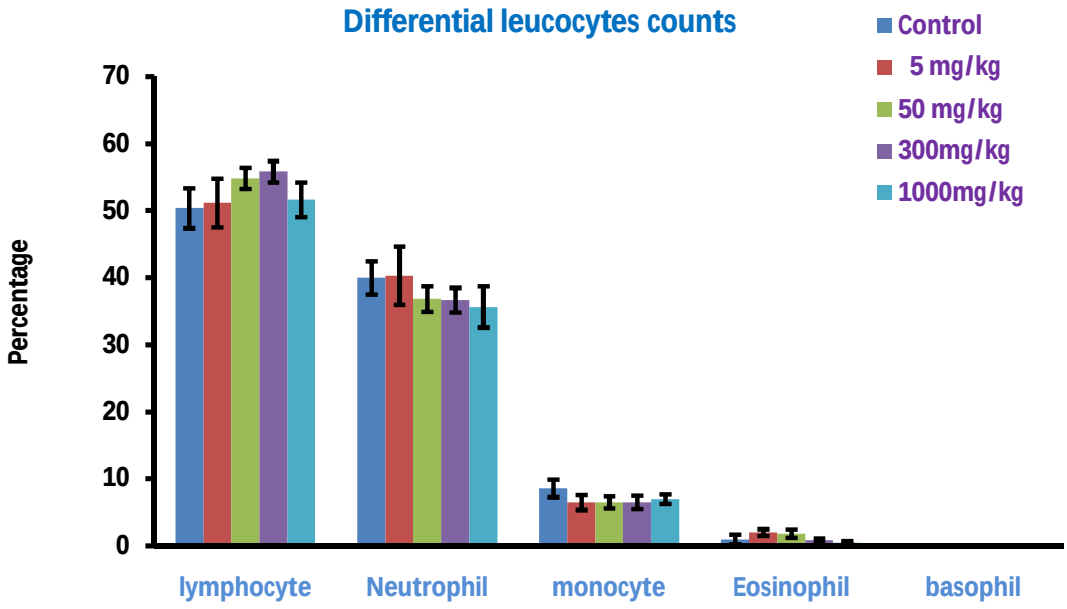


Figure (10): Effect of compound-**7D** as single acute oral dose at 5, 50, 300 mg/kg, and 1000 mg/kg body weight on differential leucocytes counts in *Swiss albino* mice (Mean±SE; n=6).

6.7. Discussion

Breast cancer possesses a complex and varied morphology. Triple negative breast cancer treatment is more challenging due to absence of suitable biomarkers. It is the most aggressive type breast cancer having poor prognosis and limited response to chemotherapy. However, clinicians are using mixed targeted therapies to tackle the disease [32].

Benzofuran and naphthofuran are important biodynamic agents exhibiting diverse pharmacological activities [33-35]. We applied fragment drug discovery approach in designing the pharmacophore using a suitable 3, 4, 5-trimethoxyphenyl fragment for inducing antitubulin effect [25]. Fragment plays an important role in quality interactions with the receptor protein while, rest of the part of pharmacophore provides a conducive environment for better quantitative affinity [18-20]. The naphthofuran derivative **7D** exhibited moderate destabilization effect. Compound **7D** inhibited tubulin polymerization inhibitions. However, this effect was less than the standard destabilizer podophyllotoxin. The dynamic equilibrium of tubulin-microtubule is an important aspect in cell growth and development which plays an

important role during mitosis. Induction of interference in this dynamic equilibrium suppresses spindle dynamics which subsequently leads to cell death [29, 36]. The modulation of tubulin-microtubule dynamics is regarded as prime target for the development of cancer chemotherapeutics [37]. The importance of tubulin binding agents in anticancer therapy is unquestionable due to emergence of successful clinical drugs like paclitaxel, vinblastine etc. In molecular docking studies the compound **7D** occupied the colchicine binding pocket in β-tubulin which was indicated by sixteen common residual amino acids. Among these interactions with β:316 and β:318 are crucial for the induction of antitubulin effects. Burns *et al.* (1992) appraised that both these interactions by a trimethoxyphenyl fragment are very crucial for microtubule destabilizers acting at colchicine binding pocket. Further, β:316 is directly involved in binding with 3,4,5-trimethoxyphenyl fragment [38]. The studied compound **7D** showed higher affinities with β-tubulin as compared to standard destabilizer colchicine. It might be due to three additional bonds *via* pi-pi stalking between the aromatic rings of the compound **7D** with another residue LYS β: 352 which otherwise is absent in colchicine interaction with β-tubulin.

Bioavailability is an important aspect in drug discovery process. ADME properties provide perception about a bioactive compound to become a successful drug candidate. Optimization of ADME properties is often the most difficult and challenging part of the drug discovery process. The ADME properties predicted through Swiss ADME software were quite satisfactory for the naphthofuran derivative. Further the naphthofuran derivative **7D** slightly disobeyed the limits of ideal bioavailability radar range. It was due to presence of high level of unsaturation and low sp3 hybridized carbons. Inadequate ADME properties can otherwise be devastating to any good drug candidate [39]. Many a times, the progress of ADME profiling has decreased the proportion of drug candidates failing in clinical trials.

Toxicity evaluation is an essential step during the development of an investigational drug [40]. Acute toxicity attributes to the adverse effects that occur on first exposure to a single dose of a substance [41]. Preclinical toxicity evaluation provides a clear indication on organ and dose specific toxic effects. It helps in assessing any adverse effect of the drug on experimental animals before initiating the clinical trial. In acute oral toxicity studies the investigational lead compound was found to be safe. Further, the compound was quite safe (Safety index>4.98) in *in vitro* evaluation against HEK-

293T normal cells. There were no significant changes in various parameters of experiment mice. However, toxicity of a test compound can be affected by several different factors like route of administration, time of exposure, physical form of compound, genetic makeup of experimental animal etc. Systematic sub-acute and chronic experiments should also be done for assessing repeated exposure of the compound to the experimental animals. Owing to its importance in drug development WHO has defined drug safety as 'Pharmacovigilence [42].

6.8 Structure activity relationship

All the derivatives were tested on both breast cancer cell lines for their activity. Their results were presented in Table (1). According to the Table (1) derivatives **5**, **6** and **6A** was not shown activity on any of the tested cancer cell lines. Then variation in functional group changes started on position of 2-aryl core and prepared seven derivatives **7A-7G.** Derivatives **7A**, **7B** and **7G** did not show activity against all cell lines, but derivatives **7C** and **7D** shown activities against both the cellines. When the acid group on the derivative **7D** was methylated and ethylated then such derivatives **7E** and **7F** were inactive against all the evaluated cell lines, it shows free acid group is essential for the activity. Similarly we prepared five more derivatives **8A-8E.** Among them two derivatives **8A** and **8E** showed very good activity against **MDA-MB-231** cell line and derivative **8A** did not show activity against **MCF-7.** All other derivatives were inactive against both the cell lines. However their activity on **MDA-MB-231** always better than the **MCF-7**.

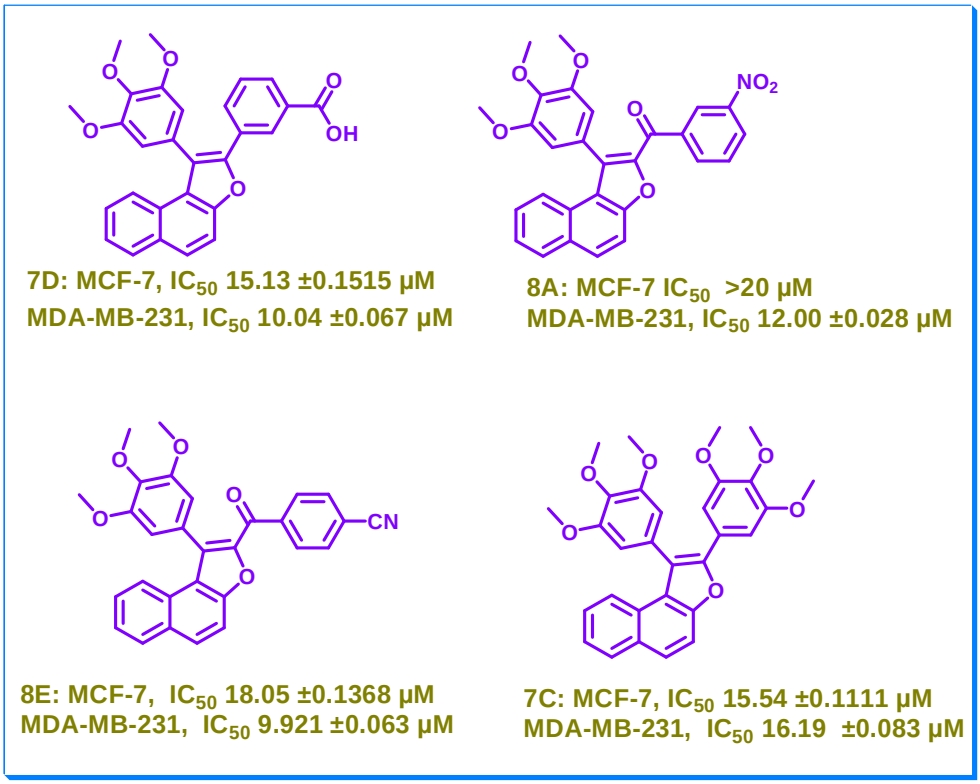


Figure (11): Active derivatives on antibreast cancer cell lines

6.9. Conclusion

Present study provided naphthofuran derivative **7D** possessing potential anticancer activity against triple negative breast cancer. The antiproliferative activity was *via* microtubule destabilization. The compound occupied colchicine binding pocket of target protein i.e. β-tubulin with comparable affinity to standard ligands. The compound **7D** possessed moderate bioavailability in *in-silico* predictions. In toxicity studies the naphthofuran derivative **7D** was well tolerated by rodents and was non-toxic to them. This naphthofuran derivative may further be optimized for better efficacy and druggability properties in future.

6.10 Experimental Section.

6.10.1 Reaction protocols.

Synthesis of 2-bromo-1-(3, 4, 5-trimethoxyphenyl) ethanone (2):- In a round bottom flask bromine (1.22 mL, 23.8 mole) was added drop wise at 0ºC to a solution of 3, 4, 5- trimethoxy acetophenone (5.00 g, 23.8 mole) in dry Et_2O (100 mL). The reaction mixture was stirred for 2 h at RT. The 100 mL of $NaHCO_3$ saturated aqueous solution was added slowly at 0°C. The Et_2O was separated, dried over Na_2SO_4 and

concentrated on rotary evaporator to get yellowish compound **2** in yield 1.95 g (88%) and used without further purification in next step.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.85 (s, 6H, 2xOCH_3), 3.87 (s, 3H, OCH_3), 4.38 (s, 2H, CH_2), 7.17 (s, 2H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.33, 60.95, 106.50, 128.98, 143.34, 153.13, 190.24.

1-(3, 4, 5-trimethoxyphenyl)-2-(naphthalene-3-yloxy) ethanone (3):- To a solution of **2** (6.0 g, 20.69 mole) and starting material of 2-naphthol (3.0 g, 20.83 mole) in dry DMF (60 mL) was added portion wise anhydrous K_2CO_3 (6.0 g, 43.478 mole). The reaction stirred at RT. On completion, reaction was quenched by adding of water (100 mL). The crude mass was extracted thrice with EtOAc, pooled EtOAc washed with water, dried over Na_2SO_4, thickened on rota evaporator and purified by column chromatography (eluent: 16% EtOAc/hexanes) to get **3** in 7.0 g (95%) yield.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.93 (s, 6H, 2xOCH_3), 3.96 (s, 3H, OCH_3), 5.34 (s, 2H, CH_2), 7.14 (d, 1H, J= 2.5 Hz, CH aromatic), 7.28 (d, 1H, J= 11.5 Hz, CH aromatic), 7.34 (s, 1H, CH aromatic), 7.37 (t, 1H, J= 8.0 Hz, CH aromatic), 7.37 (d, 1H, J= 8.2 Hz, CH aromatic), 7.45 (t, 1H, J= 8.0 Hz, CH aromatic), 7.79 (d, 2H, J= 8.8 Hz, A CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.39, 61.00, 71.09, 105.94, 107.39, 118.60, 124.11, 126.56, 126.87, 127.69, 129.40, 129.74, 134.31, 143.39, 153.26, 155.95, 193.48.

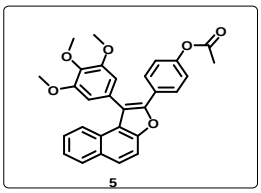
1-(3, 4, 5-trimethoxy phenyl) naphtho [2, 1-b] furan (4):- In 50 ml Round Bottom (RB) flask **3** added (2.00 g, 5.128 mol) RB flask kept in Ice bath after 10 min, temp at 2-3^{o}C. Then trifluroacetic acid was added 10 mL as a solvent cum reagent. After 10 min ice bath was removed and stirring continued at RT for 3 h. On completion, mixture poured into ice cold water and uprooted with EtOAc, pooled layer, dried over Na_2SO_4, thickened on rotary evaporator, further refined by column chromatography (2% EtOAc/hexane) to compound **4** in 1.72 g (90%) yield. Melting point: 112.3^{o}C.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.88 (s, 6H, 2xOCH_3), 3.99 (s, 3H, OCH_3), 6.83 (s, 2H, J= 2.5 Hz, CH aromatic), 7.41 (t, 1H, J= 11.5 Hz, CH aromatic), 7.46 (t, 1H, CH aromatic), 7.69 (s, 1H, J= 8.0 Hz, CH aromatic), 7.71 (dd, 1H, J= 8.0 Hz, CH

aromatic), 7.96 (d, 1H, J= 8.2 Hz, CH aromatic), 8.11 (d, 2H, J= 8.8 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.25, 61.09, 107.03, 112.67, 120.67, 123.49, 124.43, 124.46, 126.03, 126.06, 128.22, 128.99, 130.85, 137.75, 141.57, 153.11, 153.34; HRMS (ESI-TOF) m/z [M+H] calculated for $C_{21}H_{18}O_4$, 335.1230, found 335.128.

4-(-1-(3, 4, 5-trimethoxyphenyl)naphtha[2, 1-b] furan-2-yl) phenyl acetate (5):- In a round bottom flask compound **4** (1.0 g, 2.92 mol) in DMA (30 mL), 4-acetoxybromobenzene (1.35 mg, 2 equiv), $Pd(OAc)_2$ (140 mg, 0.2 equiv), and KOAc (600 mg, 2 equiv) were added at RT. After being heated at 80ºC with stirring for 4 h. The mixture was concentrated on rotary evaporator to give the residue which was refined by column chromatography (6% hexane/EtOAc) finally to yield **5** in 1.2 g (92%).



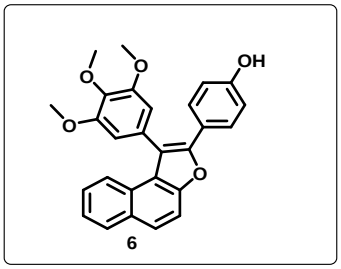
5

^{1}H NMR (500MHz, $CDCl_3$): δ 2.24 (s, 3H, CH_3), 3.73 (s, 6H, 2xOCH_3), 3.83 (s, 3H, OCH_3), 6.85 (s, 2H, CH aromatic), 7.12 (d, 2H, J= 8.8 Hz, CH aromatic), 7.28 (d, 1H, J= 8.8 Hz, CH aromatic), 7.36 (t, 1H, J= 7.2 Hz, CH aromatic), 7.42 (t, 1H, CH aromatic), 7.53 (d, 1H, J= 8.3 Hz, CH aromatic), 7.61 (d, 2H, J= 8.7 Hz, CH aromatic), 7.86 (dd, 1H, J= 9.0 Hz, CH aromatic), 8.0 (d, 1H, J= 8.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 21.30, 56.31, 60.58, 108.91, 113.25, 113.71, 115.54, 117.83, 119.02, 120.12, 123.80, 124.08, 126.16, 128.41, 129.30, 130.64, 132.35, 139.44, 150.43, 151.97, 152.45, 155.65, 171.70; Electrospray mass for $C_{29}H_{24}O_6$ (MeOH): 469 $[M+H]^+$.

4-(-1-(3, 4, 5-trimethoxyphenyl)naphtho [2, 1-b] furan-2-yl) phenol (6):- To 50 mL round bottom flask contain (200 mg, 0.427 mmol) of compound **5** was taken in methanol 25 mL then (1000 mg, 7.24 mmol) of potassium hydroxide was added to

reaction mixture. Then stirring continued at RT for 30 min. Upon completion the mixture was concentrated on rotary evaporator to get crude, further refined by filter column to get derivative **6** in 178 mg (98%).

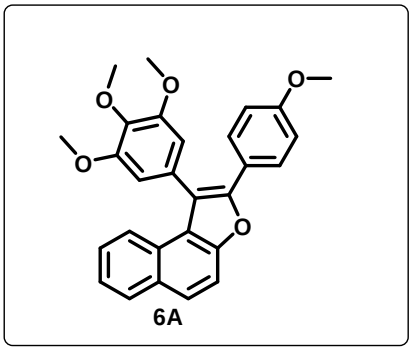


^{1}H NMR (500MHz, $CDCl_3$): δ 3.76 (s, 6H, 2xOCH_3), 3.92 (s, 3H, OCH_3), 4.87 (bs, 1H, OH), 6.71 (m, 2H, CH aromatic), 6.79 (s, 2H, CH aromatic), 7.25 (t, 1H, CH aromatic), 7.35 (t, 1H, CH aromatic), 7.43 (d, 2H, CH aromatic), 7.59 (d, 1H, J= 8.4 Hz, CH aromatic), 7.70 (dd, 2H, J= 8.9 Hz, CH aromatic), 7.89 (d, 1H, J= 8.1 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 55.40, 60.08, 109.02, 112.15, 113.02, 116.47, 118.37, 118.64, 123.58, 124.15, 124.83, 126.92, 128.89, 130.15, 132.11, 132.58, 139.31, 152.09, 152.34, 155.63, 158.98; ESI-MS (MeoH): For $C_{27}H_{22}O_5$, 465 $[M+K]^+$, HRMS (ESI-TOF) m/z [M+H] calculated for $C_{27}H_{22}O_5$, 427.1467 , found 427.1971.

1-(3, 4, 5-trimethoxy)-2-(-4methoxyphenyl)naphtha[2, 1-b] furan (6A):-

To 100 mL round bottom flask contain (100 mg, 0.234 mmol) of compound **6** was taken in dry acetone 10 mL. Then (300 mg, 2.17 mmol) of potassium carbonate, dimethyl sulfate (0.5 mL, 0.0052 mmol) was added to reaction mixture. Then stirring continued at 60ºC for 1h. Upon completion of the reaction, the mixture was concentrated on rotary evaporator to get crude, further refined by filter column to get derivative **6A** in 93 mg (90%).



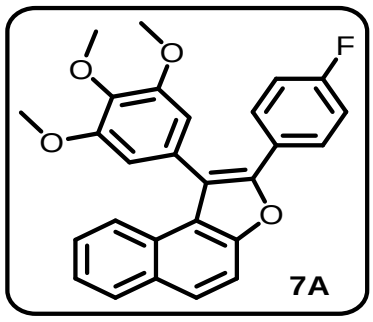
^{1}H NMR (500MHz, $CDCl_3$): δ 3.82 (s, 6H, 2xOCH_3), 3.84 (s, 6H, 2xOCH_3), 6.49 (s, 2H, CH aromatic), 6.82 (s, 2H, CH aromatic), 7.30 (d, 1H, J= 9.1 Hz, CH aromatic), 7.44 (d, 2H, J= 7.5 Hz, CH aromatic), 7.63 (d, 2H, CH aromatic), 7.67 (d, 1H, CH aromatic), 7.80 (d, 2H, J= 9.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 55.45, 55.50, 56.71, 106.53, 109.58, 113.41, 118.82, 124.65, 124.95, 125.49, 128.18, 128.25, 128.49, 128.58, 130.09, 135.12, 135.16, 159.60, 160.61, 160.63; HRMS (ESI-TOF) m/z [M+H] calculated for $C_{28}H_{24}O_5$, 441.1624, found 441.6603.

General procedure for the preparation of the substituted aryl naphthofuran (7A-7G):-

In a round bottom flask contain compound **4** (100 mg, 0.299 mmol) in DMA (25 mL), aryl benzene (65 mg, 0.37 mmol), $Pd(OAc)_2$ (40 mg, 0.17 mmol), and KOAc (60 mg, 0.61 mmol) were added at RT. After being heated at 80ºC with stirring for 2-4 h. The mixture was concentrated on rotary evaporator to give the residue which was refined by column chromatography (hexane/EtOAc) finally to yield 135-116 mg of **7A-7G** in 86-90%.

2-(-4-fluorophenyl)-1-(3, 4, 5-trimethoxy phenyl) naphtha [2, 1-b] furan (7A):-
Yield: 90%;



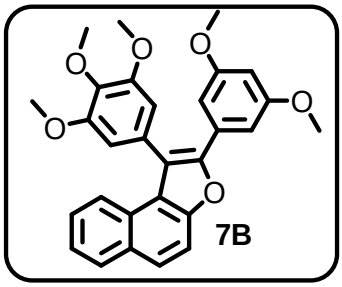
Melting point: Viscous.

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{27}H_{21}FO_4$, 429.1424, found 429.7975.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.74 (s, 6H, 2xOCH_3), 3.98 (s, 3H, OCH_3), 6.59 (s, 2H, CH aromatic), 6.80 (d, 2H, J= 5.1 Hz, CH aromatic), 7.37 (t, 1H, , CH aromatic), 7.46 (t, 1H, CH aromatic), 7.64 (d, 1H, J= 8.9 Hz, CH aromatic), 7.79 (d, 1H, CH aromatic), 7.88 (d, 2H, CH aromatic), 7.95 (d, 2H, J= 8.00 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.11, 61.05, 107.21, 112.27, 114.08, 116.49, 119.05, 121.97, 123.67, 126.29, 127.00, 128.12, 128.19, 129.09, 130.97, 137.65, 139.29, 142.61, 152.61, 153.05, 153.54, 154.25.

1-(3, 4, 5-trimethoxyphenyl)-2-(3, 5-dimethoxyphenyl)naphtho[2, 1-b] furan (7B):-



Yield: 86%;

Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{29}H_{26}O_6$ **(MeOH):** 469 $[M-H]^-$.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.71 (s, 9H, 3xOCH_3), 3.97 (s, 6H, 2xOCH_3), 6.57 (s, 4H, CH aromatic), 7.35 (t, 1H, CH aromatic), 7.44 (t, 1H, CH aromatic), 7.46 (s, 1H, CH aromatic), 7.63 (d, 1H, J= 8.90 Hz, CH aromatic), 7.80 (d, 1H, J= 8.90 Hz, CH aromatic), 7.86 (d, 1H, J= 8.35 Hz, CH aromatic), 7.94 (d, 1H, J= 8.05 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 55.32, 55.88, 56.02, 60.84, 98.99, 106.76, 107.27, 112.06, 112.45, 113.89, 120.42, 121.72, 126.83, 127.92, 127.96, 127.99, 128.31, 128.78, 130.63, 132.11, 135.02, 137.51, 141.54, 152.39, 152.82, 152.88, 153.12, 160.93.

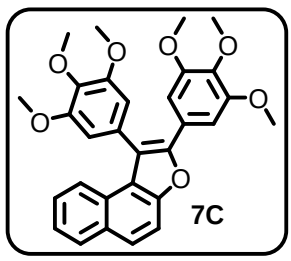
1-(3, 4, 5-trimethoxyphenyl)-2-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b] furan (7C):-

Yield: 89 %; Brown colour compound;

Melting Point: 184.6 -185.0ºC

ESI-MS (MeOH): For $C_{30}H_{28}O_7$, 523 $[M+Na]^+$ and 499 $[M-H]^-$, HRMS (ESI-TOF) m/z [M+H] calculated for $C_{30}H_{28}O_7$, 501.1835, found 501.7712.

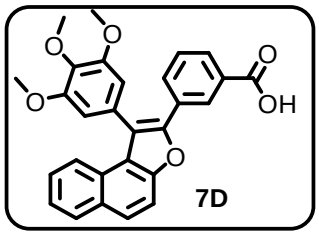
^{1}H NMR (500MHz, $CDCl_3$): δ 3.68 (s, 9H, 3xOCH_3), 3.82 (s, 9H, 3xOCH_3), 6.81 (s, 2H, CH aromatic), 7.89 (s, 2H, CH aromatic), 7.30 (t, 1H, CH aromatic), 7.37 (t, 1H, CH aromatic), 7.58 (d, 1H, CH aromatic), 7.73 (d, 1H, CH aromatic), 7.85 (m, 1H, CH aromatic), 7.86 (m, 1H, CH aromatic).



^{13}C NMR (125MHz, $CDCl_3$): δ 55.38, 55.95, 59.97, 60.63, 102.76, 107.00, 111.73, 111.88, 118.73, 122.80, 124.09, 126.71, 127.76, 128.63, 128.74, 130.58, 137.53, 137.58, 139.38, 150.74, 152.71, 152.78, 153.07, 153.98.

3-(-1-(3, 4, 5-trimethoxyphenyl) naphtho [2, 1-b] furan (7D):-

Yield: 87%; Brown colour solid compound.



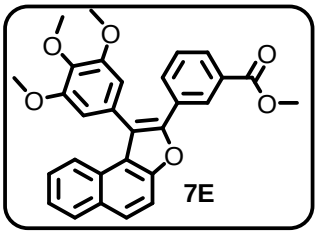
Melting Point: 211.5ºC

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{28}H_{22}O_6$, 455.1416, found 455.1792.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.73 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 6.58 (s, 2H, CH aromatic), 7.37 (t, 1H, CH aromatic), 7.41 (t, 1H, CH aromatic), 7.44 (t, 1H, CH aromatic), 7.57 (d, 1H, J= 9.05 Hz, CH aromatic), 7.61 (d, 2H, J= 8.90 Hz, CH aromatic), 7.85 (dd, 2H, J= 8.30 Hz, CH aromatic), 7.93 (d, 1H, J= 8.10 Hz, CH aromatic), 8.00 (d, 1H, J= 2.95 Hz, CH aromatic), 8.09 (bs, 1H, CH aromatic**).**

^{13}C NMR (125MHz, $CDCl_3$): δ 56.10, 61.08, 107.49, 112.28, 121.96, 123.34, 123.63, 124.68, 124.83, 126.30, 127.01, 128.15, 128.17, 128.28, 129.08, 130.24, 130.95, 131.19, 133.72, 134.67, 137.58, 142.60, 152.59, 153.03, 170.03.

Methyl-3-(-1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl) benzoate(7E):-



Yield: 88%;

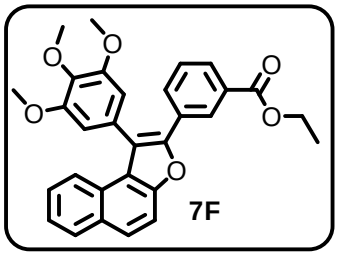
Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{29}H_{24}O_6$, 507 $[M+K]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.71 (s, 9H, 3xOCH_3), 3.97 (s, 3H, OCH_3), 6.57 (s, 2H, CH aromatic), 7.35 (d, 1H, CH aromatic), 7.36 (t, 1H, J= 5.06 Hz, CH aromatic), 7.42 (d, 1H, CH aromatic), 7.45 (t, 1H, J= 3.93 Hz, CH aromatic), 7.62 (d, 1H, J= 8.90 Hz, CH aromatic), 7.79 (d, 2H, J= 8.95 Hz, CH aromatic), 7.86 (d, 1H, J= 8.35 Hz, CH aromatic), 7.94 (d, 1H, J= 8.05 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.10, 56.22, 61.05, 107.50, 112.27, 123.34, 124.67, 126.28, 129.07, 130.95, 133.22, 137.61, 142.59, 152.59, 153.03, 172.40.

Ethyl-3-(-1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl) benzoate (7F):-



Yield: 87%;

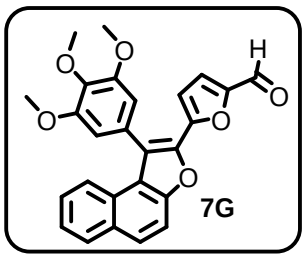
Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{30}H_{26}O_6$, 453 $[M\text{-}Et]^-$.

^{1}H NMR (500MHz, $CDCl_3$): δ 1.42 (t, 3H, CH_3), 3.72 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 4.37 (m, 2H, CH_2), 6.57 (s, 2H, CH aromatic) 7.36 (t, 1H, J= 7.87 Hz, CH aromatic), 7.40 (t, 1H, J= 7.05 Hz, CH aromatic), 7.45 (t, 1H, J= 7.87 Hz, CH aromatic), 7.53 (d, 1H, J= 10.05 Hz, CH aromatic), 7.63 (d, 1H, J= 8.95 Hz, CH aromatic), 7.84 (d, 1H, J= 8.30 Hz, CH aromatic), 7.79 (d, 1H, J= 8.95 Hz, CH aromatic), 7.92 (d, 1H, J= 8.10 Hz, CH aromatic 7.94 (d, 1H, J= 8.00 Hz, CH aromatic), 8.02 (bs, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 14.40, 56.09, 61.42, 66.22, 107.47, 112.28, 115.91, 121.96, 123.34, 123.63, 124.67, 126.30, 127.00, 128.13, 128.17, 129.08, 129.64, 129.66, 130.95, 132.23, 132.86, 137.56, 142.59, 152.59, 153.02, 174.39 .

5-(-1-(3, 4, 5-trimethoxy phenyl)naphtha[2, 1-b]furan-2-yl) furan-2-carbaldehyde (7G):-



Yield: 88%;

Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{26}H_{20}O_6$, 429 $[M+H]^+$.

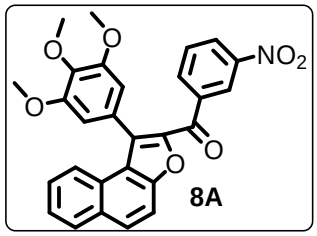
^{1}H NMR (500MHz, $CDCl_3$): δ 3.85 (s, 6H, 2xOCH_3), 4.03 (s, 3H, OCH_3), 6.36 (d, 1H, CH aromatic), 6.79 (s, 2H, CH aromatic), 7.22 (d, 1H, J= 3.80 Hz, CH aromatic), 7.38 (t, 1H, CH aromatic), 7.48 (t, 1H, CH aromatic), 7.74 (d, 1H, CH aromatic), 7.76 (s, 1H, CH aromatic), 7.85 (d, 1H, J= 9.00 Hz, CH aromatic), 7.96 (d, 1H, J= 8.05 Hz, CH aromatic), 9.64 (s, 1H, CHO).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.32, 61.25, 106.90, 110.23, 112.36, 114.07, 122.61, 123.21, 123.45, 125.03, 126.73, 127.74, 127.94, 128.05, 129.13, 131.06, 138.38, 139.30, 141.62, 150.36, 151.95, 152.60, 154.03, 177.51.

General procedure for the preparation of the substituted Benzyl aryl naphthofuran (8A-8E):-

In a round bottom flask contain compound **4** (100 mg, 0.299 mmol) in DMA (25 mL), aryl benzene (65 mg, 0.37 mmol), $Pd(OAc)_2$ (40 mg, 0.17 mmol), and KOAc (60 mg, 0.61 mmol) were added at RT. After being heated at 80ºC with stirring for 2-4 h. The mixture was concentrated on rotary evaporator to give the residue which was refined by column chromatography (hexane/EtOAc) finally to yield 124-137mg **8A -8E** in 85-89%.

(1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl)(3-nitrophenyl) methanone (8A):-



Yield: 85%; Solid compound;

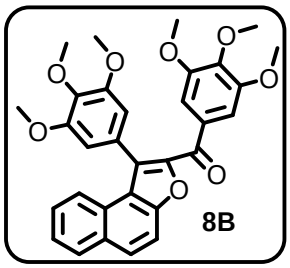
Melting Point: 175.6-176.0ºC

ESI-MS (MeOH): For $C_{28}H_{21}NO_7$, 482 $[M-H]^-$.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.72 (s, 6H, 2xOCH_3), 3.96 (s, 3H, OCH_3), 6.55 (s, 2H, CH aromatic), 7.44 (d, 1H, CH aromatic), 7.45 (d, 1H, CH aromatic), 7.46 (t, 1H, CH aromatic), 7.46 (t, 1H, CH aromatic), 7.65 (t, 2H, J= 7.97 Hz, CH aromatic), 8.39 (m, 1H, CH aromatic), 8.40 (d, 1H, CH aromatic), 8.41 (d, 1H, CH aromatic), 8.90 (s,1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.09, 61.07, 112.26, 121.11, 123.30, 124.67, 125.00, 125.72, 127.42, 132.39, 135.65, 148.28, 152.98, 167.38, 171.89.

(3, 4, 5-trimethoxyphenyl)(1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl) methanone (8B):-



Yield: 87%;

Melting Point: Amorphous.

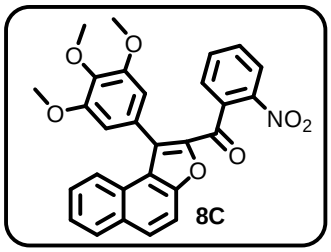
ESI-MS (MeOH): For $C_{31}H_{28}O_8$, 527 $[M-H]^-$, HRMS (ESI-TOF) m/z [M+H] calculated for $C_{31}H_{28}O_8$, 529.1784, found 529.7737.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.72 (s, 9H, 3xOCH_3), 3.96 (s, 9H, 3xOCH_3), 6.57 (s, 2H, CH aromatic), 7.28 (d,1H, CH aromatic), 7.34 (t, 1H, CH aromatic), 7.43 (t, 1H,

CH aromatic), 7.46 (d, 1H, CH aromatic), 7.62 (d, 1H, J= 8.90Hz, CH aromatic), 7.79 (d, 1H, J= 9.0 Hz, CH aromatic), 7.86 (d, 2H, J= 8.35 Hz, CH aromatic), 7.94(d, 1H, J= 8.0 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.12, 61.01, 107.54, 112.26, 114.06, 121.97, 123.34, 123.65, 124.66, 126.28, 126.99, 128.18, 130.96, 137.66, 142.60, 152.59, 153.04, 160.05, 167.00.

(1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl)(2-nitrophenyl) methanone (8C):-



Yield: 85%;

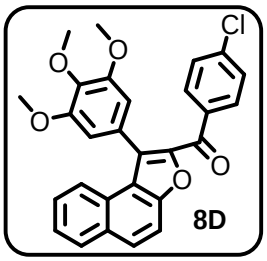
Melting Point: Amorphous.

ESI-MS (MeOH): For $C_{28}H_{21}NO_7$, 482 $[M-H]^-$.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.73 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 6.57 (s, 2H, CH aromatic), 7.37 (t, 1H, CH aromatic), 7.45 (t, 1H, CH aromatic), 7.61 (m, 1H, CH aromatic), 7.78 (d, 1H, CH aromatic), 7.80 (m, 1H, CH aromatic), 7.86 (d, 1H, CH aromatic), 7.94 (d, 1H, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.10, 61.06, 107.47, 112.28, 114.08, 121.96, 124.67, 126.30, 127.00, 128.13, 128.17, 129.08, 130.95, 137.58, 142.59, 152.59, 153.03, 161.00, 168.76.

(4-chlorophenyl)(1-(3, 4, 5-trimethoxyphenyl)naphtho[2, 1-b]furan-2-yl) methanone (8D):



Yield: 87%;

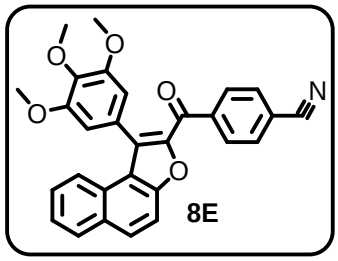
Melting Point: Viscous.

ESI-MS (MeOH): For $C_{28}H_{21}ClO_5$, 494 $[M+Na-H]^+$.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.73 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 6.57 (s, 2H, CH aromatic), 7.33 (d, 1H, CH aromatic), 7.36 (t, 1H, CH aromatic), 7.42 (t, 1H, CH aromatic), 7.45 (d, 2H, CH aromatic), 7.45 (d, 1H, CH aromatic), 7.62 (d, 1H, J= 8.95 Hz, CH aromatic), 7.78 (d, 1H, J= 8.95 Hz, CH aromatic), 7.84 (d, 2H, J= 8.35 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.10, 61.05, 103.96, 111.25, 119.52, 124.82, 127.08, 130.69, 131.62, 134.03, 135.37, 140.72, 142.24, 150.98, 151.94, 162.83, 166.17.

(4-benzonitrle)(-1-(3, 4, 5-trimethoxyphenyl)naphtha[2, 1-b]furan-2-yl)furan-2-yl) methanone (8E):-



Yield: 89%; Solid compound.

Melting Point: 201.3-202ºC

HRMS (ESI-TOF) m/z [M+H] calculated for $C_{29}H_{21}NO_5$, 464.1420, found 464.3072.

^{1}H NMR (500MHz, $CDCl_3$): δ 3.73 (s, 6H, 2xOCH_3), 3.97 (s, 3H, OCH_3), 6.57 (s, 2H, CH aromatic), 7.35 (t, 1H, CH aromatic), 7.44 (t, 1H, CH aromatic), 7.63 (d, 1H, J= 8.90 Hz, CH aromatic), 7.71 (d, 1H, J= 8.45 Hz, CH aromatic), 7.78-7.76 (m, 4H, CH aromatic), 8.19 (d, 2H, J= 8.50 Hz, CH aromatic).

^{13}C NMR (125MHz, $CDCl_3$): δ 56.10, 61.08, 107.50, 112.28, 117.02, 117.87, 121.96, 123.33, 123.60, 124.68, 126.30, 127.01, 128.16, 129.08, 130.61, 130.96, 133.30, 137.56, 142.61, 152.60, 153.01, 168.76.

6.11 Biological assays.

6.11.1 Antiproliferative activity by Sulphorhodamine assay

Human cancer cell lines, MCF-7 (Hormone dependent breast cancer), MDA-MB-231 (Triple negative breast cancer), and normal cell HEK-293 were originally obtained from American type of cell culture collection (ATCC), USA and grown at 37ºC in DMEM supplemented with 10% FBS and Ab-Am (antibiotic-antimitotic) solution in a CO_2 incubator (New Brunswick/Eppendorff, Germany) under 5% CO_2 and 95% relative humidity. Tamoxifen and doxorubicin were used as standard drugs (positive control) for cytotoxicity.

Briefly, 10^4 cells/well were grown overnight in 96 well culture plates at 37ºC in 5% CO_2. Cells were then incubated with serial dilutions of test compounds for 48 h and were subsequently fixed with ice cold 50% (w/v) tri-chloro acetic acid (100 µL/well) at 4ºC for 1h. After removing fixative, 50 µL sulphorhodamine dye solution (0.4% w/v in 1% acetic acid) was added to each well and washed after 30 min incubation at room temperature. Bound dye was solubilised with 10 mM Tris base (150 µL/well)

and absorbance was read at 540 nm on a plate reader (Biotek, USA). The cytotoxic effect of compound was calculated as % inhibition in cell growth as per formula: [1-(absorbance of drug treated cells/absorbance of untreated cells) x100]. Dose dependent curves were used to determine 50% inhibitory concentration (IC_{50}).

6.11.2. Tubulin polymerization assay

Tubulin polymerization assay was performed on porcine brain tubulin (>99% pure) using 'assay kit-BK006P' from Cytoskeleton, USA, as per manufacturer's reported protocol [43, 44]. Podophyllotoxin (PDT) was used as standard inhibitor and paclitaxol as standard stabilizer of tubulin polymerase and DMSO as negative control. Briefly, tubulin protein (3 mg/mL) in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP and 15% glycerol) was placed in pre-warmed 96-well micro liter plates at 37°C in the presence of naphthofuran derivative **7D** at three different concentrations at half IC_{50}, IC_{50} and double IC_{50}. All samples were mixed well and polymerization was monitored kinetically using Spectramax plate reader at 340 nm every min for 1h. Absorbance Vs time graph was plotted as tubulin kinetics for polymerization of tubulin protein.

6.11.3. Molecular docking studies

The site directed docking of the compound **7D** was performed at colchicine binding pocket of β-tubulin [45] using AutoDock Vina (http://www.vina.scripps.edu/), (Molecular Graphics Lab at The Scripps Research Institute, USA) [46]. The binding site was determined based on the binding pocket of colchicine co-crystallized with tubulin. The 3D-crystal structure of β-tubulin (PDB: 4O2B) was downloaded from RCSB (Protein Data Bank) to serve as docking template for the docking experiment. The box dimensions were taken as x: 17.0614, y: 65.2932, z: 42.8437. ADME properties of the naphthofuran derivative was predicted on Swiss ADME online software [47].

6.11.4 Safety studies

Considering significant anti-cancer activity the safety assessment for the naphthofuran derivative **7D** was done as per reported method [48].

The study was carried out in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987) and following the

IAEC (Institutional Animal Ethical Committee) approved protocols vide ref. no. CIMAP/IAEC/2016-19/01, dated 09/2/2017. Method described in chapter-2A in section (2.7.8).

6.12 References

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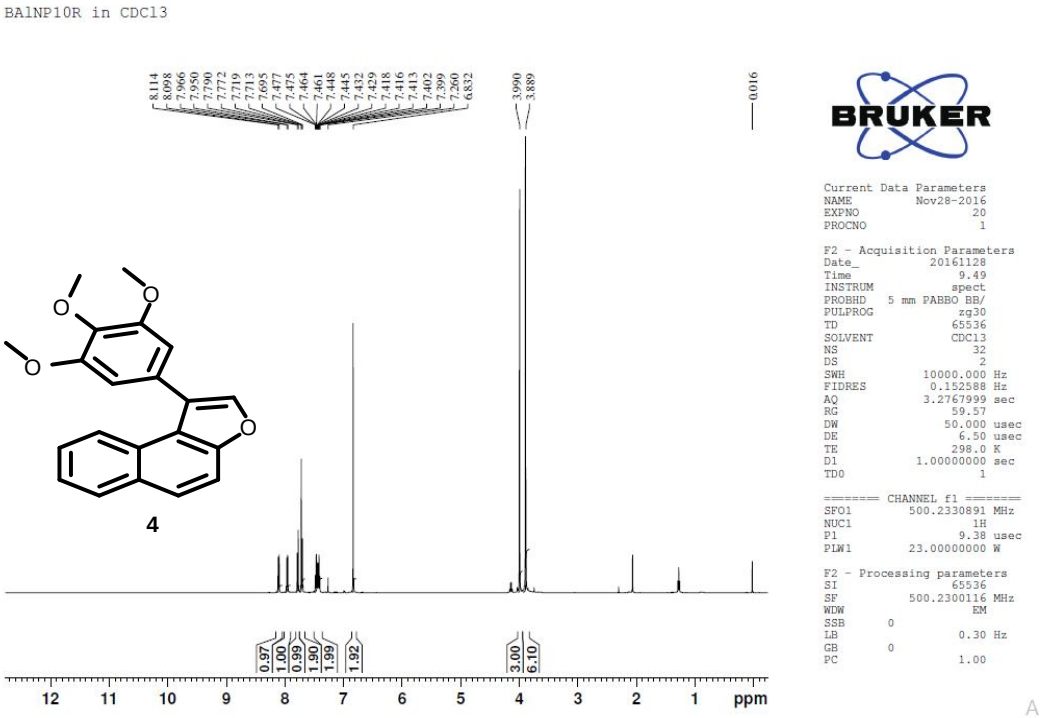
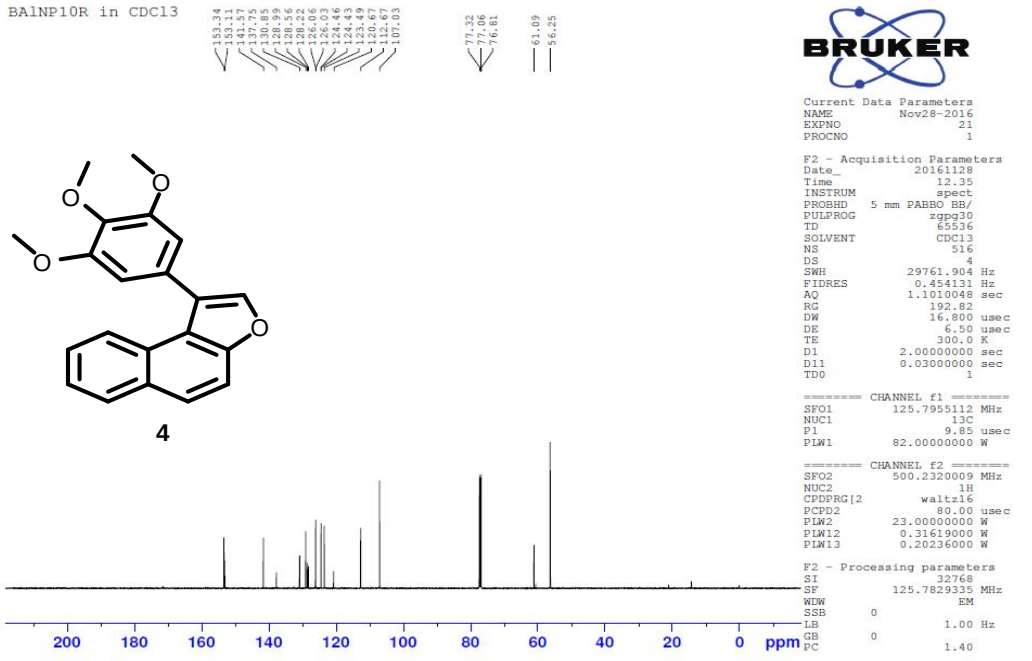
6.13 Spectra of final compounds

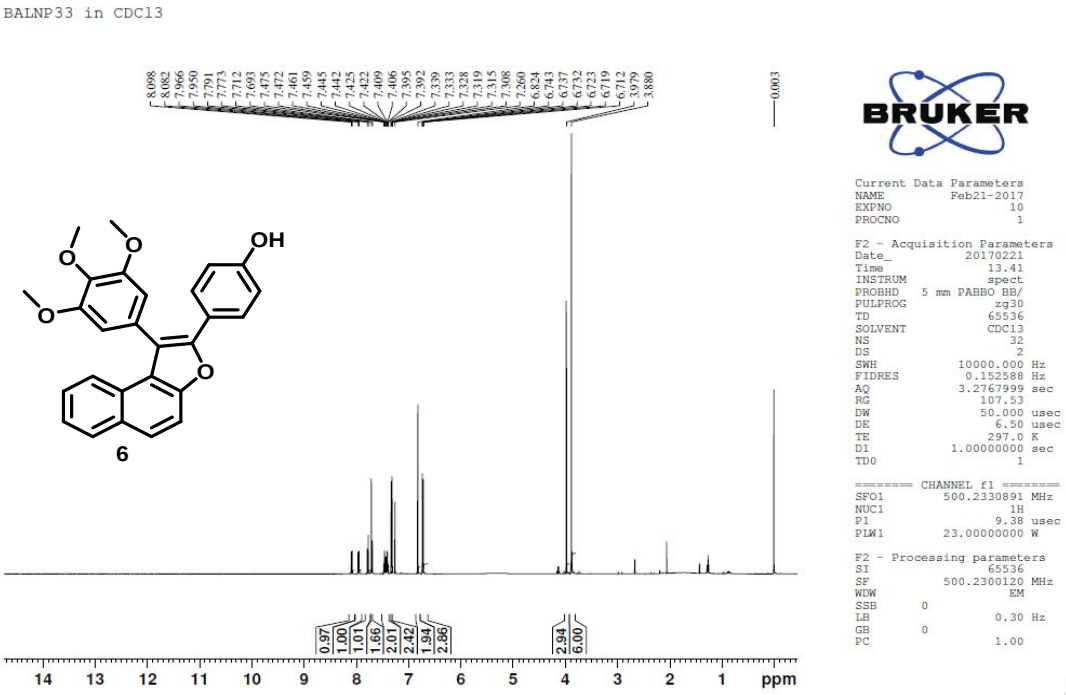
Compound 4: ^{1}H NMR



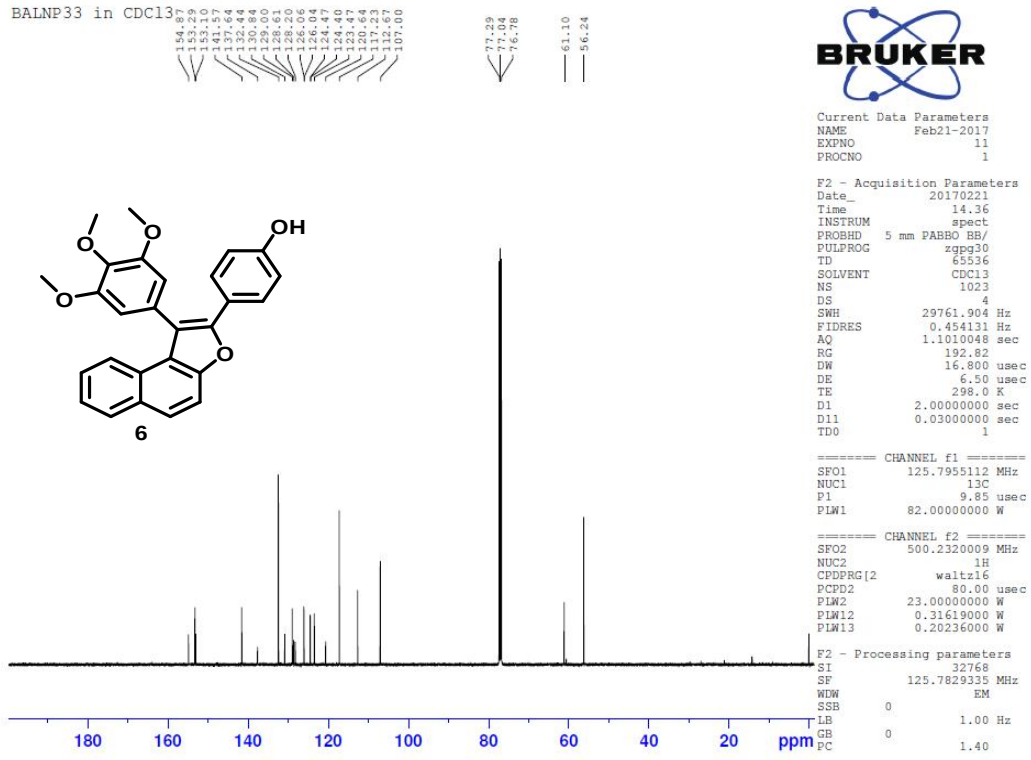
Compound 4: ^{13}C NMR



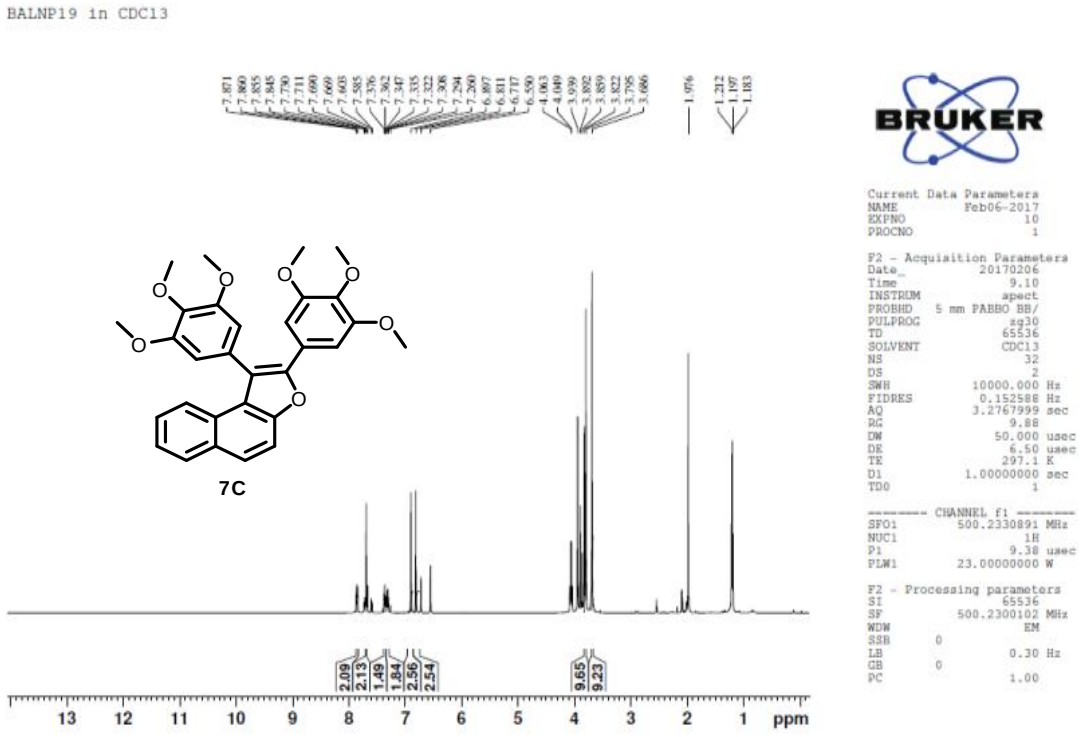
Compound 6: ^{1}H NMR



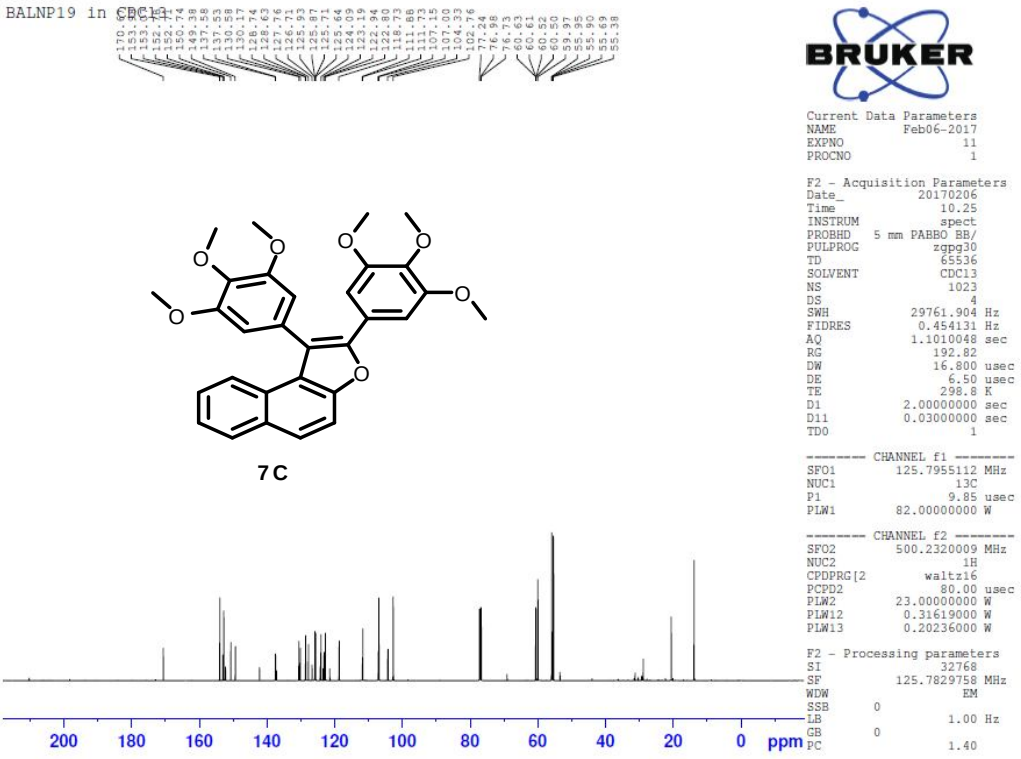
Compound 6: ^{13}C NMR



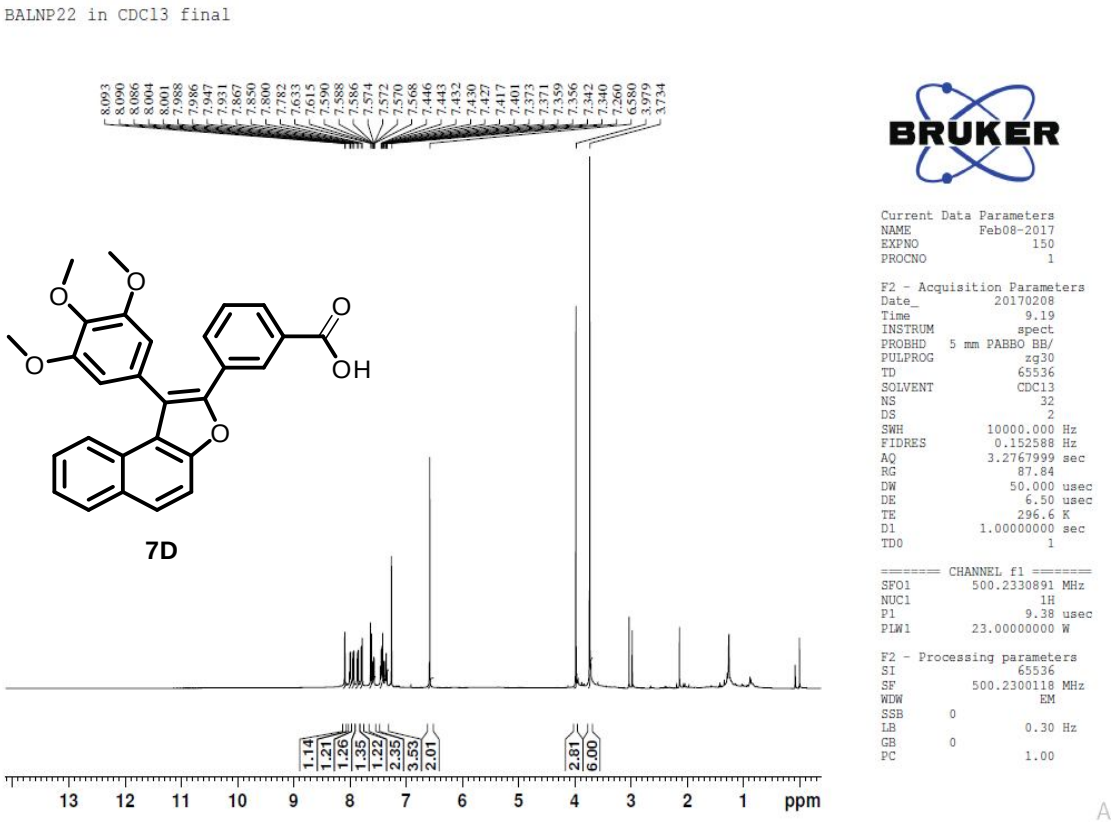
Compound 7C: ^{1}H NMR



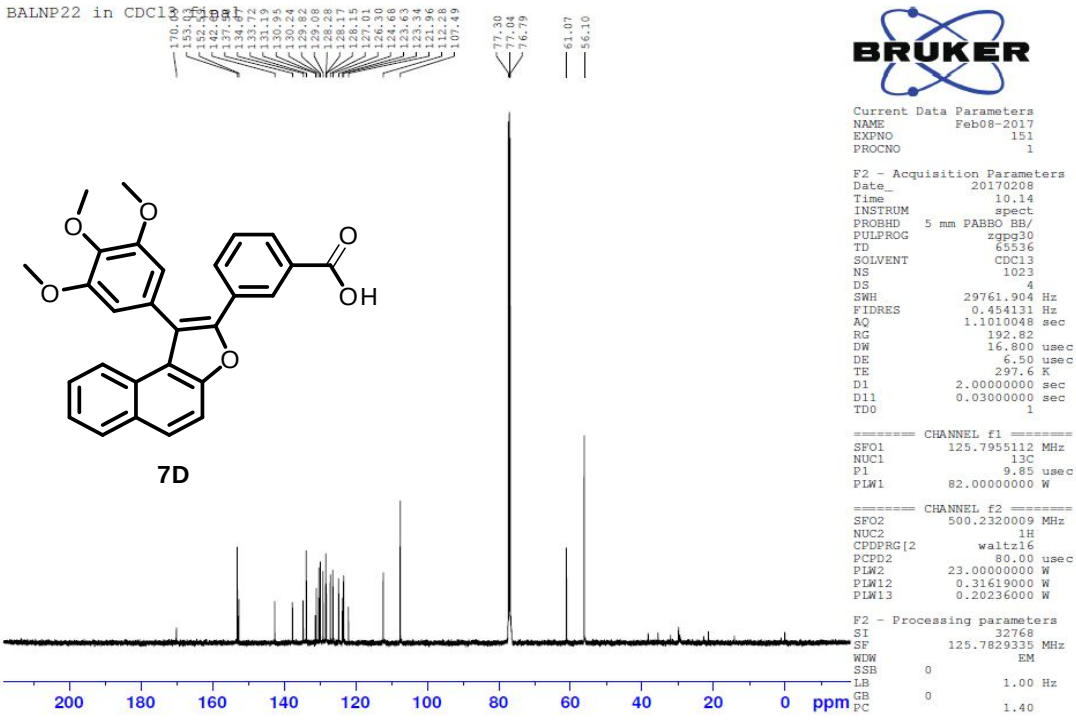
Compound 7C: ^{13}C NMR



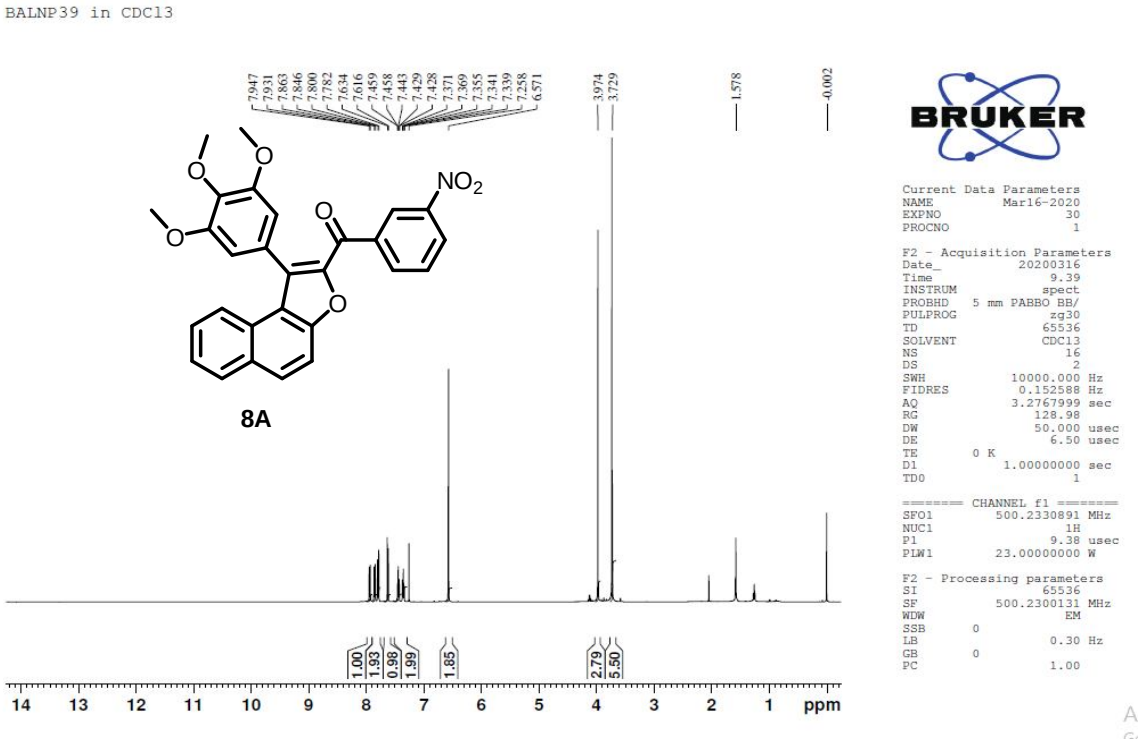
Compound 7D: ^{1}H NMR



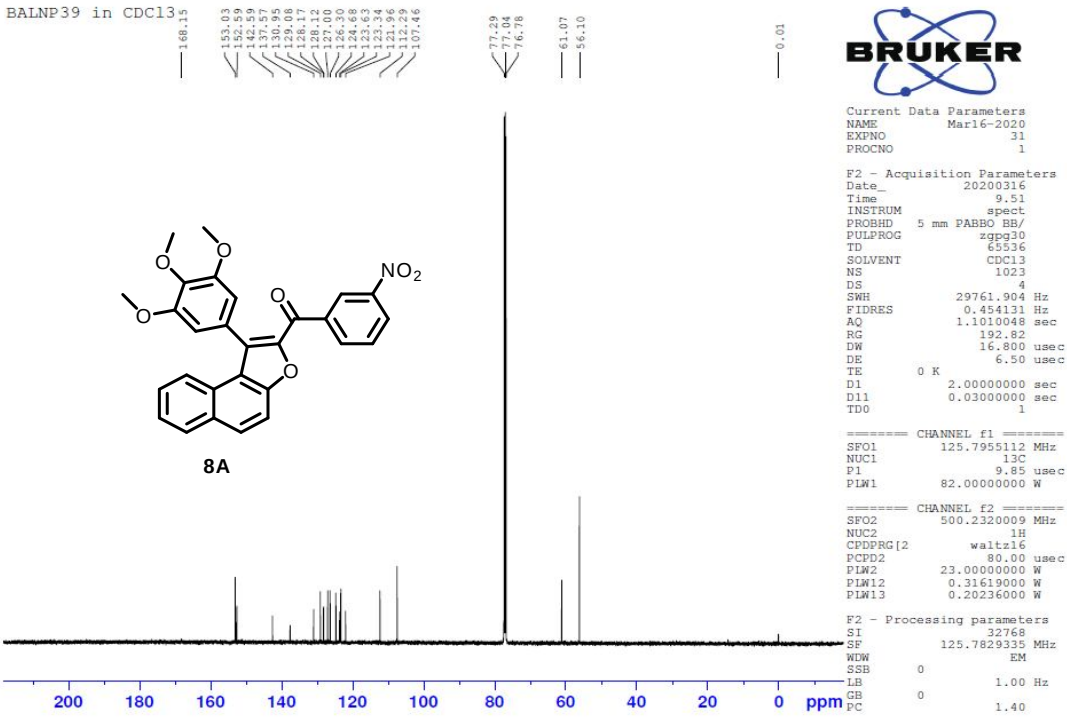
Compound 7D: ^{13}C NMR



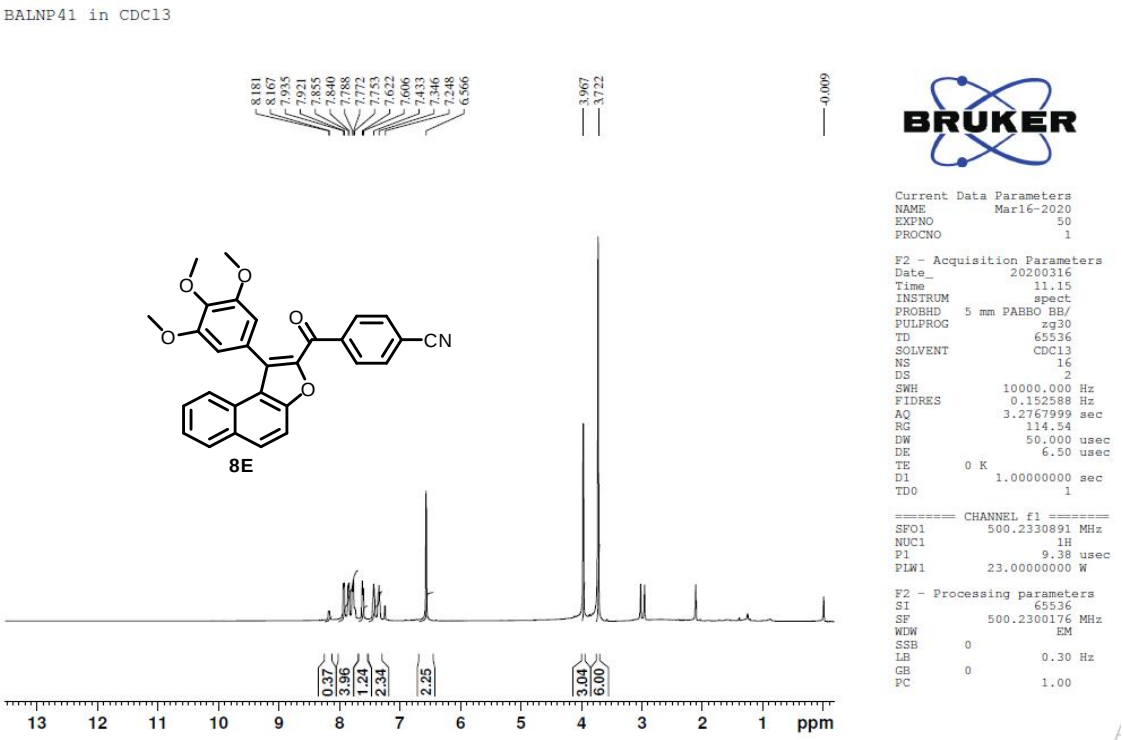
Compound 8A: H NMR



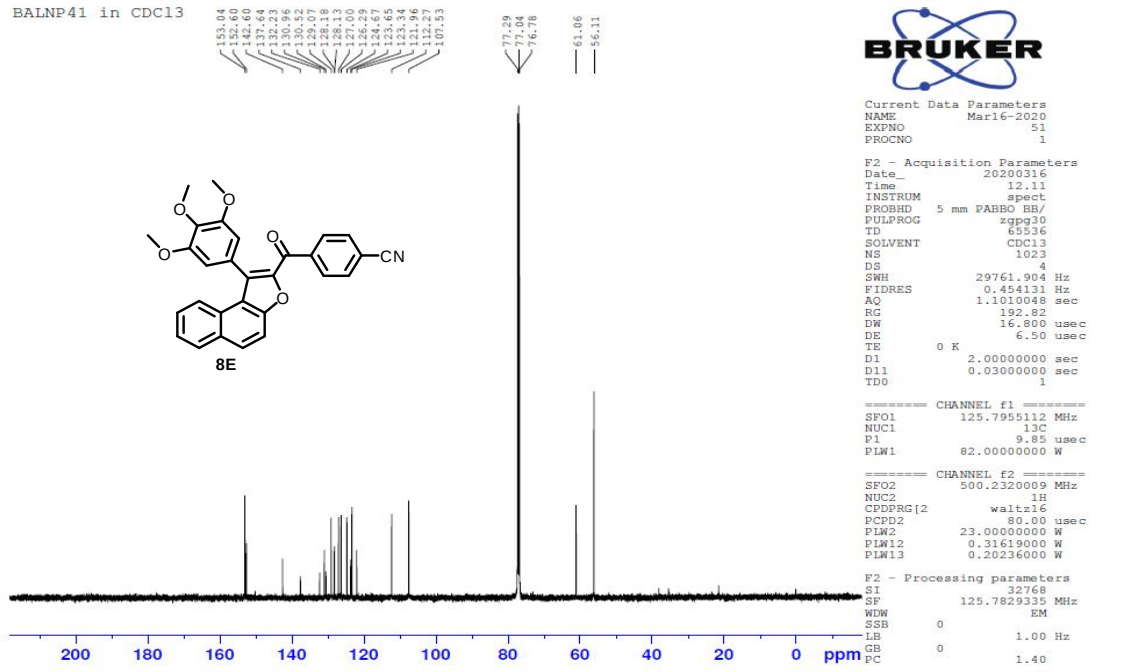
Compound 8A: ^{13}C NMR



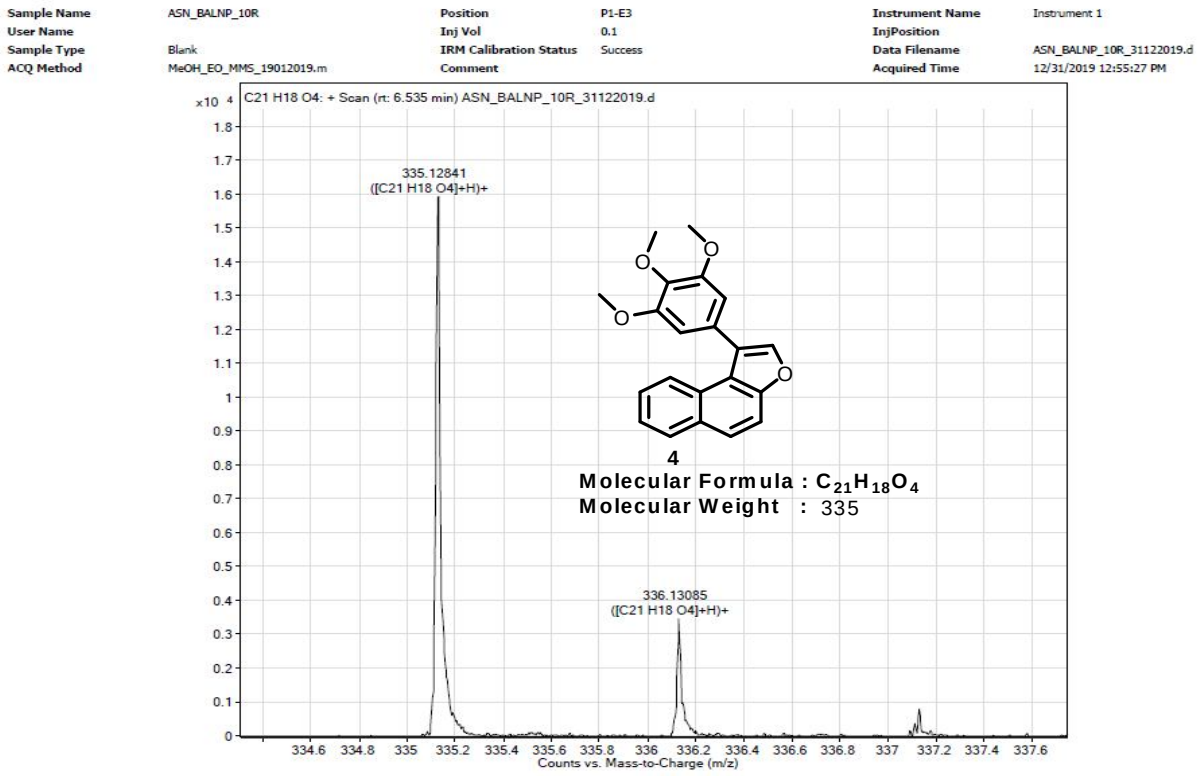
Compound 8E: ^{1}H NMR



Compound 8E: ^{13}C NMR



Intermediate 4: HRMS (ESI-TOF) m/z [M+H] calcd for $C_{21}H_{18}O_4$, 335.1239, found 335.1284.



Compound 6: ESI-MS (MeOH): for $C_{27}H_{22}O_5$ 427 $[M+H]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

Sample Code : ASN_BALTS_1FC

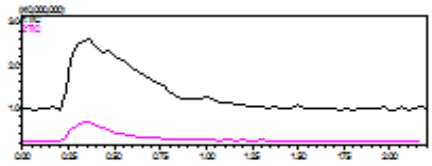
Solubility :Methanol

Name of the Scientist : Dr. A.S.NEGI

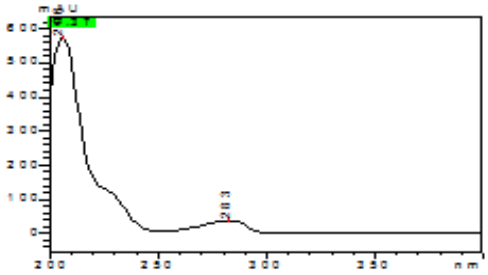
Project Code: MLP-02

Mass Range: 100-600

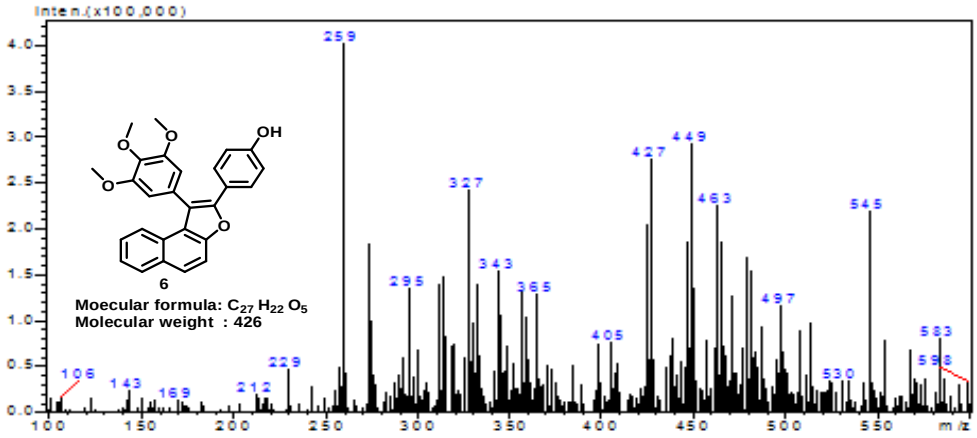
Mass TIC Chromatogram



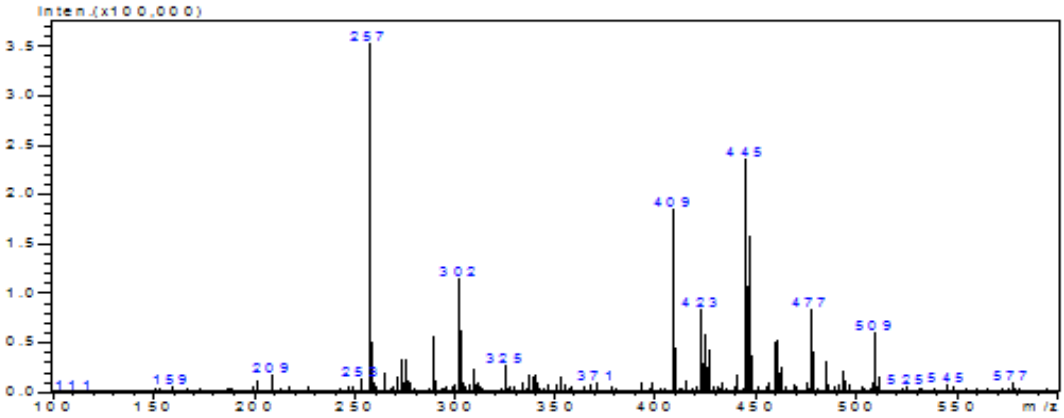
UV-Spectra



ESI+



ESI-



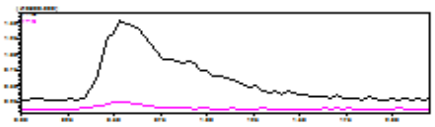
Compound 7C: ESI-MS (MeOH): For $C_{30}H_{28}O_7$, 523 $[M+Na]^+$ and 499 $[M-H]^-$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule

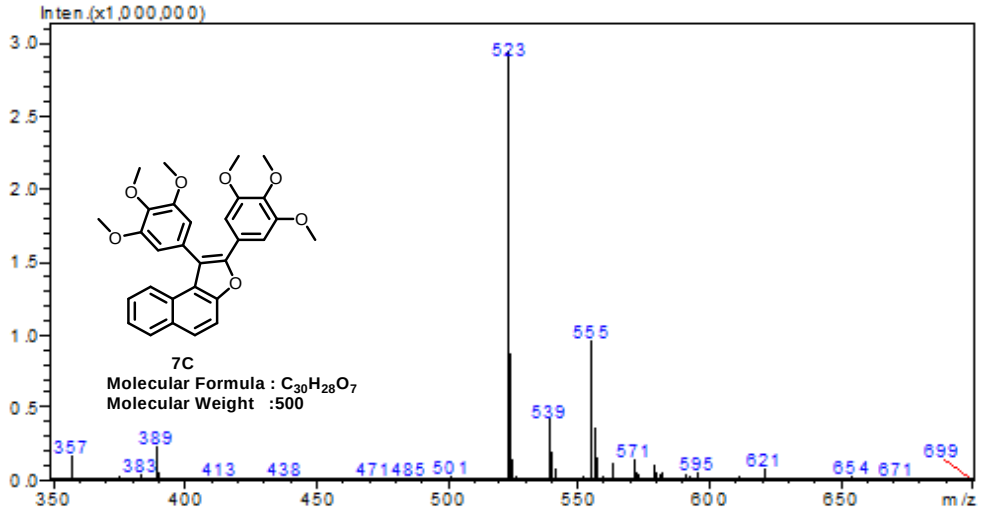
Sample Code : ASN_BALNP19
Solubility : MeOH
Name of the Scientist : Dr A S NEGI

Project Code: MLP-02
Mass Range: 350-700

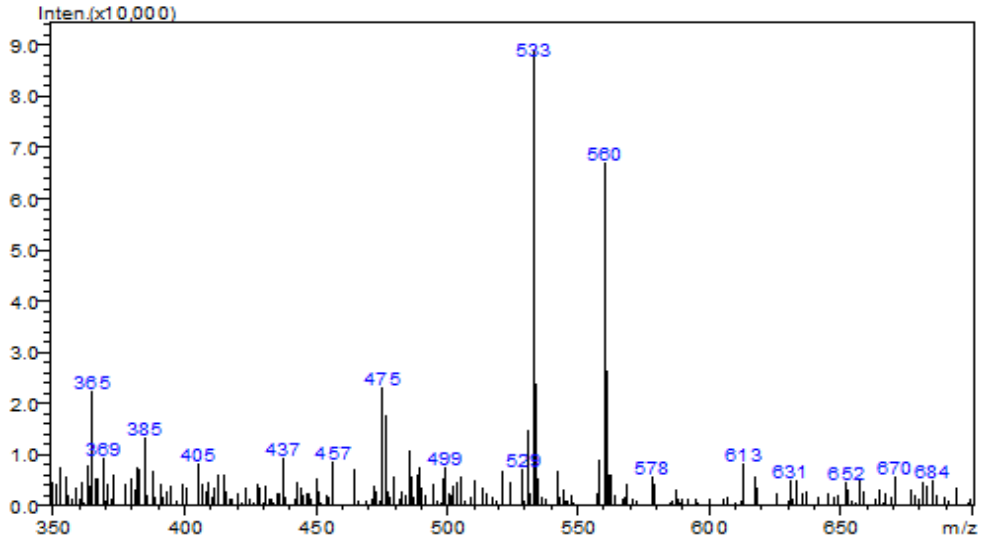
Mass chromatogram



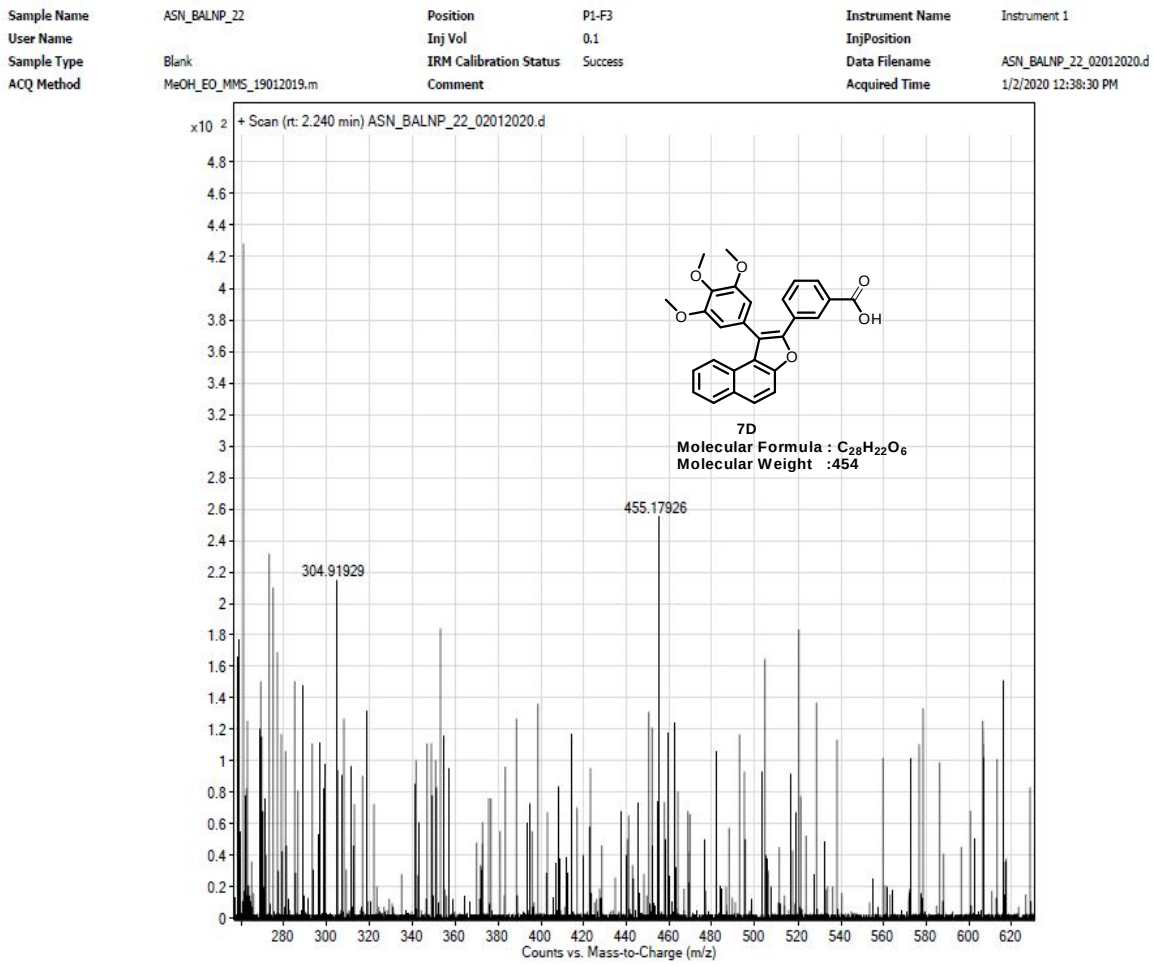
ESI+



ESI-



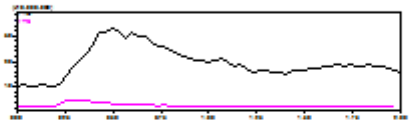
Compound 7D: HRMS (ESI-TOF) m/z [M+H] calcd for $C_{28}H_{22}O_6$, 455.1416, found 455.1792.



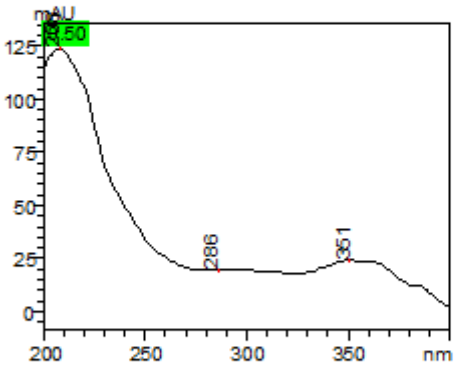
Compound 8E: ESI-MS (MeOH): for $C_{29}H_{21}NO_5$ 464 $[M+H]^+$.

Sample Information for Direct Mass Analysis of Isolates/synthetic molecule
Sample Code : ASN_BALNP41
Name of the Scientist : Dr. A.S.NEGI
Project Code: MLP-02
Mass Range: 300-700

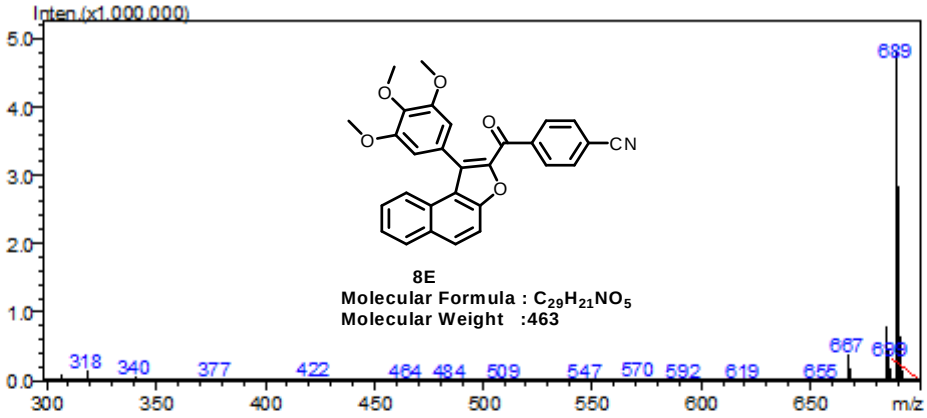
Mass chromatogram



UV-Spectra

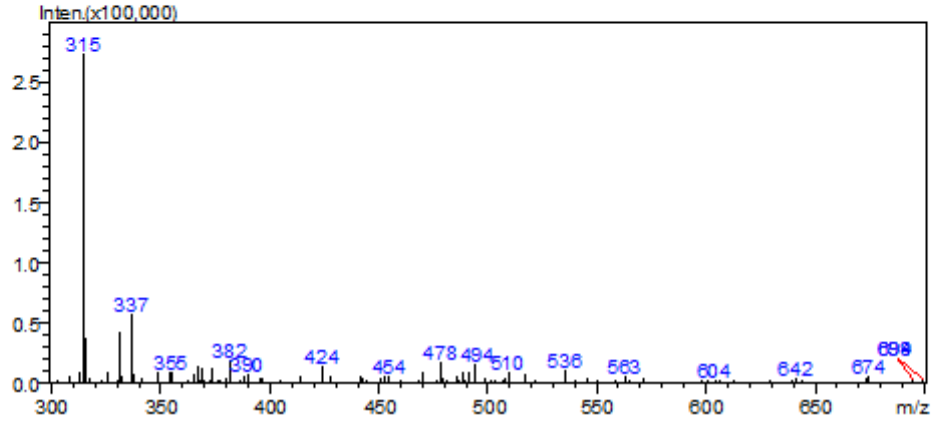


ESI+



8E
Molecular Formula : $C_{29}H_{21}NO_5$
Molecular Weight :463

ESI-



Summary of Research Work

Summary

The thesis accredit, **"Studies on synthesis of designed and isolated anticancer molecules"** involves designing of novel colchicine and estradiol based 2, 3-diarylnaphthofuran and 2, 3-diarylbenzofuran core, various prototypes, their synthesis and biological evaluation. Apart from these prototypes value addition of natural product of brevifoliol and total synthesis of biologically active natural molecule rugosa flavonoid-B have been undertaken. The studies mainly involve tubulin and caspase proteins as biological targets for anticancer agents. While, polyketide synthase-13 and enoyl-ACP reductase were biological targets for antitubercular agents. All these investigations have been carried out by the author under the supervision of Dr. Shailesh Kumar, Assistant professor, Department of Applied Chemistry, Babasaheb Bhimrao Ambedkar University, Lucknow and Dr. Arvind Singh Negi, Senior Principal Scientist, Chemical Science Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, UP, India. The work embodied in this thesis has been covered in five Chapters.

Chapter-1: "Recent advances in the chemistry and pharmacology of taxol and its derivatives"

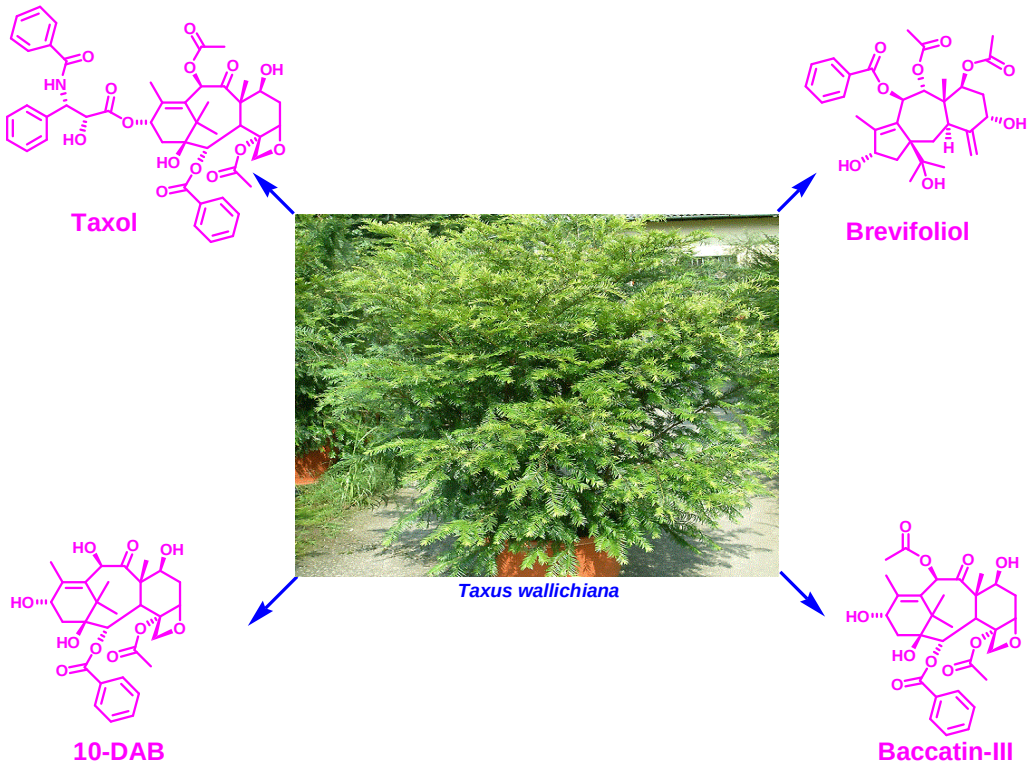


Figure (1): Important molecules of *Taxus wallichiana*

The chapter-1 covers review of natural molecules isolated from the *Taxus spp.* like taxol, 10-DAB, 10-deacetyl baccatin-III, and brevifoliol etc. Further, detailed coverage on their medicinal use, isolation of essential oil, non-taxoid isoprenoids, lignan, and isolation of taxanes from various parts of the plant (leaves, stem, twig, barks, hardwood and roots). Different sources of taxanes, *T. wallichiana* usage in various forms (herbal formulation, stem cell usage, marketed drugs). Some important molecules brevifoliol, 10-DAB, and taxol and diverse pharmacology of their derivatives have also been provided. Important biological activity of the plant given in the figure (2).

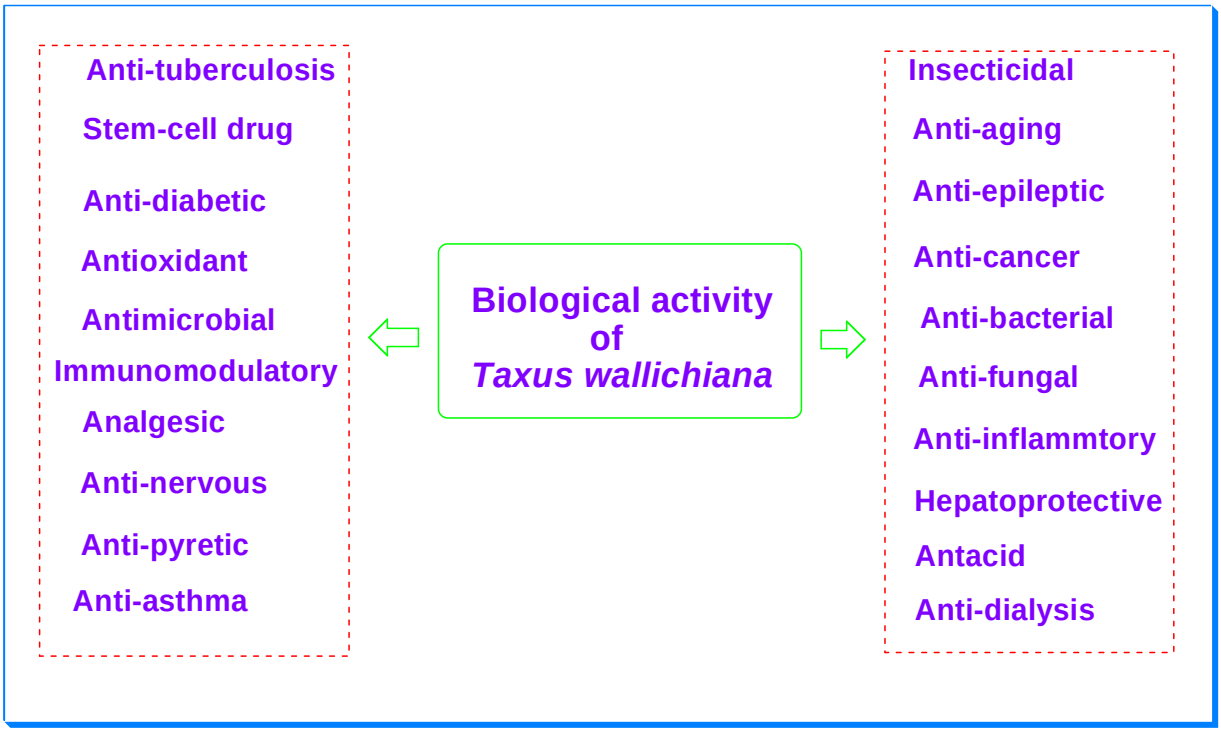


Figure (2): Various activities of *Taxus wallichiana*

Chapter-2A: "C5 Derived esters of brevifoliol as anticancer agents against prostatic adenocarcinoma"

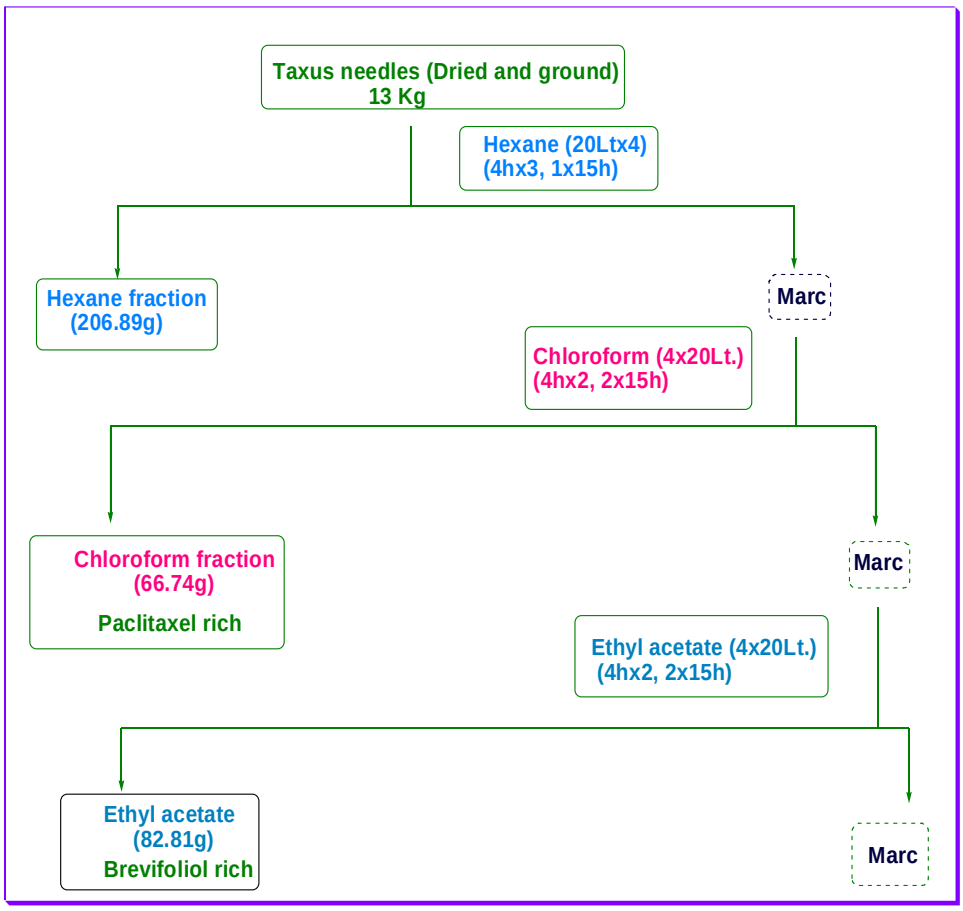
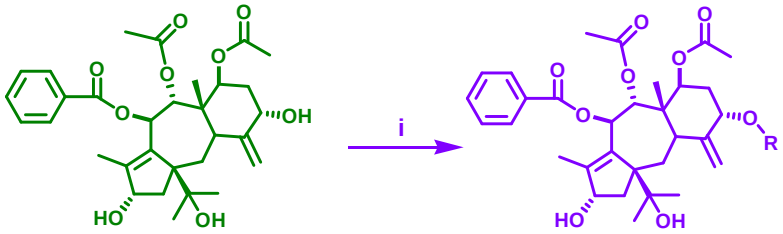


Figure (3): Isolation procedure for brevifoliol

The chapter-2A focuses mainly on development of anticancer agents against prostatic adenocarcinoma. Prostate cancer mainly effects the male. Prostate cancer incidences occupied fourth place around the world. It can be screened with the prostate-specific antigen (PSA) blood test. Prostate cancer cases were 60% more in blacks than white. However it causes remains unclear. Usually prostate cancer grows slowly and firstly attacks to prostate gland. However, critical and spreading type prostate cancer must be treated early. For the hunting of lead molecules, Selected the brevifoliol was the starting compound which was isolated from *Taxus wallichiana* needles has been derivatized as C5 esters using steglich esterification reaction. Eighteen diverse analogues were evaluated against a panel of human cancer cell lines like breast (MCF-7), colon (COLO-205), lung (A549) and prostate (PC-3) by MTT Assay.

Preparation of brevifoliol derivatives



1: Brevifoliol **Derivatives**

2: R= Angelic acid;
3: R=4-Ethylbenzoic acid;
4: R=3,4,5-Trimethoxyphenyl benzoic acid;
5: R=3-Chlorophenyl benzoic acid;
6: R=3,5-Dinitrophenyl benzoic acid;
7: R=(4-Methoxyphenyl) acetate;
8: R=(2,4-Dimethoxyphenyl)acetate;
9: R=(3,4,5-Timethoxyphenyl)acetate;
10: R=(4-Chlorophenyl)acetate;
11: R=(4-Nitrophenyl)acetate;
12: R=(3Trifluoromethylphenyl)acetate;
13: R=(4-Aminophenyl)acetate;
14: R=4-Methoxycinnamyl;
15: R=3,4,5-Trimethoxycinnamyl;
16: R=4-Methylcinnamyl;
17: R=4-Methoxyphenyl benzoic acid ;
18: R= Piperonyl;
19: R= Angelic acid ;13-Acetoxy;
20: R= Pivalic acid;

Scheme-1: **Reagents and conditions:** ROOH, DCC, DMAP, CH_2Cl_2, RT, 4-8 h, 85% -95%

Among these, two of the semi-synthetic analogues i.e. **11** and **14** exhibited potent cytotoxicity, selectively against PC-3, prostate cancer cell lines, at IC_{50} 3.89 µM and 5.02 µM respectively. In cell cycle analysis, analogue **11** induced S and G2/M phase arrest and induced apoptosis by activating caspase-3. Compound **11** showed moderate efficacy in *in-vivo* ehrlich ascites carcinoma in *Swiss albino* mice by reducing 55.85% tumour at 100 mg/kg i.p. dose. Further, compound **11** was well tolerated and found to be safe in *Swiss albino* mice up to 1000 mg/kg dose in acute oral toxicity. [B. Bhukya, *et al*, *Chem. Biol. Drug Design* **2020**; 95: 150-161]

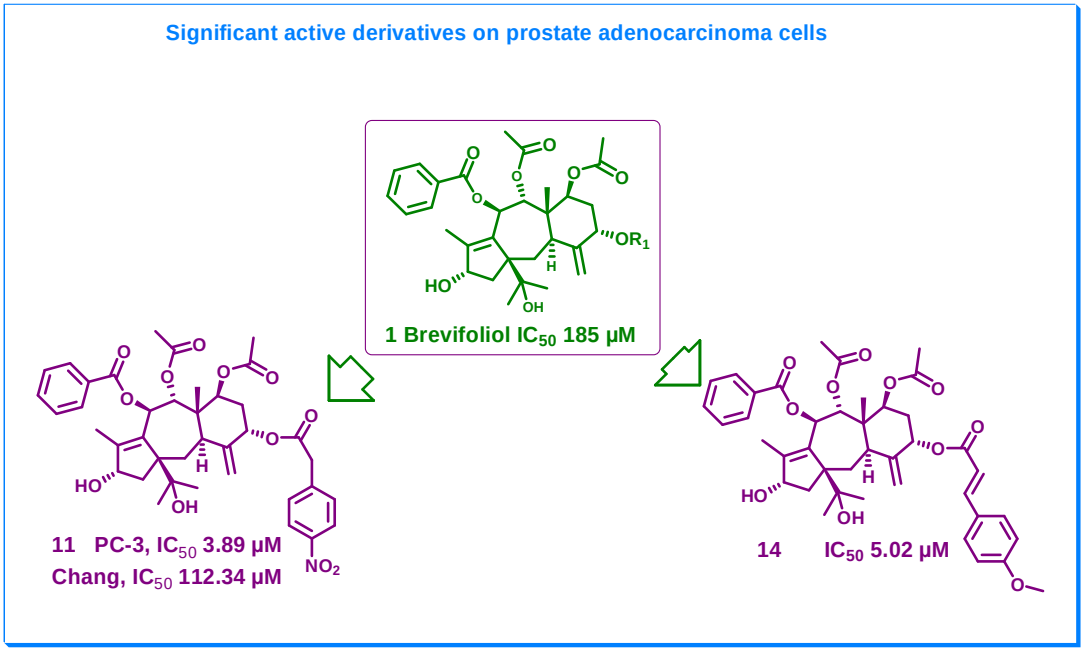


Figure (4): Potent derivatives on PC-3 Cell.

Chapter-2B: "Brevifoliol and its analogs: A new class of antitubercular agents"

Tuberculosis (TB) is one of the major causes of impermanence globally. It is caused by *Mycobacterium tuberculosis* (Mtb). It is a communicable disease. Nowadays these bacterial strains have become resistance to many of the first and second line antitubercular drugs. Day by day global HIV cases have increased in TB patients that boosted the incidences of the TB. There were many MDR cases which have to be taken special concern to reduce impermanence rate globally. Hence, special care to be taken to overcome MDR in order to get novel anti-TB drugs which can have higher activity to reduce consumption, better activity, potential permeability and rapid recovery from the disease.

Phytomolecules give new directions to reduce TB because of their structural diversity and adaptation. Hence, the moto of derivatization is that to get number of hits which may provide lead molecules with increase selectivity too. Over the past few decades, taxanes from the genus *taxus* (yews), have exhibited broad range of biological properties. A detailed literature search revealed that paclitaxol, 10-deacetylbaccatin (10-DAB) and their derivatives possess significant antitubercular activity. This boosted us to search antitubercular property of similar taxanes available in Himalayan yew i.e. *Taxus wallichiana*. Brevifoliol is an abeo-taxane and rearranged taxoid having an 11 (15→1) abeo-taxane system isolated from above plant and belongs to the same group of taxol. Himalayan yew needles relatively contain high content of

brevifoliol as compared to paclitaxel. It would be worth mentioning that taxol is obtained from the bark (which is a destructive mode of harvesting) of the plant while brevifoliol is isolated from the dried needles of the plants, which is a renewable, sustainable and a non-destructive mode of harvesting. This is first report antitubercular activity of brevifoliol and its eighteen semi-synthetic ester derivatives for their anti-tubercular potential against *Mycobacterium tuberculosis* H37Ra, an avirulent strain. The 3-(chlorophenyl) benzoic acid and 3, 5-(dinitrophenyl) benzoic acid ester derivatives (**7, 8**) were most active (MIC 25 µg/mL) against the pathogen. Further, *in silico* docking, target, ADMET risk, pharmacokinetic, toxicity effect studies were also performed for the active derivatives **7** and **8**. Additionally, both the derivatives (**7** and **8**) showed no cytotoxicity towards the healthy liver cell lines CHANG.

[B. Bhukya, *et al, Curr Topic Med Chem* 2020; 99:103784.]

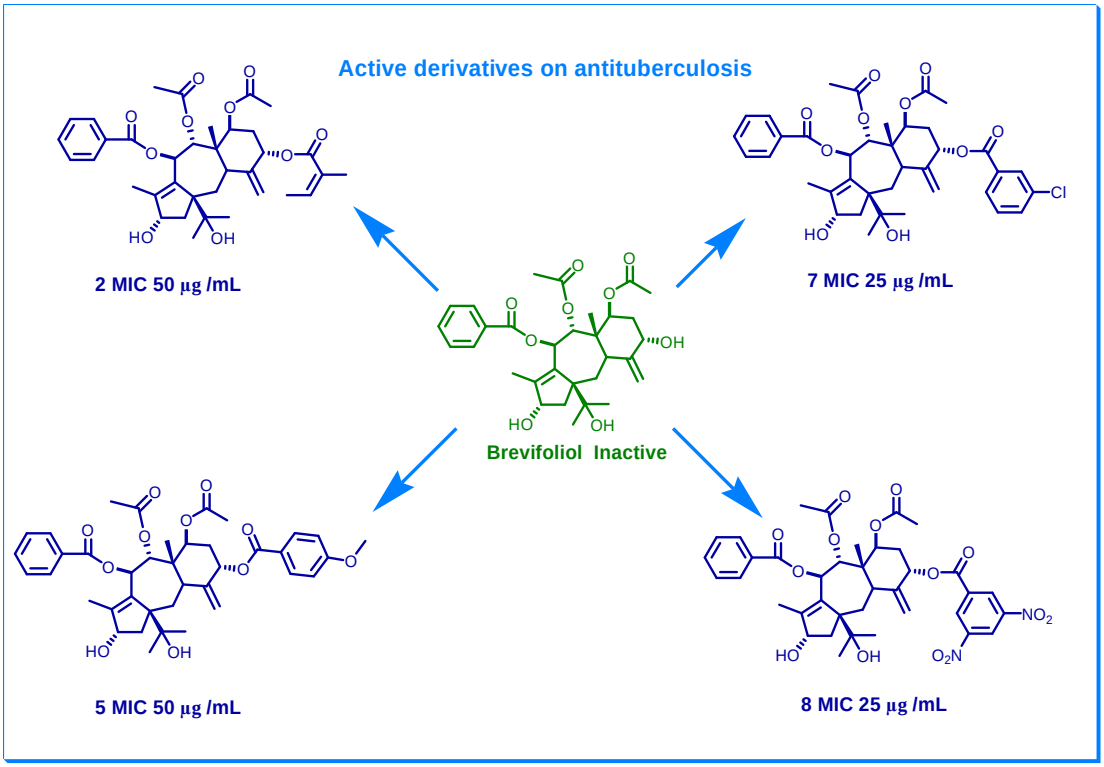


Figure (5): Potent antitubercular derivatives.

Chapter-3A: "2, 3-Diaryl benzofuran as an anti-tubercular agent"

In this chapter combretastatin A4 based benzofuran prototypes were designed and synthesized. A total of seventeen 2, 3-diaryl benzofuran hybrids were prepared and screened for their anti-tubercular potential against *Mycobacterium tuberculosis* H37Ra, a virulent strain. Out of seventeen, four derivatives showed significant activity against *M. tuberculosis* H37Ra avirulent strain (ATCC 25177) with MIC value ranging from 12.5-50 µg/mL but out of four, one derivative **11E** was significantly active (MIC 12.5 ug/mL), which was further supported by the *in silico* docking, target, pharmacokinetic, toxicity effect studies also performed for the active derivatives **8C, 10, 11C** and **11E**. All the derivatives were also evaluated for their cytotoxicity against the normal lung cell line L-132 by the MTT assay and no toxicity was observed up to 27.4 µg/mL concentration. This report on the antitubercular potential of benzofuran derivatives may be of great help in anti-tubercular drug development. [B. Bhukya, *et al*, *Bioorg Chem* 2020; 99:103784.]

Detailed synthesis strategy has been presented below schemes.

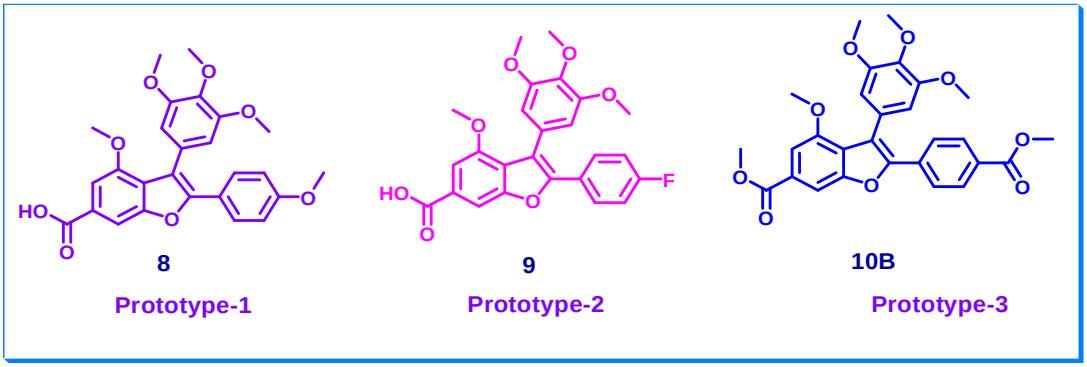
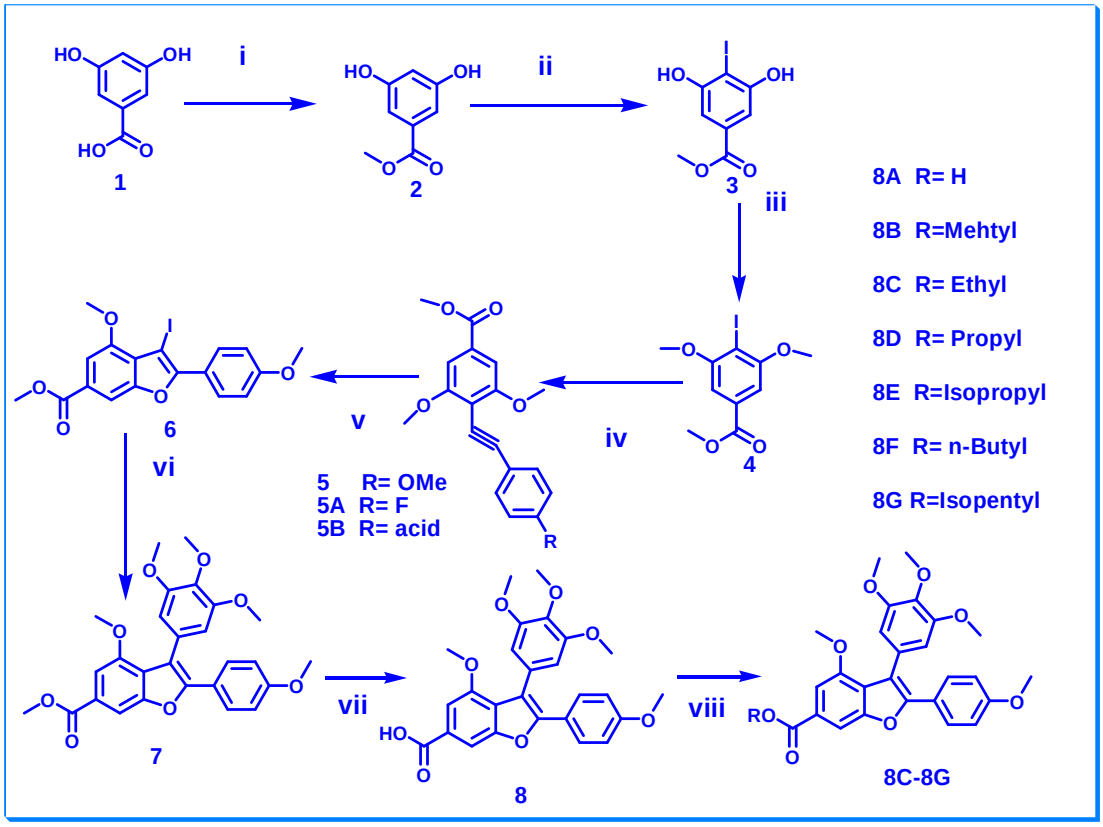


Figure (6): Designed prototypes

Scheme-2

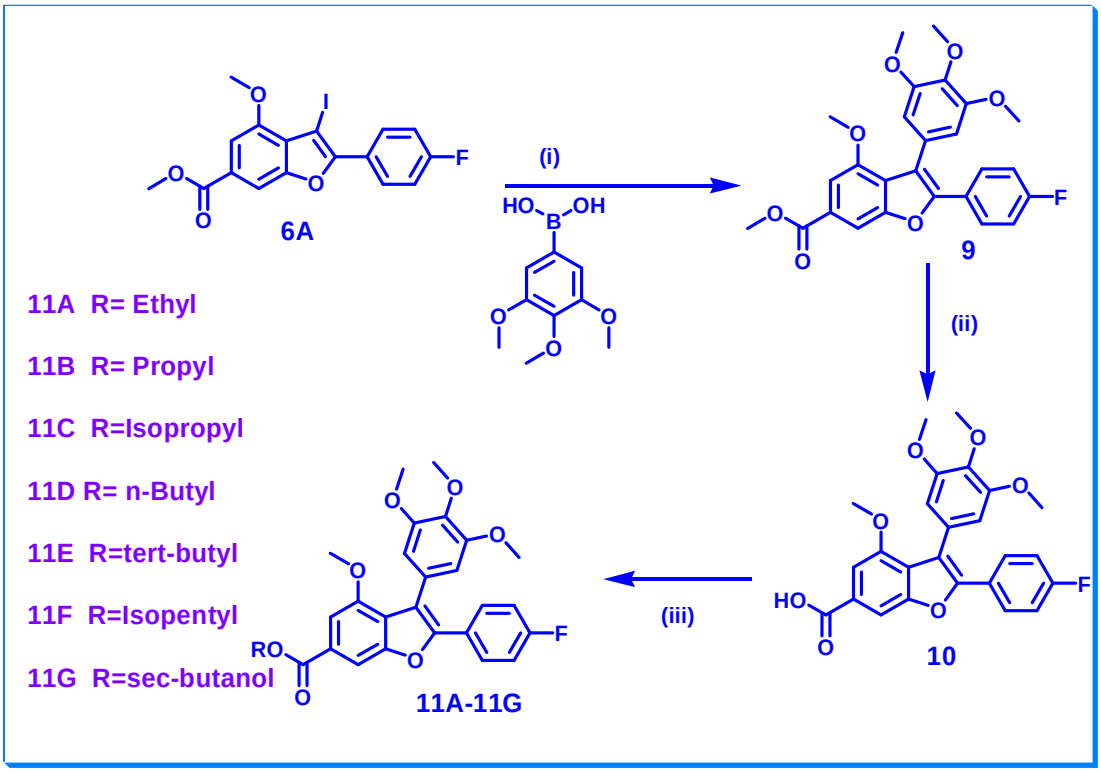
In scheme-2 provided synthesis of designed 2, 3-diarylbenzofuran pharmacophore. Synthesis started by using 3, 5-dihydroxybenzoic acid as a starting substrate and within seven steps final pharmacophore prepared and by using it seven derivatives were prepared.



Scheme-2: Reagent and condition: (i) Conc-H_2SO_4, Methanol, temp 80°C, 4h, 94%; (ii) NIS, Methanol, RT, 16h, 98%: (iii) K_2CO_3, Methyl Iodide, DMF, RT, 15h 81% : (iv) $(Ph_3P)_2PdCl_2$, CuI, Aryl iodide, TEA, 60ºC, 5h, 66%: (v) Iodine, CH_2Cl_2, RT, 23h, 96%: (vi) Trimethoxy phenyl boronic acid, $NaHCO_3$, $(Ph_3P)_2PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 89%: (vii) KOH, MeOH/ H_2O (9:1), 80ºC, 6h, 97%. (viii) ROH, H_2SO_4, 80ºC, 1-2h, 80-90%.

Scheme-3

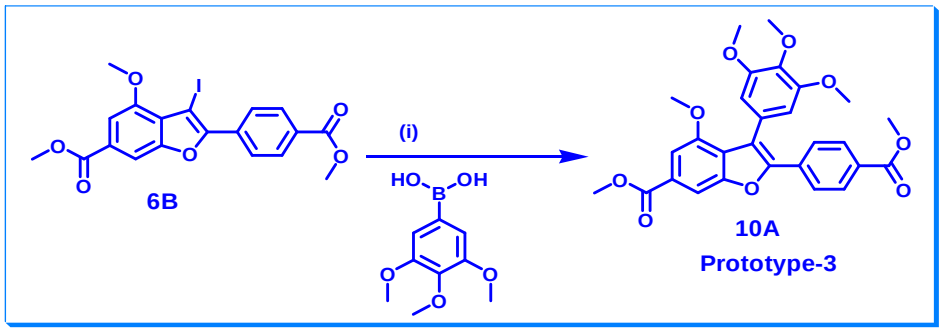
In scheme-3 benzofuran derivative **6** and 3, 4, 5- trimethoxy phenylboronic acid were reacted *via* suzuki cross-coupling reaction to get fluorinated diaryl benzofuran derivatives **9.** Ester group of **9** was saponified with 10% KOH in methanol and water (9:1) to get final pharmacophore **10**. Compound **10** used further derivatization purpose to get derivatives from **11A-11G** and confirmed by spectroscopy.



Scheme-3: Reagent and condition: (i) $NaHCO_3$, $(Ph_3P)_2PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 93%: (ii) KOH 5% in methanol/ water (9:1), 80ºC, 6h, 91%. (iii) Conc-H_2SO_4, methanol, 80ºC, 4h, 98%.

Scheme-4

In scheme-4 benzofuran derivative **6B** underwent suzuki cross-coupling reaction with 3, 4, 5- trimethoxy phenylboronic acid to get compound **10A**.



Scheme-4: Reagent and condition: (i) $NaHCO_3$, $(Ph_3P)_2PdCl_2$, DMF/H_2O (4:1), 80ºC, 7h, 80%:

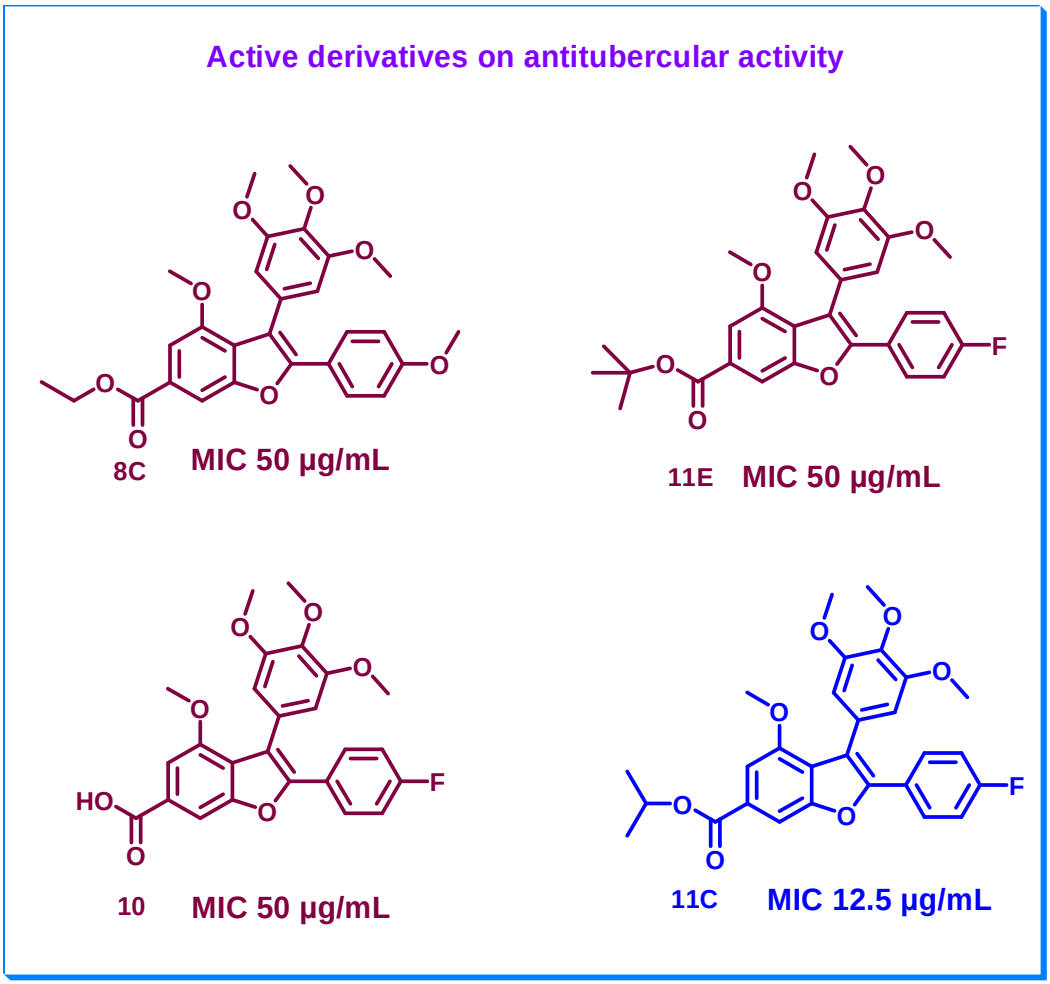


Figure (7): Active derivatives on antituberculosis

Chapter-3B: "Total synthesis of natural product rugosaflavonoid-B, and its derivatization"

Phytomolecules have great importance in our day to day life. Most of the natural products taking as medicine, supplements, aroma, in the pure form as well as in the form of herbal. Natural molecules may be from any source like medicinal, aromatic plants, marine, microbes etc. For example taxol, vincristine, podophyllotoxin and camptothecin are natural molecules, having many semi synthetic derivatives like taxotere-docetaxel and cabazitaxol, navelbine (vinorelbine) *tenifoside-VM-26, etoposide-vepesid,* topotecan-hycamtin, Irinotecan as successful drugs. Natural products and their derivatives effectively served as medicines since last 50 years. Almost 61% of the FDA approved and pre-NDA candidates are natural product or their derivatives.

By keeping the importance of natural products we have selected the rugosa flavonoid for the total synthesis. Rugosaflavonoids-B isolated from the common ornamental

flower plant *Rosa rugosa* (Rosaceae) which is distributed in the temperate regions of eastern asia and widely cultivated in yunnam province. *Rosa rugosa* plant petals and buds are used in the food, incense in china. Its medicinal uses also there like diarrhea, stomach-ache and gynecological problems. Xue-Mei Gao group isolated rugosa flavonoid-B from this plant and reported its anticancer activity on three human cancer cell lines. We have selected it for total synthesis and its derivatization to generate more lead molecules to see its activity improvement. Its synthetic strategy is given below.

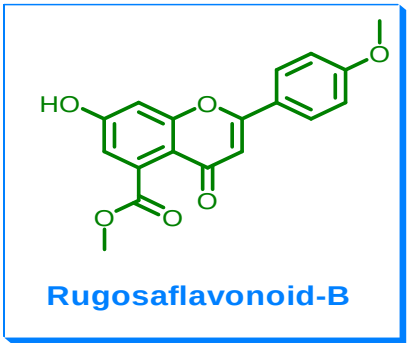
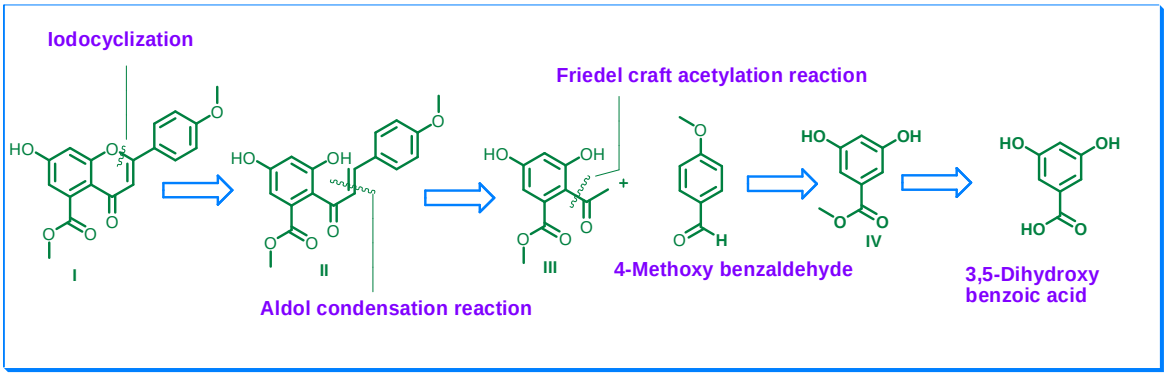


Figure (8): Rugosaflavonoid-B isolated from the *Rosa rugosa*.

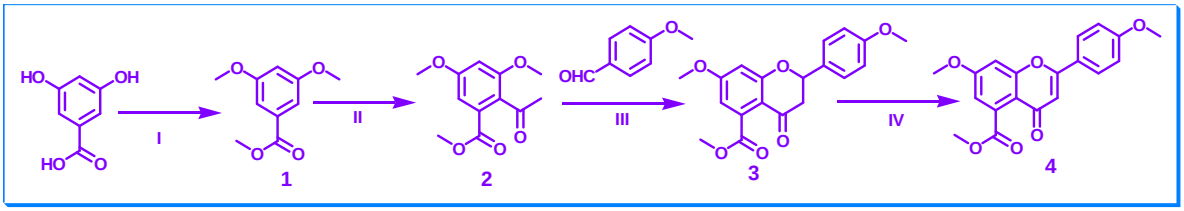
Retro-synthetic approach for the rugosaflavonoid-B presented in the scheme-1.The targeted molecule synthesis can be started with the readily available molecules is 3,5-dimethoxy benzoic acid esterfication followed by protection of one hydroxyl group can be provide synthon-IV. Then the synthesis of synthon-III can be done through friedel craft acetylation using boron trifluoride-etherate. By using synthon-III and 4-methoxy benzaldehyde to prepare synthon-II envisioned *via* aldol condensation reaction to get final target-I.

Scheme -5: Retrosynthesis of rugosaflavonoids-B.



Scheme -6

In scheme -5 we started the total synthesis of the rugosaflavonoid-B by using 3, 5-dihydroxy benzoic acid and 4-methoxy benzaldehyde as starting material. We have completed the compound **3**. Final step and its derivatization under progress in our lab.



Scheme-6: Reagents and conditions: I). 3, 5-dihydroxybenzoic acid, dry acetone, dimethyl sulphate, K_2CO_3, 80ºC, 8h, 93%. (II). Compound-1, Acetyl chloride, CS_2, $AlCl_3$, 46%. (III) Compound-2, Anisaldehyde, I_2, Pyrrolidine, Dimethyl sulphate, 8h reflux, 35%. (IV) Compound-3, Iodine, DMSO.

Chapter-4: "Design, synthesis of 2, 3-diaryl naphthofuran and its derivatives as an antibreast cancer agent"

Breast cancer is the most common invasive cancer which is second most leading cause of cancer deaths in women. To treat the breast cancer and to get more hits designing of novel colchicine and estradiol based 2, 3-diarylnaphthofuran core, prototypes, their synthesis and biological evaluation started. Designed the pharmacophore by expecting that a dual action molecule like estradiol carrier type and tubulin binder in order to show the potent anticancer activity. Out of sixteen new analogs, four analogs exhibited significant antiproliferative activity against both hormone dependent (MCF-7) and hormone independent (MDA-MB-231) breast cancer cell lines. Among them the most active compound **7D** showed antitubulin effect. In molecular docking studies compound 7**D** occupied colchicine binding pocket with high affinity with crucial residual amino acids at β-tubulin. The

compound **7D** was found to be safe and non-toxic in *Swiss albino* mice up to 1000 mg/kg oral dose. The optimization of new lead compounds may yield some better candidates in future. [Manuscript submitted *Bioorganic Chemistry*-2020]

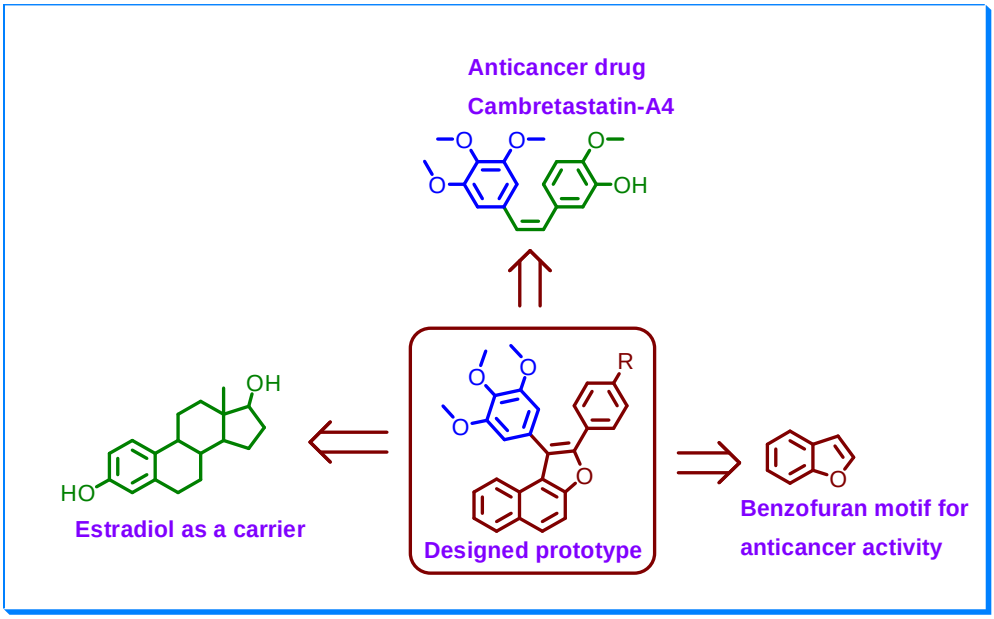


Figure (9): Designed prototype

The study provided naphthofuran derivative **7D** possessing potential anticancer activity against triple negative breast cancer. The antiproliferative activity was *via* microtubule destabilization. The compound occupied colchicine binding pocket of target protein i.e. β-tubulin with comparable affinity to standard ligands.The compound **7D** possessed moderate bioavailability in *in-silico* predictions. In toxicity studies the naphthofuran derivative **7D** was well tolerated by rodents and was non-toxic to them. This naphthofuran derivative may further be optimized for better efficacy and druggability properties in future.

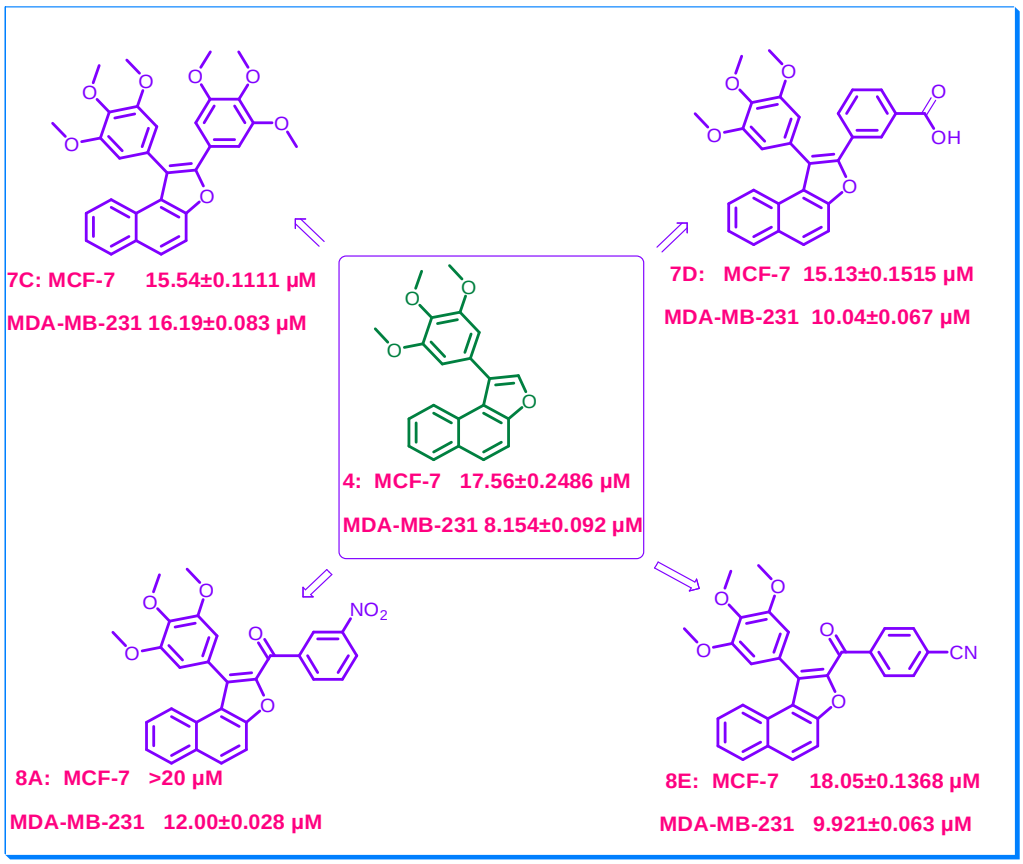
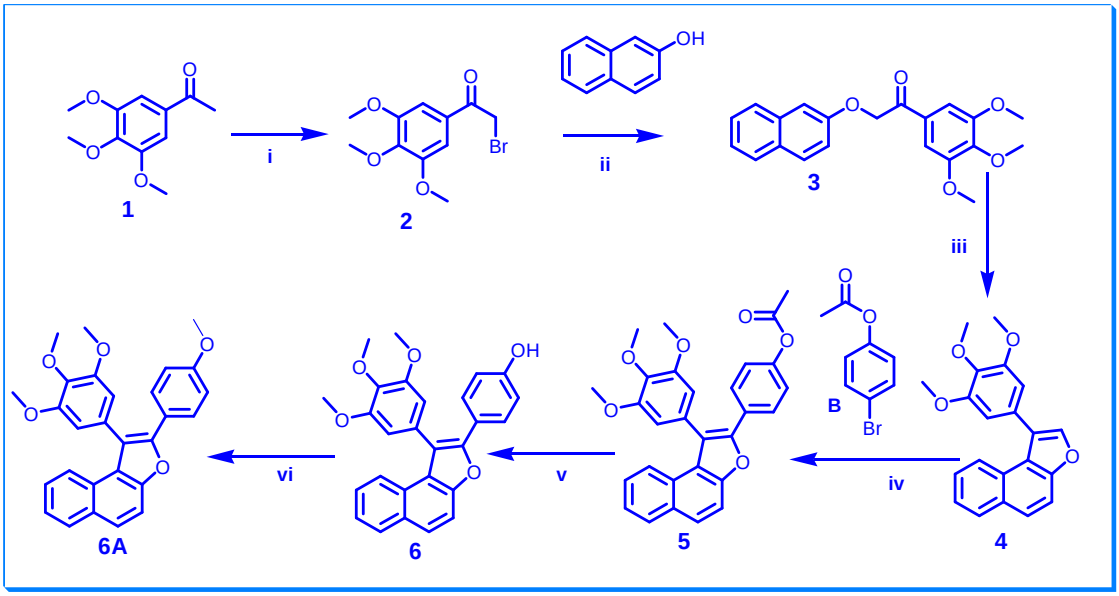


Figure (10): List of potent derivatives

Scheme-7

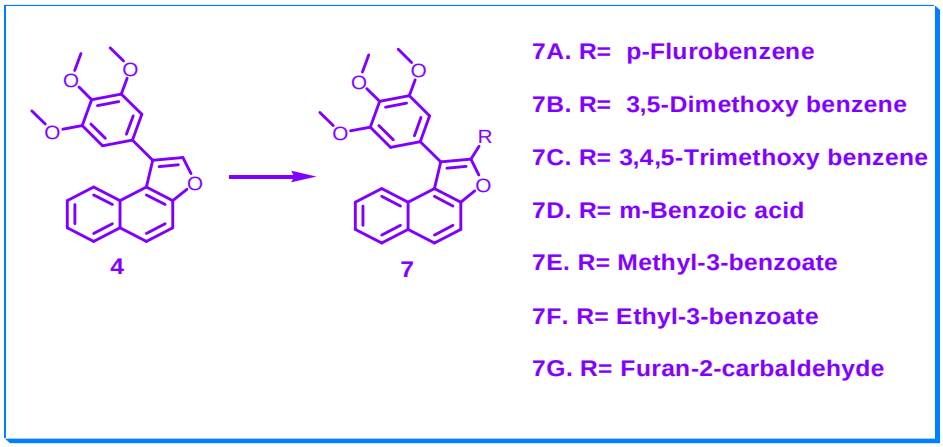
In the scheme-7 synthesis of designed 2, 3-diaryl naphthofuran pharmacophore started with readily available starting materials of 3, 4, 5-trimethoxyacetophenone and β-naphthol. Synthesis of pharmacophore was completed in five steps with good yields.



Scheme-7: Reagent and condition: **(i)** Br_2, Diethyl ether, RT, 2h, 88% **(ii)** 2-Naphthol, K_2CO_3, DMF, RT, 4h 95% **(iii)** Trifluroacetic acid, RT, 5h, 90 % **(iv)** Para-acetoxy bromobenzene, DMA, $Pd(OAc)_2$, 80ºC, 4h, 92 % **(v)** KOH, Methanol / H_2O (9:1), 80ºC, 30min, 98%. (vi) Dimethyl sulfate, K_2CO_3, Acetone, 80ºC, 1h, 90%.

Scheme-8

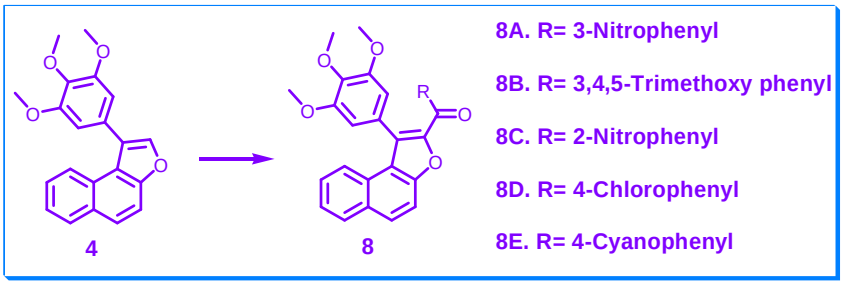
1-(3, 4, 5-trimethoxy phenyl) naphtho [2, 1-b] furan **4** was taken as a starting substrate in the scheme-8. By using the heck cross-coupling reaction prepared seven derivatives from **7A-7G** and their yields were in the range of 85-90%.



Scheme-8: Reagent and conditions: Halo benzene, DMA, $Pd(OAc)_2$, KOAc, 80ºC, 4h, 86-90%.

Scheme-9

1-(3, 4, 5-trimethoxyphenyl) naphtho [2, 1-b] furan **4** was taken as a starting substrate in the scheme-9. By using the heck cross-coupling reaction prepared five derivatives from **8A-8E** and their yields are in the range of 85-89%.



Scheme-9: Reagent and conditions: Benzoyl halide, DMA, Pd $(OAc)_2$, Potassium acetate, 80ºC, 4h, 85-89%.

Overall, the present work developed few anticancer and antitubercular agents. These bioactive molecules exhibited pharmacological activity *via* well defined biological targets. Total synthesis of a natural product was also developed. Several new chemical entities (NCEs) were also developed. Three research papers in international journals of repute have been published and one in under communication. Further optimization of identified leads should be taken up in future.

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List of Publications

LIST OF PUBLICATIONS

1. **Balakishan Bhukya**.; Kaneez Fatima .; Abhishek Nagar .; Vijaya Lakshmi .; Poornima Dubey .; Shailesh Kumar .; Yogesh Kumar .; Suaib Luqman .; Debabrata Chanda .; Sudeep Tandon .; Karuna Shankar .; Feroz Khan .; Arvind S Negi .; Brevifoliol Ester Induces Apoptosis in Prostate Cancer Cells by Activation of Caspase Pathway. *Chemical Biology Drug Design* 2020:95(1):150-161. DOI: 10.1111/cbdd.13631.

2. **Balakishan Bhukya**.; Aparna Shukla.; Vinita Chaturvedi.; Priyanka Trivedi.; Shailesh Kumar.; Feroz Khan.; Arvind S. Negi.; Santosh Kumar Srivastava.; Design, synthesis, in vitro and in silico studies of 2, 3-diaryl benzofuran derivatives as antitubercular agents. *Bioorganic Chemistry* 2020; 99:103784. DOI: 10.1016/j.bioorg.2020.103784

3. **Balakishan Bhukya**.; Sarfaraz Alam.; Vinita Chaturvedi.; Priyanka Trivedi.; Shailesh Kumar.; Feroz Khan.; Arvind S. Negi.; Santosh Kumar Srivastava.; Brevifoliol and its Analogs: A New Class of Anti-tubercular Agents. *Current Topics in Medicinal Chemistry.* DOI: 10.2174/1568026620666200528155236

4. **Balakishan Bhukya**.; Tanu Kaushal.; Deepak Kumar.; Diksha Singh.; Shahnaz Parveen.; Sana Khan.; Gauri Pathak.; Suaib Luqman.; Rituraj Konwar.; Debabrata Chanda.; Feroz Khan.; Shailesh Kumar.; Abha Meena.; Arvind S. Negi.; Diarylnaphthofurans exhibit antiproliferative activity *via* microtubule destabilization. *Chemical Biology Drug Design.* 2020:

Poster presented in national and international conferences

5. **Balakishan Bhukya**.; Kaneez Fatima.; Rekha Tyagi.; Vineeta Gupta.; Abhishek nagar.; Shailesh Kumar.; Suaib Lugman.; Sudeep Tandon.; Karuna Shankar.; Arvind Singh Negi.; (2018) Poster presented in International Conference on **"Cell Death in Cancer and Toxicology (CDCT-2018)"** CSIR-Indian Institute of Toxicology Research, Lucknow, India **February 20-22, 2018** The title of the poster was "C5 Derived esters of Brevifoliol as anticancer agents against prostatic adenocarcinoma".

6. **Balakishan Bhukya.;** Arvind Singh Negi.; Hariom Gupta.; Karuna Shankar.; Namita Gupta.; Anju Yadav.; Manju Singh.; SudeepTandon.; Shailesh Kumar.;

(2019) Poster presented in the **Conference on magnetic resonance in medicine and 25th National Magnetic Resonance Society meeting on 13th-16th February-2019 at the auditorium, Indian National Science Academy (INSA) New Delhi**. The title of the poster was "^{1}H-NMR based metabolic profiling in five Chemotypes of *Cymbopogon flexuous* (lemongrass)".

7. **Balakishan Bhukya**.; Kaneez Faitma.; Vijayalakshmi.; Poornima Dubey.; Yogesh Kumar.; Suaib Luqman.; Debabrata Chanda.; Feroz Khan.; Sudeep Tandon.; Arvind Singh Negi.; Shailesh Kumar.; **(2019)** Poster presented in **7th International Symposium on Current Trends in Drug Discovery Research 20th- 23rd February-2019 at the CSIR-Central Drug Research Institute**. The title of the poster was "BRE-13 exhibits antiproliferative activity through modulation of caspase pathway in prostate cancer cells".

8. **Balakishan Bhukya.;** Arvind Singh Negi.; Hariom Gupta.; Karuna Shankar.; Namita Gupta.; Anju Yadav.; Manju Singh.; Sudeep Tandon.; S. K.Srivastava.; Shailesh Kumar.; Raja Roy.; (2019) Poster presented in **National Conference on Mints-Prospects, Challenges and Threats 24th- 26th February-2019. In the CSIR- Central Institute of Medicinal and Aromatic Plants**. The title of the poster was "^{1}H-NMR based metabolic profiling in Twelve Varieties of *Mentha arvensis* (Field mint)".

Oral presentations in national and international conferences

9. **Oral presentation** delivered in the National Seminar on Chemical Sciences: Advancing Frontier **15thand 16th March-2019 at the Department of chemistry, university of Lucknow**. The title of the Oral presentation was "New Brevifoliol derivatives as Antitubercular and Anticancer Agents".

10. **Oral presentation** delivered in the Global conference on the control of green house gases at the source by physical and chemical technology on **22-24 April-2019 at the Babasaheb Bhimrao Ambedkar University of Lucknow**. The title of the Oral presentation was "Synthesis of 2, 3-diaryl benzofuran derivatives by incorporating greener steps".

Training Acquired

1. Training attended National workshop on **"Small Molecule Analysis by NMR Spectroscopy and Mass Spectrometry" from 16th-18th March, 2016** at Sophisticated Analytical Instrument Facility in Central Drug Research Institute (**SAIF-CDRI**) Lucknow.

2. One day Hands-on Training/Workshop on **"Structure-activity relationship studies for lead identification by using 3D QSAR tool and Techniques" on 20th June, 2016**. At CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP) Lucknow.