Chapter 1

Synthesis and Bioactivities of Betulinan C and its Analogs
1.1 Introduction

1.1.1 Natural products in drug discovery

Indeed, natural products have been undoubted eternal source of drug for mankind. Before establishment of drug discovery methodology i.e. high-throughput screening and genomic age more than 80% of the drugs were inspired by natural products.

![Pie chart showing classification of drugs approved from 1981 to 2010.](image)

Nearly half of the existed drugs from the last two decades in US are either natural product or their derivatives. These natural products are mostly secondary metabolites from animals, bacteria, fungus and plants. In addition, deep sea is rich source of marine natural products isolated from brown algae, bryozoans, cnidarians, echinoderms, green algae, mangroves, microorganisms, molluscs, phytoplankton, red algae, sponges and tunicates. In 2012 Newman and Cragg published a review article having details of all the organic compounds that are used as therapeutic solution in human diseases and reported in 1981 to 2010 (Figure 1). In a 2006 research article, Gunatilaka reviewed the natural products from plant-associated microorganism, their distribution, structural diversity, bioactivity and its relevance with biological and ecological evolution. In 2009, Molinari shaded some light on natural product and its present status and perspectives in drug discovery.

In last decade and half, due to the advancement in techniques such as combinatorial chemistry, computer assisted drug discovery, NMR, pharmaceutical companies drug programs, rational drug discovery and X-ray there is increase in the number of lead molecules in clinical trials. Presently, thousands of natural products have been screened and under testing for became a drug still there is need to explore the more number of candidates for the spectrum of diseases faced by the human being.
Chapter 1

in today's modern world. This would be an endless journey in human civilization to proliferate their race.

1.1.2 Betulinan A, B and C

In 1996, Lee et al. reported the two lipid peroxidation inhibitor with IC\textsubscript{50} value 0.46 and 2.88 μg/mL in rat liver microsomes respectively, named betulinan A 1 and betulinan B 2 were isolated from the methanol extract of fungus Lenzites betulina (Source: basidiomycetes). Betulinan A 1 having molecular formula C\textsubscript{20}H\textsubscript{16}O\textsubscript{4} CAS (55458-24-7) and IUPAC name: 2,5-dimethoxy-3,6-diphenyl-p-Bezoquinone and trivially known as Di-O-methylpolyporic acid. Betulinan B 2 having C\textsubscript{20}H\textsubscript{14}O\textsubscript{4} CAS (184092-49-7) and IUPAC name: 2-methoxy-3-phenyl-6H-benzo[c]chromene-1,4-dione. In Lipid peroxidation assay, betulinan A 1 was found four times active as vitamin E 1.68 μg/mL (Control). This demonstrate the free radical scavenger activity of betulinan A 1 and betulinan B 2.\textsuperscript{9-12}

\[
\begin{align*}
\text{Betulinan A 1} & \quad \text{Betulinan B 2}
\end{align*}
\]

In 2003, Wijeratne et al. reported the isolation of betulinan A 1 along with the three structurally new acids and other four known natural products from Aspergillus terreus in the rhizosphere of Opuntia versicolor using bioassay-guided fractionation. The Betulinan A 1 had exhibited cytotoxicity on human tumor cell line with IC\textsubscript{50} value 58.4, 8.7, 28.0 μM against NCI-H460, MCF-7 and SF-268 respectively.\textsuperscript{13, 14} Betulinan A 1 is reported for its generation via direct C-H arylation of arylphenol using aryloboronic acid and iron as catalyst in the form of Fe\textsubscript{2}(SO\textsubscript{4})\textsubscript{3}.\textsuperscript{15}

In October 2012, Liu et al. reported isolation of 2-methoxy-3,6-diphenyl-4H-pyran-4-one 3 (Molecular formula C\textsubscript{18}H\textsubscript{14}O\textsubscript{3}), named betulinan C 3 from fungus Lenzites betulinan along with three other compounds, out of which two compounds were firstly isolated from macrofungi.\textsuperscript{16}
In January 2013, El-Elimat et al. reported the isolation of compounds BTH-II0204-207\(^5\) and \(6\) from an organic extract of an ascomycete fungus order of Chaetothyriales (MSX 47445) along with betulinan A \(1\) and betulinan C \(4\). The name betulinan C has been repeated for structurally resembling isolated compounds from the same source fungus \textit{Lenzites betulina} in vicinity time. However, name betulinan C \(4\) assigned by El-Elimate for compound appears to be more rational and the same is taken for present study. Betulinan C \(4\) is having molecular formula \(\text{C}_{19}\text{H}_{14}\text{O}_3\) and IUPAC name: 3-methoxy-2,5-diphenyl-[1,4]benzoquinones. These three isolated compounds were evaluated for cytotoxicity against human cancer cell, antimicrobial activity against \textit{S. aureus} and \textit{C. albicans} and phosphodiesterase (PDE4B2) inhibition activity. Betulinan A \(1\), BTH-II0204-207 \(5\), betulinan C \(4\) and rolipram (Positive control) were found with IC\(_{50}\) value 44, 31, 17, 0.4 \(\mu\)M respectively for PDE4B2 inhibition activity. The docking studies of these compounds with validated protocol were performed on PDB:1RO6.\(^1\)

The docking results were found in best correlation between docking score and PDE4B2 inhibition activity. Betulinan A \(1\), BTH-II0204-207 \(5\), betulinan C \(4\) and rolipram (positive control) were shown docking score -8.071, -8.277, -8.732, -11.396 kcal/mol respectively for PDE4B2 inhibition activity. The \(^{13}\)C NMR and \(^1\)H NMR values for structure of betulinan C \(4\) were assertively assigned by using HMBC and NOSEY spectroscopic experiments. The crystal structure was resolved with X-ray spectroscopy data. HT-II0204-207 \(5\) and betulinan C \(4\) were equipotent against \textit{S.}}
**aureus** with MIC values of 25 μg/ml and none of the isolated compounds were found active against *C. albicans*.

In present studies, betulinan C 4 and its analogs have been synthesized; to the best of our knowledge this is the first synthesis of betulinan C 4. Betulinan C 4 and its analogs were tested for their biofilm inhibition activity, individual and synergetic anti-bacterial activity with eugenol isolated from *O. tenuiflorum*.

### 1.2 Review of Literature

#### 1.2.1 *p*-Terphenyls

Historically, polyporic acid 7 astromentin 8 and telephoric acid 9 have importance as they were the first *p*-terphenyls isolated in 1877 in beginning of chemical exploration of fungal pigment. The structural illustration of polyporic acid 7 and astromentin 8 by Kögl symbolize the noteworthy advance in organic chemistry. *p*-Terphenyls are aromatic hydrocarbon consist of three benzene rings in its structure. Interesting facts about terphenyl are as follows: Most of terphenyls occur in nature are *p*-Terphenyls and *α*-Terphenyls have not been found in nature until now. Very few naturally occurring *m*-terphenyls are reported. No *p*-terphenyls, *m*-terphenyls have been reported from kingdom plant and fungi respectively. *p*-Terphenyls are the metabolites from biogenesis of shikimate to chorismate and the best example is polyporic acid 7.

![Polyporic acid 7](image1.png)  ![Astromentin 8](image2.png)  ![Telephoric acid 9](image3.png)

Recently, it has been found and reported that some of the naturally occurring terphenyls demonstrate significant biological properties such as anticoagulant, antithrombotic, cytotoxicity immunosuppressant, neuroprotective, specific 5-lipoxygenase inhibitory.

The two *p*-terphenyls 10 and 11 were isolated from the fruiting bodies of *S. leucopus* having antibacterial activity on an average MIC >100 μg/mL against Gram
negative *P. mirabilis, E. coli, H. alvei, A. hydrophi la* and Gram positive *S. aureus S. faecalis, M. luteus*.\(^{21}\)

Seven \(p\)-terphenyl derivatives terrestrins A-G were isolated from the methanol extract of fruiting bodies of the Japanese inedible mushroom *Thelephora terrestris* (Family: Thelephoraceae).\(^{22}\) In another such finding, Saprolmycins A–E, a quinone containing five new antibiotics was isolated from the culture broth of *Streptomyces* sp. strain TK08046.\(^{23}\)

The number of synthesis has been reported for the \(p\)-terphenyls, polyhydroxylated \(p\)-terphenyls, \(p\)-terphenylquinone.\(^{24-26}\) Isomer specific synthesis has been reported for polychlorinated \(p\)-terphenyl as standards for environment analysis.\(^{27}\) Number of terphenyls and quaterphenyl derivatives was prepared by the Ni-NHC catalyzed cross coupling of the corresponding biphenyl and terphenyl-sulfonates with arylmagnesium bromides.\(^{28}\) In 2012, Kamal *et al.* reported the synthesis of terphenyl benzimidazoles having tubulin polymerization inhibition activity.\(^{29}\) \(p\)-terphenyls isolated from fungus *Paxillus crustisii* chelate irons a hypothesis in search of the possible role of \(p\)-terphenyls in fungus.\(^{30}\)

A novel series of \(p\)-terphenyl derivatives was synthesized and its *in-vitro* anticancer activities were evaluated one of the compound 12 from the series, showing the best antiproliferative activity with IC\(_{50} < 1 \mu M\) against MDA-MB-435 cell.\(^{31}\) In recent times, Rahman *et al.* reported the synthesis of terphenyls and biphenyl derivatives by using Iron (III) catalyzed coupling reaction of aryl halides.\(^{32}\)
1.2.2 Synthesis and bioactivities of quinones

The treatment of phenyl diazonium salt with 1,4-benzoquinone and sodium acetate in alcohol practically yielded 55-85% of arylquinones, these arylquinones were optically studied for their redox properties.\textsuperscript{33} Ironically, arylquinones are an organic metals having redox potentials and this property in particular makes them very important for their bioactivities and material properties. The disodium salt of hydroquinone on treatment with 30% hydrogen peroxide gave up to 70% of 2,5-dihydroxyquinone \textsuperscript{55}.\textsuperscript{34}

![Figure 2: A General mechanism of microsomal activation of quinone anti-cancer agents to free radical species. Figure 2 adopted from Bachur \textit{et al.}\textsuperscript{35}](image)

The extremely active, quinone-containing anticancer drugs, aclacinomycin A, adriamycin, carminomycin, daunorubicin, nogalamycin, rubidazone, steffimycin are from class benzantraquinone; mitomycin C and strptonigrin are from class N-heterocyclic quinone, and lapachol is naphthoquinone. These drugs interact with microsomes in mammalian cell and function as free radical species. The proposed mechanism for the action of these quinone drugs is summarized in the Figure \textit{2}.\textsuperscript{35,36}

The few natural and synthetic naphthoquinones are found active against \textit{Trypanosoma cruzi} and could be possible drug for chagas disease.\textsuperscript{37} The quinone containing alkylating agent are used for treatment cancer since 1940, the article by Beall and Winski shade some light on the mechanism of action of such drugs in NQO1-directed drug development approach.\textsuperscript{38}
In 2000, Spyroudis reviewed the hydroxylquinones, quinoid compounds in perspective of their synthesis and reactivity pattern. Similarly, Zeidan noted application of quinones in synthesis of natural products. Fiag and coworkers explored the aziridinylbenzoquinone 21 and Indolequinone 22 and 23 in structure based drug development for cancer treatment.

![Aziridinylbenzoquinone 21](image)

![Indolequinone 22](image)

![Indolequinone 23](image)

Xylariaquinone 25, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione 24, and 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione 26 were isolated from endophytic fungus Xylaria sp. Compound 24 and 25 were found active (antimalarial) against *P. falciparum* and cytotoxicity against African green monkey kidney fibroblast. The presence of the antibacterial, antitumor, perkinamycin 27 was studied using technique of spectral global fitting and $^{13}$C isotope labeling of the reactive center. Hoyt in his thesis excellently account on the anticancer mode of action of the quinone natural product Cribostatin 6 28, this manuscript would certainly be landmark in reactive oxygen species and as cancer therapy.
Chapter 1

The phytotoxic and anticancer 2,5-Bis(alkylamine)1,4-benzoquinones were prepared in yield ranging from 9-58% by treating secondary amine with 1,4-benzoquinone in ratio of 2:3. These quinones were found phytotoxic against C. sativus and S. bicolor at 1 mmol/L concentration. In addition, they also exhibit cytotoxicity against HL-60 (Leukemia), MDA-MB-435 (melanoma), SF-295 (brain) and HCT-8 (colon) human cancer cell lines and human peripheral cells (PBMC).

The naturally occurring 1,4-benzoquinones and its derivative have been used in diverse organic synthesis, the review by Abraham and coworkers focus on the computational chemistry, cycloaddition reactions, methodologies and pulse radiolysis of 1,4-benzoquinone. Interesting, recent report states lawsone (2-hydroxy-1,4-naphthoquinone) is found having unique characteristic property in class of quinones of cyanide and acetate sensing.
1.2.3 Biofilm and quorum sensing

In nature, planktonic bacteria may exist as single cell floating, swimming independently in a liquid, eventually they may attached to each other usually on solid surface, this latter mode of activities in the complex collection of microbe (Figure 3) is called as biofilm. Over 90% of the bacteria reside in biofilm. The biofilm provides them mechanical stability and resistance to extreme pH, temperature conditions and ecological stress such as antibiotics. The biofilm is normally consisting of extracellular polysaccharides, also known as exopolysaccharides (EPS). *P. aeruginosa* O1 is planktonic and non-toxic as non-colonized bacteria. However, its colonized forms of biofilm are highly toxic and found to develop multidrug resistance. The polymeric gel is non-toxic until the size of it is as big as to devastate in the immune system. The extracellular DNA is required to form the biofilm in PAO1. The Gram negative organisms such as *P. aeruginosa* make use of N-aceyl-L-Homoserine Lactones 30 and 31 (AHLs) to converse with each other.

Bacteria altruistically put their efforts to make resources available to their community. Biofilm is like a small town in which bacteria are remain together for wholesale period of time. Quorum sensing is an ability of bacterial colony to sense the size of the colony and regulates its activities and responses. *Pseudomonas*
Aeruginosa, Vibrio fisheri, myxococcus are few examples of organisms those form biofilm and act quorum sensing.

The biofilm causes serious impacts on human health, about 60% bacterial infections are origin by biofilms, like dental plaques and cystic fibrosis. Biofilm extent to cause contamination to water supply, contact lenses, medical implantation. In addition, it is also responsible for financial losses in industries, cooling towers, oil refineries, ships industries and food processing due to contamination of biofouling.

Biofilm Inhibition

In 2002, Hentzer et al. assessed 5-Bromomethylene-5H-furan-2-one 32, a halogenated furan compound for its biofilm inhibition activity against P. aeruginosa PAO1 and found interfering AHL-mediated quorum sensing mechanism.

![5-Bromomethylene-5H-furan-2-one 32](image)

In another study, L-cysteine, L-cysteine sulfoxide, sulfide and disulfide derivatives were studied as biofilm inhibition candidates. Thai traditional medicinal recipes, THR-SK011, THR-SK010, THR-SK004 which contains various part of the plant were studied for their anti-biofilm inhibition against S. epidermidis. The glass coated these biofilm were analyzed under SEM, crystal violet staining. The study further reveals that ethanolic extract of THR-SK010E (0.63-5 µg/mL) decrease the 30-40% biofilm development; whereas THR-SK004E and THR-SK011E having concentration 200-500 µg/mL, 10-20 µg/mL respectively were found to demolish 7 days old Staphylococcus biofilm within 24 h.

Based on the previously reported observation that human peptide LL-37 is having an ability to prevent the biofilm formation at concentration below its MIC. Recently, Fuente-Nunez et al. screened few synthetic small cationic peptides having low antimicrobial activity but found 1/30th MIC the peptide was able to prevent the >50% of biofilm formation against clinical pathogens P. aeruginosa O1, B. cenocepacia and L. monocytogenes. The six known natural products including reserpine (MIC 15.3 µg/mL), linoleic acid (MIC 31.2 µg/mL), berberine (MIC 63.5 µg/mL), chitosan (MIC 63.5 µg/mL), eugenol (MIC 63.5 µg/mL) and curcumin (MIC 250 µg/mL) were identified to have biofilm inhibition activity against opportunistic
The nine new polyhydroxyanthraquinones 33-41 are isolated for endophytic fungus *P. restrictum* uncovered as the potential quorum sensing inhibition against clinical isolates of *S. aureus* with IC\(_{50}\) values ranging from 8-120 \(\mu\)M. Phelan and coworker in their review discussed in detail regarding different class of chemical compounds acting as biofilm inhibiting agents.

The toluene extract of seaweed *C. crispus* along with (+) usnic acid 42 and juglone 43 isolated for terrestrial source were examined against marine bacteria, *C. marina* and *M. hydrocarbonoclasticus* for biofilm inhibition potency. Out of these three natural products solitary juglone 43 shows anti-biofilm performance at concentration range 5-20 ppm.

The small molecules and its analogs showing bacterial biofilm inhibition via various mechanisms approximately belongs to broad chemical class viz. (-)-agelasine-D, (-)-ageloxime-D bromoageliferin, carolacton, disulfide, fatty acid, flustramine, halogenated aryl, halogenated furan, halogenated heterocycle, indole, lactone, metal salt, nucleoside, oroidin, polyhydroxylated phenol, steroid acid, sulfathiazole, sulfone, walkmycin. Recently, chlorinated metabolites 44-46 from *Leptolyngbya crossbyana*
were found potent bioluminescence inhibitor of bacterial quorum sensing in *Viberio harveyi*. Polyhydroxyanthraquinone, juglone are the only compounds having 1,4-benzoquinone moiety in their structure and accountable for the quorum sensing inhibition.

Significantly, in present study betulinan C 4 and its analogs haven been studied for their biofilm inhibition activity.  

1.3 Present Work  
1.3.1 Objectives and methodology

The literature is documented for the isolation of betulinan A 1, B 2 and C 4 from the fungus *Lenzites betulinan*. In particular, betulinan C 4 is reported for its antioxidant and cytotoxicity and PDE4B2 inhibition activity. As increase in resistance of antibiotics in clinically pathogenic bacteria, there is need for new bio-film inhibitor candidates. Betulinan C 4 and its analogs were seen as the potential candidates for above mentioned activities. However, the synthesis of betulinan C 4 has not yet been reported, and hence there is need of short and practical route that acquiescent its large scale preparation too.

![Scheme 1: Retrosynthetic analysis of betulinan C 4](image)

The retrosynthesis of betulinan C 4 is sketch in Scheme 1. As shown in Scheme 1, we visualized 3-chloro-2,5-diphenylcyclohexa-2,5-diene-1,4-dione 47 as the potential intermediate for the synthesis of betulinan C 4. 3-chloro-2,5-diphenylhydroquinone 48 can be easily obtained by addition of hydrochloric acid to 2,5-diphenyl-[1,4]-benzoquinone 49. 2,5-diphenyl-[1,4]-benzoquinone 49 is commercially available for synthesis. The earlier imagined three steps synthesis to accomplish the TM compound starting from 2,5-diphenyl-[1,4]-benzoquinone 49, was actually achieved in two steps due to elegant modification of reagents and conditions.
In order to explore the substituent effect in the mentioned bioactivities the hydrophobic analog 50 was designed. The reports such as, a series of lipophilic 2-substituted 5,7-di-tert-butylbenzoxazoles was prepared by the reaction of 3,5-di-tert-butyl-1,2-benzoquinone with amino acids, is the example of studying t-butyl group substituent compounds for their antibacterial activity are found in literature. In consistency with strategy shown in Scheme 1, 3-methoxy-2,5-di-tert-butyl-[1,4]benzoquinone 50 was planned for its synthesis.

![Scheme 1: Retrosynthetic analysis of 3-methoxy-2,5-di-tert-butyl-[1,4]benzoquinone 50](image)

The precursor 3-chloro-2,5-di-tert-butylhydroquinone 51 was crucial intermediate in synthesis of 50. 2,5-di-tert-butylhydroquinone 53 and 2,5-diphenyl-[1,4]-benzoquinone 52 are the literature known compounds in Scheme 2. The hydroquinone 54 is easily available starting material.

1.3.2 Biofilm inhibition activity:

The betulinan C and its analogs were evaluated for their biofilm inhibition against clinical pathogen *P. aeruginosa* 01.

1.3.2.1 SEM analysis:

The phenomenon, biofilm and its inhibition has got remarkable attention in current clinical research. *P. aeruginosa* 01 is an obvious opportunistic pathogen found almost at every place. In current study the betulinan C and its analogs were evaluated for their potency in biofilm inhibition. Generally the quantification of formation of the biofilm is done by spectrophotometric method. However, the same
can be studied in detail by observing the biofilm under scanning electron microscope. This method facilitates the investigator to observe the effect of the compounds at unicellular level. The morphological changes in the biofilm are compared to that of control and the difference between them can well distinguish in SEM images as shown in Figure 12.

In present study, the effects of betulinan C and its analog on the biofilm of \textit{P. aeruginosa} have been evaluated.

1.3.3 Synergetic activity of betulinan C and its analogs with eugenol isolated from \textit{O. tenuiflorum} L.

In search of the effective therapeutic solution to the clinical problem numbers of drugs and their formulations have been tried and studied. The drug can be solely effective in particular aliment or the combination of the two or more drug together eradicate diseases. In particular this synergetic therapy has been used in alternative medicinal from the ages; studying synergetic effects enhance the understanding of the mechanism of action of the drug and broaden the new horizon in drug discovery.\textsuperscript{64} The drug approach now is more systematic and reached to the level of finding functional group and its role in the bioactivity.\textsuperscript{65} The synergetic drug therapy is demand time since the golden age of antibiotics which was started in 1928 in now at its end due to resistance generated by the pathogenic bacteria.\textsuperscript{66,67}

Recently, the water extracts of \textit{L. nobilis}, \textit{M. syriaca}, \textit{O. bascilucum}, \textit{P. guajava}, \textit{R. domascena}, \textit{R. officinalis}, \textit{S. aromaticum} and \textit{S. fruitcosa} evaluated individually and synergistically with clinically recognized antimicrobial agents having different mode inhibition action on pathogen \textit{S. aureus} isolates. The inhibition was recorded by well diffusion and microdilution methods, both the method demonstrate the enhancement of the activity in both sensitive and resistant strain of \textit{S. aureus}.\textsuperscript{68}

In another such case, the nineteen pathogenic, infectious, opportunistic \textit{candida sp.} and isolates have been studied with extract of \textit{O. sanctum} essential oil and the established, known proven drugs fluconazole and ketoconazole. This has been observed that \textit{O. sanctum} is proved to be one of the promising candidates in combinational treatment of candidosis,\textsuperscript{69} similar results were observed in case of eugenol and methyl eugenol.\textsuperscript{70}
Essential oils from the leaves of *L. multiflora*, *M. piperita* and *O. basilicum* from place Brukina Faso were analyzed by GC-FID. The nine Gram negative and Gram positive bacterial strains were studied with alone and in combination of these isolated compounds. The best results were obtained for the combination having eugenol from *O. basilicum*. Linalool, a essential oil isolated from *O. sanctum* was found as lead candidate for fungal treatment, having proposed mechanism of inhibition of H⁺ extrusion. In 2010, Khanna and co-worker found that the treatment of hyperlipidemia with *C. mukul* and *O. sanctum* along with folic acid and ramipril can be a promising synergetic combination. Redasani and Bari in their recent article, mention the synthesis of prodrugs by esterification reaction with ibuprofen with volatile natural products menthol, thymol and eugenol. These prodrugs acts slight differently to that of the formulation synergetic effect in sense that acidity caused by ibuprofen counter balanced by the natural alcohols. The same is been supported by the reduction in gastric ulceration compared to that of ibuprofen alone.

In present study, eugenol isolated from the plant *O. tenuiflorum* has been used for evaluating the individual and its synergetic effect with betulinan C and analogs against *P. vulgaris* and *S. aureus*.

1.4 Results and discussion

**Synthesis of betulinan C**

![Image of 48]

The synthesis of the betulinan C was successfully achieved by Scheme 1. The intermediate 3-Chloro-2,5-diphenyl-p-hydroquinone 48 would be an ideal intermediate for the synthesis of betulinan C, which can be easily achieved from 49 using addition of hydrochloric acid to it. The structure of the 48 was confirmed by spectroscopic data. In IR spectrum it showed –OH stretching at 3500 cm⁻¹, 3061 cm⁻¹ for aromatic C-H, 764 and 948 cm⁻¹ for mono-substituted benzene ring, 702 cm⁻¹ for Ar-Cl. In ¹H-NMR spectrum, single at 6.86 δ, a downfield proton confirmed the presence of penta-substituted benzene ring in its structure. The two broad singlets at
4.58 δ and 5.37δ were assigned for the phenolic -OH at C1 and other for -OH at C4 in vicinity of chlorine atom respectively. The aromatic envelop in range 7.30 - 7.35 for two sets of five protons were assigned to two phenyl ring protons.

Scheme 3. Reagents and conditions: i) CHCl₃, Dry HCl, overnight, 88%; ii) Dry CH₃OH, KBrO₃, cat. conc. H₂SO₄, reflux, 2 h, 59%; iii) KBrO₃, glacial acetic acid, 60-70°C, 1.5 h, 84%.

Plausible mechanism for the synthesis of betulinan C 4

PTO .....
Figure 4: Figure showing plausible mechanism for the synthesis of betulinan C 4.
Figure 4 is showing the number of intermediates and possible mechanism for the synthesis of betulinan C 4. Compound 49 on treatment with dry HCl gave Lewis acid-base complex 49a and consequently stabilized via canonical structures 49b, 49c and 49d. The stable intermediate 49d is attacked by the nucleophile Cl⁻ to yield semiquinone compound 49e. The semiquinone 49e is tautomerized to the stable aromatic compound 48. The compound 48 was isolated and its structure was confirmed by various spectroscopic techniques. Recently, Benites et al. studied similar HCl addition to the quinone in the preparation of 6-substituted dihydroxybenzaldehyde.⁷⁵

Compound 48 was converted to the 47 by oxidation with KBrO₃ in presence of catalytic acid. KBrO₃ in acidic condition liberates molecular bromine which oxidizes chlorohydroquinone into chloroquinone; however complete mechanism of the oxidation is not yet known in literature. Compound 47 forms Lewis acid-base complex with sulfuric acid, the 47a, 47b, 47c and 47d are the canonical structures. The intermediate 47d is attached by the solvent methanol and led to formation 47e which further subsequently transform to 47f and betulinan C 4.

In ¹³C NMR spectrum of compound 48 fourteen distinguish peaks were observed and tentatively assingend in Figure 5 for their respective carbon in its structure. Molecular weight for 48, C₁₈H₁₃ClO₂ [M⁺] calculated is 296 and was found m/z 292(75) [(M-4)⁺], 296(100). Compound 48 shows purity 96.6% on GC and
sensitive to air and light. The UV maxima 210 nm (541), 242 nm (297), 268 nm (149), 315 nm (129) was recorded. (The values in round brackets are extinction coefficient at that $\lambda_{\text{max}}$). The compound was recrystallized from aqueous alcohol and used for further reactions. Betulinan C 4 was achieved in one step less to that of planned retrosynthesis Scheme 3, by altering solvent and reaction conditions. The compound 48 was subjected to oxidation by mild oxidizing reagent KBrO$_3$ in dry methanol and catalytic concentrated sulfuric acid. Compound 4 was obtained as orange crystals. The molecular weight was determined as C$_{19}$H$_{15}$O$_3$ via HRMS. The UV maxima of 196, 243 and 338 nm advised the structural similarity with betulinan A 1. $^1$H NMR revealed the presence of ten aromatic protons around 7.37-7.48 $\delta$ suggesting two mono-substituted benzene rings, one olefinic proton at 6.84 $\delta$ and set of three protons of methoxy at 3.76 $\delta$. In $^{13}$C NMR spectrum of compound 4 fifteen distinguish peaks were observed and assigning in Figure 6 for their respective carbon in its structure. The all spectroscopic data was found in consistent with previously reported literature data.

![Figure 6](image)

**Figure 6:** $^{13}$C NMR spectroscopic data of 47

The intermediate compound 47 formed in conversion of 48 to 4 was isolated by treating 48 with KBrO$_3$ in glacial acetic acid and catalytic sulfuric acid. The molecular weight was determined as C$_{18}$H$_{11}$ClO$_2$ via HRMS. The UV maxima of 242 and 331 nm advised the structural similarity with betulinan C 4. $^1$H NMR revealed the presence of ten aromatic protons around 7.40-7.50 $\delta$ suggesting two mono-substituted benzene rings, one olefinic proton at 7.02 $\delta$. In $^{13}$C NMR spectrum of compound 47 eleven distinguish peaks were observed and assigning in Figure 7 for their respective carbon in its structure.
**Scheme 4.** Reagents and conditions: i) t-BuOH, Conc. H_2SO_4, reflux, 2 h, 95%; ii) KBrO_3, 2N H_2SO_4, 90 °C, 2 h, 90%; iii) CHCl_3, Dry HCl, overnight.

**Synthesis of betulinan C analogs**

The betulinan C analogs 52 and 53 were successfully achieved by Scheme 4. The compound 2,5-di-t-butyl-1,4-hydroquinone 53 was obtained by treatment of hydroquinone in t-butanol and concentrated sulfuric acid. Molecular weight for 53 C_{14}H_{22}O_2 [M^+] calculated is 222 and was found m/z 207(100), 222(61). The structure of the 52 was confirmed by spectroscopic data.

**Figure 8:** ^1H NMR spectroscopic data of 53

**Figure 9:** ^13C NMR spectroscopic data of 53

In IR spectrum it showed –OH stretching at 3412 cm⁻¹, 2992 cm⁻¹ for aromatic C-H, 2869 cm⁻¹ for aliphatic C-H stretch, 1400, 1528 cm⁻¹ phenyl ring and 1118 cm⁻¹ suggested C-O functional group in its structure. The ^1H NMR showed three distinct peaks at 1.24 δ (18H), 6.55δ (2H), 8.31 δ (2H) and assigned as in Figure 8. In ^13C NMR spectrum of compound 53 five distinguish peaks were observed and assingend in Figure 9 for their respective carbon in its structure. The crude compound 53 was
recrystallized using ethyl alcohol. The white crystalline compound 53 was for further reactions.

The compound 2,5-di-t-butyl-p-benzoquinone 52 was obtained by oxidation of 53 with KBrO₃ in 2N sulfuric acid. Molecular weight for 52 C₁₄H₂₀O₂ [M⁺] calculated is 220 and was found m/z 205(97), 220(100) [M⁺]. The structure of the 52 was confirmed by spectroscopic data. In IR spectrum it showed, 3046 cm⁻¹ for aliphatic C-H stretch, and 1018 cm⁻¹ suggested presence C-O functional group in its structure. The ¹H NMR showed two distinct peaks at 1.25 δ (18H), 6.46 δ (2H) and assigned as in Figure 10. In ¹³C NMR spectrum of compound 52 five distinguish peaks were observed and assingend in Figure 11 for their respective carbon in its structure. The crude compound was recrystallized using ethyl alcohol. The yellow amorphous compound 52 was for further reactions.

![Figure 10: ¹H NMR spectroscopic data of 52](image)

![Figure 11: ¹³C NMR spectroscopic data of 52](image)

The desire compound 51 was not obtained even after number of attempts, this may be due to the hydrophobicity of the penultimate compound 52 which is responsible for the inhibition of the reaction. The compound 55 was prepared by using Scheme 5 for some other preparation in our laboratory and used in present study for sake of curiosity. The compound 55 was confirmed with comparing the obtained physical constant with literature.³⁴

![Scheme 5. Reagents and conditions:](image)

³⁴ i) NaOH, 27% H₂O₂, HCl, 1.5 h, 68%.
Biofilm formation assay

The values of percentage of biofilm and compounds are tabulated in the following Table 1. The standard deviation is noted for each observation and mark on the right side. In the present work the synthesized betulinan C and its analogs were explore for their clinical application of to obstruct quorum sensing mediated the formation of the biofilm in *P. aeruginosa*.
Figure 12: SEM images of the biofilm of \textit{P. aeruginosa} 01 at 5000X magnification (left) and the structure of the compound (right). The dense biofilm can be seen in case of control, while rupture of the biofilm can be clearly seen in case of compound 48 and 53.

Table 1: Biofilm formation by \textit{P. aeruginosa} 01, cells of \textit{P. aeruginosa} were subjected to various concentrations of compounds and observed for the biofilm formation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>100.0 ± 0.0</td>
<td>92.9 ± 1.5</td>
<td>88.4 ± 1.0</td>
<td>79.0 ± 1.5</td>
<td>77.0 ± 1.5</td>
<td>70.8 ± 1.4</td>
</tr>
<tr>
<td>55</td>
<td>100.0 ± 0.0</td>
<td>94.6 ± 3.2</td>
<td>90.1 ± 1.6</td>
<td>84.9 ± 3.5</td>
<td>76.8 ± 2.3</td>
<td>69.2 ± 3.2</td>
</tr>
<tr>
<td>4</td>
<td>100.0 ± 0.0</td>
<td>91.3 ± 1.2</td>
<td>88.1 ± 1.6</td>
<td>76.4 ± 1.9</td>
<td>71.6 ± 0.6</td>
<td>68.4 ± 1.9</td>
</tr>
<tr>
<td>47</td>
<td>100.0 ± 0.0</td>
<td>92.7 ± 1.6</td>
<td>89.9 ± 1.3</td>
<td>85.4 ± 2.2</td>
<td>80.5 ± 0.7</td>
<td>80.3 ± 1.0</td>
</tr>
<tr>
<td>53</td>
<td>100.0 ± 0.0</td>
<td>93.1 ± 0.8</td>
<td>44.2 ± 1.3</td>
<td>18.2 ± 0.9</td>
<td>11.8 ± 1.8</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>52</td>
<td>100.0 ± 0.0</td>
<td>95.8 ± 1.4</td>
<td>85.7 ± 2.3</td>
<td>79.5 ± 3.6</td>
<td>74.4 ± 4.7</td>
<td>70.2 ± 1.0</td>
</tr>
<tr>
<td>48</td>
<td>100.0 ± 0.0</td>
<td>92.8 ± 2.7</td>
<td>70.7 ± 4.4</td>
<td>23.3 ± 2.7</td>
<td>15.0 ± 1.0</td>
<td>9.4 ± 1.0</td>
</tr>
</tbody>
</table>

\((n = 3)\)
Figure 13: Quantification of biofilm formation. Cells of *P. aeruginosa* at various concentrations of benzoquinone showed a reduction in formation of biofilm. At 0 μg/mL, a 100 percent biofilm was formed (0% inhibition), at 25 μg/mL of 48 and 53, 20 to 17% biofilm was formed (80 to 83% inhibition). The error bars represented standard deviation (*n* = 3).

The minimum inhibition concentration (MIC) was determined by spectrophotometric method. Empirically the compounds 48 and 53 having phenolic functional group in their structure show the MIC (Figure 13) close to 25 μg/ml. The SEM images (Figure 13) clearly showed that the biofilm is totally shattered in case of compounds 48 and 53.

**Zone diffusion assay**

In the present work the synthesized betulinan C and its analogs were explore for their clinical application of inhibit the growth of the Gram negative *P. vulgaris* and Gram positive *S. aureus*. The zone diffusion method was deployed for the evaluation of the antibacterial potency of the compounds. The Tables 2, 3, 4 and 5 represents the zone of inhibition value and expressed in mm. The eugenol used in this assay was isolated from the *O. tenuiflorum*, a natural source. (Chapter 3)
Figure 14: The radial representation of the structures verses antibacterial activity in term of zone of inhibition against *P. vulgaris*.

Table 2: Anti-bacterial activity of betulinan C and its analogs against *P. vulgaris* (*n*=3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>4</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>52</th>
<th>53</th>
<th>55</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of inhibition in mm</td>
<td>0</td>
<td>0</td>
<td>16 ± 3.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Individually, the compound 48 (Figure 14) was found showing zone of inhibition 16 ± 3.5 against the *P. vulgaris*. These observations can some extent correlate to biofilm inhibition assay where compound with phenolic function group was found active. However, the compound 53 having phenolic group along with hydrophobic t-butyl was found inactive for its antibacterial activity.
Figure 15: The radial representation of the structures verses synergetic antibacterial activity with eugenol in term of zone of inhibition against *P. vulgaris*.

Table 3: Anti-bacterial activity of betulinan C and its analogs against *P. vulgaris* (*n* = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>4</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>52</th>
<th>53</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of inhibition in mm</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The compounds 4, 47, 48, 49, 52, 53, 55 (Figure 15) were not showed any synergistic activity with eugenol against the *P. vulgaris*. The compound 48 (Table 3) was found negative synergetic activity with eugenol against *P. vulgaris*. 
Figure 16: The radial representation of the structures verses antibacterial activity in term of zone of inhibition against S. aureus.

Table 4: Anti-bacterial activity of betulinan C and its analogs against S. aureus (n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>4</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>52</th>
<th>53</th>
<th>55</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of inhibition in mm</td>
<td>8 ± 0.6</td>
<td>0</td>
<td>12 ±0.6</td>
<td>15 ±2.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The compounds 4, 48, 49 (Figure 16) individually were found showing zone of inhibition 8 ± 0.6, 12 ±0.6, 15 ±2.9 respectively (Table 4) against the S. aureus. Empirically, the hydroquinone and quinone having phenyl group in their structure except 47 were found active with substantial extent of zone of inhibition.
Figure 17: The radial representation of the structures versus synergetic antibacterial activity with eugenol in term of zone of inhibition against S. aureus.

Table 5: Anti-bacterial activity of betulinan C and its analogs against S. aureus (n=3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>4</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>52</th>
<th>53</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of inhibition in mm</td>
<td>0</td>
<td>0</td>
<td>11 ± 1.2</td>
<td>0</td>
<td>19 ± 1.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The compounds 48, 52 (Figure 17) were found synergistically active with eugenol showing zone of inhibition 11 ± 1.2 and 19 ± 1.0 (Table 5) respectively against the S. aureus. Surprisingly, the compound 52 was found elevation in synergetic activity with eugenol against S. aureus. The betulinan C, 4 and 48 showed negative inclination in case of synergetic activity with eugenol against S. aureus. Compounds 4, 47, 49 were showed null synergetic activity with eugenol against S.
General observations from the current study were as follows; hydroquinone with phenyl group was found active against Gram negative *P. vulgaris*. Hydroquinone and quinone with phenyl group were found active against Gram positive *S. aureus*. However, the trend of the synergetic activity could not be predicted with current studies.

1.5 Conclusion:

In conclusion, synthesis of betulinan C and its analogs were successfully achieved except analog 51; the major advantage of the Scheme 3 was the simple starting and mild reaction condition render the betulinan C 4 in quite good yield and required no further purification. We visualized the simple method that may be viable way to analogs compound even at industrial production scale. Compounds 3-chloro-2,5-diphenyl-*p*-hydroquinone 48 and 2,5-di-*tert*-butylhydroquinone 53 exhibited significant bio-film inhibition properties against *P. aeruginosa* O1 and afforded IC$_{50}$ value less than 25 μM. Quinone and hydroquinone having phenyl group are empirically found active against *P. vulgaris* and *S. aureus*.

1.6 Experimental Section

3-Chloro-2,5-diphenyl-*p*-hydroquinone 48

![3-Chloro-2,5-diphenyl-p-hydroquinone](image)

2,5-diphenyl-*p*-hydroquinone 49 (0.200 gm, 0.79 mmol) was dissolved in 100 ml dry chloroform and cooled at 0-5 °C under anhydrous condition. To this solution dry hydrochloric acid gas was purged for 10-15 min and then solution left stirring for overnight at room temperature. The reaction was monitor by thin layer chromatography; after no starting material found on TLC, the nitrogen gas was purged in the solution for 5 min and solvent evaporated in vacuum. The resulting crude product was crystallized from aqueous ethyl alcohol.

**TLC** : Ethyl Acetate:Hexane (20:80), $R_f$: 0.54 at UV$_{254}$ nm.
Appearance: White, crystalline

Yield: 88%

Physical Constant: mp 138-140 °C

UV(λ_{max}(ε)):

IR (v_{max}, cm^{-1} KBr):

GC:

HNMR:

13C NMR:

Mass (GC-MS):

3-Methoxy-2,5-diphenyl-p-benzoquinone (Betulinan C) 4

3-Chloro-2,5-diphenyl-p-hydroquinone 48 (0.050 g, 0.17 mmol) was rigorously stirred in methanol having 0.2 ml conc. H_2SO_4. To this solution potassium bromate (0.011 g, 0.20 mmol) was added in one lot and refluxed for another half an hour. On cooling, orange crystals were separated from reaction mixture. Crystals were separated and washed with 2 mL cold methanol.

TLC: Chloroform, R_f: 0.69 at UV_{254nm}.

Appearance: Orange, crystalline
Yield : 59%

**UV(λ<sub>max</sub>)** : 196, 243, 338

**IR (ν<sub>max</sub>, cm<sup>-1</sup> KBr)** : 765, 1072, 1089, 1266, 1330, 1442, 1492, 1597, 1639, 1662, 2934

**<sup>1</sup>H NMR** : (400 MHz, CDCl<sub>3</sub>) δ 7.48 (2H, dd, J = 8.0 Hz, J = 1.6 Hz), 7.42 (2H, m), 7.40 (1H, m); 7.30 (2H, dd, J = 8.0 Hz, J = 1.6 Hz), 7.39 (1H, m), 7.37 (2H, m), 6.84 (1H, s), 3.76 (3H, s)

**<sup>13</sup>C NMR** : (50 MHz, CDCl<sub>3</sub>) δ 187.13, 183.09, 155.22, 144.29, 132.76, 132.44, 130.50, 130.01, 129.22, 129.14, 128.70, 128.61, 128.53, 127.91, 61.41

**HRMS** : Calculated for C<sub>19</sub>H<sub>15</sub>O<sub>3</sub> [M + H]<sup>+</sup> 291.1015, Found 291.1020

3-Chloro-2,5-diphenyl-p-benzoquinone 47

![Image]

2-Chloro-3,5-diphenyl-p-hydroquinone 48 (0.050 g, 0.17 mmol) was stirred in 5 ml glacial acetic acid having 0.1 ml conc. H<sub>2</sub>SO<sub>4</sub>. To this solution potassium bromate (0.011 g, 0.20 mmol) was added in one lot and maintained at 70-80 °C. Resulting clear solution was stirred for another hour. On cooling, yellow crystals were obtained from acetic acid. Crystals were separated and wash with 2 mL cold water and dried under infra red.

**TLC** : Chloroform, R<sub>f</sub> : 0.84 at UV<sub>254nm</sub>

**Appearance** : Yellow-red

**Physical Constant** : mp 188-189 °C
Chapter I

Yield : 84%

UV(λ_{max}) : 242, 351

IR (ν_{max}, cm^{-1} KBr) : 695, 755, 794, 890, 1176, 1200, 1201, 1309, 1484, 1485, 1577, 1643, 1670, 2924

^1H NMR : (200 MHz, CDCl$_3$) δ 7.50(6H, m), 7.40(4H, m), 7.02(1H, s),

^13C NMR : (100 MHz, CDCl$_3$) δ 184.50 179.49, 140.98, 145.95, 132.80, 143.46, 129.82, 130.98, 130.57, 132.47, 129.70, 129.36, 128.74, 128.24

HRMS : Calculated for C$_{18}$H$_{11}$ClO$_2$Na [M + Na]$^+$ 317.0345, Found 317.0337

2,5-Di-t-butyl-1,4-hydroquinone 53

Hydroquinone 54 (0.500 g, 5.45 mmol) was dissolved in t-Butanol (10 g, 135 mmol) and heated to reflux. To this hot solution 0.5 ml conc. H$_2$SO$_4$ was added cautiously. The mixture was vigorously stirred and solidifies after some time. 25 ml water was added to this solid. The slurry was filtered and washed with water till neutral to litmus. The crude product was obtained and crystallized from ethyl alcohol to afford white puffy solid.

TLC : Ethyl Acetate:Pet Ether [fraction 60-80°C] (20:80), $R_f$: 0.72 at UV$_{254nm}$.

Appearance : White, crystalline

Physical Constant : mp 216-218 °C

Yield : 95%
2,5-Di-\(t\)-butyl-\(p\)-benzoquinone 52

\[ \text{52} \]

2,5-Di-\(t\)-butyl-1,4-hydroquinone 53 (0.400 g, 1.8 mmol) was stirred in 20 ml 2N H\(_2\)SO\(_4\) at 60-70 °C. To this solution potassium bromate (0.120 g, 2.16 mmol) was added in three lots and suspension was stirred for another hour. The resulting crude yellow product was filtered, washed with water and crystallized from ethyl alcohol.

**TLC** : Chloroform, \(R_f\): 0.88 at UV\(_{254\text{nm}}\).

**Appearance** : Yellow, crystalline

**Physical Constant** : mp 152-154 °C

**Yield** : 90%

**IR (\(v_{\text{max}}, \text{cm}^{-1}\) KBr)** : 842, 946, 1018, 1076, 1182, 1245, 1259, 1349, 1366, 1456, 1481, 1597, 1649, 2870, 2910, 2990, 3064

**\(^1\)H NMR** : (400 MHz, CDCl\(_3\)) \(\delta\) 6.46(2H, s), 1.25(18H, s)

**\(^{13}\)C NMR** : (400 MHz, CDCl\(_3\)) \(\delta\) 188.63, 154.36, 133.69, 34.72, 29.16
Mass (GC-MS) : Calculated for C\textsubscript{14}H\textsubscript{20}O\textsubscript{2} [M\textsuperscript{+}] 220, Found m/z 205(97), 220(100) [M\textsuperscript{+}]

2,5-dihydroxyquinone 55

The compound 55 was prepared as per the procedure mentioned in ref\textsuperscript{34} and confirmed by the physical constant.

Physical Constant : mp 212-214 °C

Quantification of biofilm:

The \textit{P. aeruginosa} O1 was incubated overnight in Luria bertani broth at 37 °C. 10 μL solution having 0 to 50μL/mL of betulinan C and its analogs and 50 μL diluted culture having optical density value 0.02 at 600 nm was mixed and diluted to 1000 μL medium. The resultants plated were incubated for 18 h at 37 °C. Consequently, the planktonic bacteria were removed and biofilm was washed with phosphorus buffer saline and fixed with methanol. The dried biofilm was stained by crystal violet (0.1%) and incubated for 15 minutes at room temperature. Excess of crystal violet was washed with water and dissolved by adding 2 ml 30 % acetic acid to stained biofilm. The absorbance of the solution along was recorded on spectrophotometer (UV-1700, Shimadzu) at 590 nm. The same protocol was followed for the control culture.\textsuperscript{19}

SEM of biofilm

The biofilm samples prepared were prepared as per above procedure were washed with cacodylate buffer at 4 °C. Subsequently, samples were fixed with 2% glutaraldehyde for 4 h at 4 °C and dehydrated with absolute ethanol for 15 min each. The dried biofilms were mounted on the conductive carbon cemented aluminum studs. The sample were analyzed under SEM (S-4800, Hitachi).\textsuperscript{19}
Disk diffusion assay

The bacterial culture of *Staphylococcus aureus* (Gram Positive) NCIM No. 2079 ATCC No. 6538P and *Proteus vulgaris* (Gram Negative) NCIM No. 2813 ATCC No. 9484 strain were procured from NCIM, National Chemical Laboratory, Pune-08. The nutrient broth was prepared in the 15 mL Borosil glass tube having 0.070 g of nutrient dehydrated powder, 1% agar agar and 5 mL distilled water. The resultant solution was stirred well and autoclave for 15 min at 121 °C. The bacterial culture was inoculated in the above nutrient broth in between two lighted Bunsen burner separated by less than 6 inches with the help of sterile nichrome wire loop at room temperature. The inoculated broth was incubated for 24 h at 37 °C. The base agar plates were prepared with nutrient agar. Dehydrated nutrient agar powder was added in distilled water and sterilized at 121 °C for 15 min. 20 ml of the medium was poured into sterile empty petri plates. The seed agar was prepared by adding 1% agar agar powder in nutrient broth. 5 ml of this mixture was taken in dilution tubes. It was sterilized at 121 °C for 15 min. 0.1 ml culture was added into 5 ml of seed agar, when the temperature drops up to 45 °C. The seed agar was poured on base agar plate and allowed to solidify. The stainless steel cork borer of 6 mm diameter was used to bore the wells. 3 wells were bored on single plate.

The compounds to be tested were diluted in DMSO such that it results in 1 mg/mL concentration. The 40 μL of the sample compound was added in the well, in separate well control solvent DMSO was added. The plate was incubated for 24 h at 37 °C. On the next day the zone of inhibition in mm were recorded.

1.7 Spectra

Spectra of betulinan C and its analogs are on following pages.
S1: $^1$H NMR spectrum of 3-chloro-2,5-diphenyl-$p$-hydroquinone 48
S2: $^{13}$C NMR spectrum of 3-chloro-2,5-diphenyl-$p$-hydroquinone 48
$^1$H-NMR 500 MHz
Solvent: CDCl$_3$

![Chemical Structure: Betulinan C 4](image)

S3: $^1$H NMR spectrum of Betulinan C 4
$^{13}$C-NMR 500 MHz
Solvent: CDCl$_3$

Betulinan C 4

S4: $^{13}$C NMR spectrum of Betulinan C 4
S5: $^1$H NMR spectrum of 3-chloro-2,5-diphenyl-p-quinone 47
S6: $^1$H NMR spectrum of 2,5-di-$t$-butyl-$p$-hydroquinone 53
S7: $^{13}$C NMR spectrum of 2,5-di-$t$-butyl-$p$-hydroquinone 53
S8: $^1$H NMR spectrum of 2,5-di-$t$-butyl-$p$-quinone 52
$^{13}$C-NMR 500 MHz
Solvent: CDCl$_3$

S9: $^{13}$C NMR spectrum of 2,5-di-$t$-butyl-$p$-quinone 52
References:

36. Lou, K. *Synthesis of novel antitumor 1,4-anthracenediones and functionized cyclododecicycne based molecular gears.* Kansas State University, Kansas, 1996, 297.