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Research Article

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Synthesis and Antimalarial Evaluation of a Dihydroartemisinin - Histone Deacetylase Inhibitor Conjugate

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Abstract Malaria is a tropical disease caused by several species of the *Plasmodium* genus with *P. falciparum* being the most prevalent and dangerous specie. Despite the plethora of antimalarial drugs available, there is still the pressing need to develop new agents to treat the disease; this is due to problems with existing medications such as toxicity and parasite resistance to previously effective drugs. Histone deacetylase enzyme catalyzes the removal of the acetyl group from histone lysine residues and has been shown to play a key role in the regulation of gene expression in the plasmodium parasite. In the present study, the concept of covalent bitherapy was employed to synthesize a compound in which the Artemisinin pharmacophore is chemically conjugated to a histone deacetylase inhibitor via an ester linkage. The antiplasmodial activity of the compound was evaluated against a standard sensitive strain of *Plasmodium falciparum*. They cytotoxicity of the compound was also evaluated using the Brine Shrimp lethality assay. The conjugate showed good activity against the parasite with IC_{50}: value of 5.369 nM as compared to the standard drugs Chloroquine (IC_{50}: 13.003 nM) and Dihydroartemisinin alone (IC_{50}: 9.968 nM). The conjugate showed good activity and provides a good starting point for the development of a new class of antimalarial agents.

Keywords Histone deacetylase inhibitor, Dihydroartemisinin, Phenylbutyric acid, Malaria, Hybrid

Introduction

Malaria is one of the leading causes of ill health and death in the tropical areas of the world with the disease accounting for about two hundred and sixteen million clinical cases and roughly half a million fatalities yearly [1].Several species of the *Plasmodium* parasite can cause the disease but *Plasmodium falciparum* is responsible for the most severe form of the disease in humans. Despite the fact that substantial progress has been made in the search for an effective vaccine for the disease [2], antimalarial drugs still remain the cornerstone for preventing and treating the disease. However, global malaria control efforts employing chemotherapeutic agents have been hampered by the increasing emergence of resistant strains of the *Plasmodium* parasites to currently used antimalarial drugs [3, 4]. This further highlights the need to develop newer and more effective drugs acting by novel mechanisms of action.

Histone deacetylase enzymes (HDACs) are Zinc dependent enzymes which play critical roles in modulating cell chromatin structure, transcription, and also contribute to the regulation of gene expression. They are an essential

group of epigenetic regulator and are crucial for normal cell growth and development. In the *Plasmodium* parasites, they represent potential new targets for antimalarial drug development. So far, five different sub-types of HDACs have been characterized in *Plasmodium falciparum* and they have been demonstrated to play key roles in transcriptional regulation of the parasite life cycle [5]. Several inhibitors of histone deacetylase enzyme are presently undergoing clinical trials for the therapy of diverse conditions ranging from parasitic and infectious diseases, tumours and hemoglobinopathies [6 - 7]. Some HDAC inhibitors have also been shown to possess antimalarial activity. For example, Apicidin a natural compound with a cyclic tetra-peptide nucleus was isolated from *Fusarium spp*. and is one of the earliest known compounds to demonstrate *in vitro* inhibitory effects on plasmodial HDAC. It was however found to be toxic to mammalian cells thereby ruling out its potential development as an antimalarial agent [8 – 9]. Trichostatin, hydroxamic acids, benzamides and a 2-aminosuberic acid derivative were also found to inhibit HDAC enzymes by binding in the zinc-containing active site [10 – 16]. Similarly, a number of cinnamate containing compounds were synthesized and some of them were demonstrated to induce hyperacetylation of *Plamodium falciparum* histones and this was associated with potent antimalarial activity [17]. HDAC inhibitors have also been shown to have potent activity against other parasites such as *Trypanosoma brucei* [18]. Other examples of HDAC inhibitors include: Short chain fatty acids e.g. Butyric and Valproic acids [19–24].

The Artemisinin group of compounds form the backbone of the currently recommended Artemisinin combination therapies (ACT's) for malaria chemotherapy. They were originally isolated from *Artemisia annua* but a number of semi-synthetic derivatives have also been developed such as Dihydroartemisinin, Artemether etc [25]. They all possess the endoperoxide bridge which is essential for activity and are active against both the sexual and asexual blood stage of the parasite. Their target is still the subject of debate but recent evidence suggests that the Iron (II) activated form of the Artemisinins potently inhibits *PfATP6*, a key parasite Ca^{2+} transporter [26].

One of the innovative strategies that has recently been employed in the quest for new antimalarial agents is the concept of molecular hybridization. It entails combining two antimalarial scaffolds into a single molecule with a number of attendant benefits such as improved antimalarial activity, fewer side effects and most importantly, the potential to overcome drug resistant parasite strains [27 - 28] In furtherance of this concept, a number of hybrid molecules have previously been synthesized and evaluated with some of them showing promising antimalarial activity. These include: Artemisinin-Quinine [29], Artemisinin-Triazine [30], Pyrimidine-Quinoline hybrids [31], Coumarin-Triazole analogues [32], Artemisinin – Ferrocene [33] and a Quinine-Triazole hybrid [34].

To further extend on-going efforts at developing safer and more effective antimalarial drugs, the present study aims at synthesizing a new hybrid in which the Artemisinin scaffold is chemically linked to 4-Phenylbutyric acid (a plasmodial histone deacteylase inhibitor) with the objective of obtaining a dual acting molecule with enhanced antimalarial activity. To the best of our knowledge, this study represents the first report of a hybrid molecule combining these two pharmacophores.

Materials and Methods

Chemistry

Materials

Chloroquine diphosphate was purchased from Greenfield Pharmaceutical Limited, Jiang Su, China; Dihydroartemisinin (analytically pure) was procured from Nanjing Zelang Medical Technology Company, (Nanjing, China). Oxalyl Chloride, Triethylamine, RPMI-1640 Medium, Triton X-100, Sodium L-Lactate, Trishydroxymethylamino methane (Trizma base), 3-Acetylpyridine adenine dinucleotide, Nitro Blue Tetrazolium (NBT), Phenazine Ethosulfate (PES), HEPES, 4-Phenylbutyric acid, Silica gel 60a,230-400 mesh,40-63u, Silica gel on TLC aluminium foils silica gel matrix with fluorescent indicator 254nm were all obtained from Sigma-Aldrich (Germany). Albumax (Gibco, Invitrogen, USA), All the chemicals and reagents used in the study were of analytical grade.

Equipment

Ultraviolet-Visible double beam Spectrophotometer (model 1250, Shimadzu, Japan), Fourier Transform Infrared spectrophotometer (Cary 630 model, Agilent Technologies), Agilent Liquid Chromatography (model 1260) with Agilent 6130 single quadrupole mass spectrometer. Agilent Chemstation software was used for initial processing and MassHunter was used for reporting the results, Bruker Advance III 400MHz spectrometer with an ultrashield magnet equipped with a Bruker B-ACS-60 autosampler. The software used is Bruker Topspin – the internal standard used was tetramethylsilane (TMS). Chemical shifts are reported in parts per million (ppm). The peak splitting patterns are described as singlet (s), doublet (d), triplet (t), and multiplet (m).

General Methods

The synthetic reactions were monitored by TLC using pre-coated silica gel aluminium plates; Visual detection of spots was done using a UV (ultraviolet) lamp and also by spraying with a 0.5% solution of vanillin in Sulphuric acid.

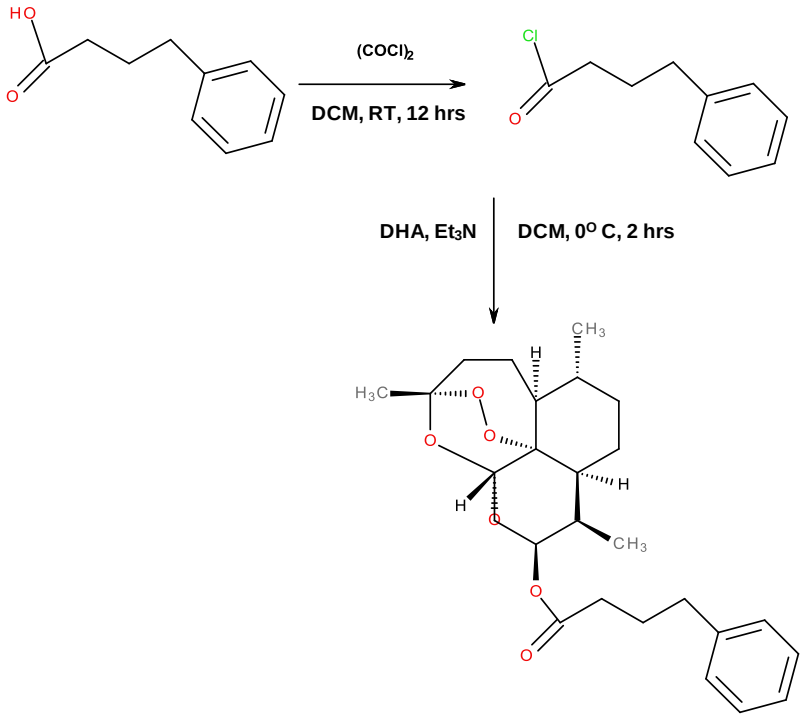
Synthesis of 4-Phenylbutyric acid - Dihydroartemisinin Ester Conjugate

(a) Synthesis of 4-Phenylbutyric Acid Chloride:

Oxalyl chloride (0.6 mL, 6.7 mmol) was added drop wise to a solution of 4-Phenylbutyric acid (1.0 g, 6.09 mmol) in dry CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature of 25° C for twelve (12) hours, and the solvent removed *in vacuo*. The product obtained was washed with n-hexane (3 x 25 mL) and dried under vacuum.

(b) Coupling of the acid chloride to dihydroartemisinin [35]

Triethylamine (0.52 ml, 3.8 mmol) was added in a drop wise manner at 0° C to a solution of dihydroartemisinin (1.0 g, 3.52 mmol) and the crude acid chloride (0.71 g, 3.57 mmol) dissolved in dry dichloromethane (30 mL). The mixture was stirred for two (2) hours while maintaining the temperature at 0° C for 2 hours after which the reaction was terminated with saturated sodium bicarbonate solution (25 mL) and extracted with dichloromethane (3 x 25 mL). The organic layer was washed with 10% aqueous Hydrochloric acid solution (2 x 20 mL), followed with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure (Scheme 1).



Scheme 1: Synthesis of 4-Phenylbutyric Acid - Dihydroartemisinin Ester Conjugate

Purification of Conjugate

The conjugate was purified using a form of medium pressure chromatography with gradient elution.

Characterization of Conjugate

The structure of the conjugate was confirmed using Ultraviolet-visible absorption spectroscopy, Fourier transform infrared spectroscopy, LC-MS, ^{1}H and ^{13}C NMR spectroscopic analyses.

Biological Evaluation

Ethical consideration

Ethical approval was sought and obtained from the Institutional Health Research Ethics Committee of the Bingham University Teaching Hospital (BHUTH) before the study began.

***In vitro* antimalarial screening (parasite Lactate dehydrogenase (pLDH) assay)**

Plasmodium falciparum Culture and Maintenance.

The chloroquine sensitive (3D7) strain of *P. falciparum* was used for the *in vitro* assay. The parasites were grown and maintained in culture by means of a previously reported method [36] with some modifications [37].

Analysis of test results from the LDH assay

The optical density (OD) values generated from the assay at various concentrations of the drug were transformed into percentage parasite growth using the formula shown below. Log dose response graphs were plotted with the aid of GraphPad Prism Software and IC_{50} values determined.

$$\% \text{ Parasite Growth} = \frac{\text{(Average OD (no drug: negative control) – Average OD (test))}}{\text{Average OD (no drug: negative control)}} \times 100$$

Brine Shrimp Lethality Assay

The cytotoxicity of the compound was evaluated according to an earlier method with slight modifications [38]. The eggs of *Artemia salina* were placed in a tank containing 1% solution of Sodium Chloride for two days to hatch the shrimp and allowed to mature as nauplii. Graded concentrations of the compound were prepared and used in the assay. Cyclophosphamide served as the positive control and a negative control without any drug was also used. The concentration-mortality data obtained was analysed statistically using probit analysis for LC_{50} determination.

Statistical Analysis

Numerical data (IC_{50} values) obtained from the assay are expressed as the mean value ± standard error of the mean. Statistical analysis were performed using the analysis of variance (ANOVA) followed by Tukey post-hoc test with the aid of IBM Statistical Package for Social Scientist (SPSS 20) software. P values < 0.05 were considered statistically significance.

Results

Results of the Synthetic Reaction

The physicochemical properties of the conjugate are presented in Table 1 below.

Table 1: Physicochemical Properties of the Synthesized Conjugate

Compound Code	Molecular Formula	Appearance	Melting Point (° C)	% Yield
4-PBEC	$C_{25}H_{34}O_6$	Off white crystals	154 - 155	75.02

(Key: 4-PBEC = 4-Phenylbutyric acid – Dihydroartemisinin ester conjugate)

Spectroscopic characteristics of the synthesized conjugate

(4-Phenylbutyric acid – Dihydroartemisinin ester conjugate) 3,6,9-trimethyldecahydro-12*H*-3,12-epoxypyrano[4,3-*j*][1,2]benzodioxepin-10-yl 4-phenylbutanoate

UV(λ_{max}): 258 nm
IR (Neat): 2926 cm^{-1} (C-Hstr. aliphatic), 1736 cm^{-1} (C=Ostr ester), 1204 cm^{-1} (C-Cstr), 1013 cm^{-1} (C-Ostr)
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.19 (m, 3H, 18, 19, 21), 7.18 (m, 2H, 20, 22), 5.81 (dq, J = 6.9, 1.5 Hz, 1H, 8), 5.42 (s, 1H, 9), 2.77 (m, 1H, 15'), 2.63 (m, 1H, 27''), 2.56 – 2.46 (m, 1H, 4), 2.50 – 2.40 (m, 1H, 5), 2.24 (m, 1H, 15''), 2.10 – 1.43 (m, 14H, 1, 2, 3, 7, 13, 14, 27', 28), 1.44 (s, 3H, 29), 0.95 (dd, J = 6.8, 1.5 Hz, 3H, 24), 0.86 (dt, J = 6.7, 1.5 Hz, 3H, 23).
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.69 (C – 12), 141.46 (C – 16), 128.61 (C - 18, C – 21), 128.58 (C - 20, C – 22), 128.53 (C – 19), 98.33 (C – 9), 98.29 (C – 26), 98.26 (C – 8), 81.64 (C – 6), 50.49 (C – 5), 50.46 (C – 4), 36.96 (C – 3), 36.94 (C – 7), 36.92 (C – 15), 36.90 (C – 13), 36.88 (C – 1), 35.19 (C – 28), 25.94 (C – 14), 25.91 (C – 29), 25.87 (C – 2), 25.84 (C – 27), 23.96 (C – 24), 12.74 (C – 23).
HRMS (ESI): m/z $[M + H]^+$431.6121

Antimalarial activity of the conjugate

The *in vitro* antimalarial activity of the synthesised compound against the 3D7 strain of *Plasmodium falciparum* determined using parasite Lactate dehydrogenase assay is presented in Table 2 below

Table 2: *In vitro* Antimalarial Activity of the Synthesized Compounds against the 3D7 Strain of *Plasmodium falciparum* Determined Using Parasite Lactate Dehydrogenase Assay.

S/n	Compound	IC_{50} (nM)
1.	4-PBEC	5.369*$^{\#}$± 0.045
2.	Dihydroartemisinin (DHA) alone	9.968 ± 0.114
3.	Chloroquine (CQ) alone	13.003 ± 0.758

The IC_{50} values are expressed as mean ± SEM, n = 3 in each group.
*indicates significant difference compared to DHA, while # indicates significant difference compared to CQ (ANOVA followed by Tukeys post hoc test, $p < 0.05$).

Brine Shrimp Lethality Assay

Cytotoxic effects as illustrated by LC_{50} values of the compound and the standard drug Cyclophosphamide are summarized in Table 3 below.

Table 3: Brine Shrimp Lethality Assay of the Synthesized Compound.

S/n	Compound	LC_{50} (µg/mL)
1.	4-PBEC	139.25* ± 5.46
2.	Cyclophosphamide (Standard)	1.08 ± 0.20

The LC_{50} values are expressed as mean ± SEM, n = 3 in each group.
* indicates significant difference compared to Cyclophosphamide (ANOVA followed by Tukeys post hoc test, $p < 0.05$).

Discussion

Spectroscopic Characteristics of the Conjugate

The 4-Phenylbutyric acid ester conjugate of Dihydroartemisinin (4-PBEC) exhibited a peak on the UV absorption spectrum at 258 nm which can be ascribed to the conjugated pi electron system of the Bezenoid chromophore specifically the B band of the Benzene ring [39]. In the infrared spectrum of the conjugate, the disappearance of the broad OH stretch absorption band which usually occurs between 3100 to 3300 cm^{-1} together with the presence of the carbonyl absorption band of the ester linkage at 1736 cm^{-1} are key features that indicate formation of the ester. Other bands that are visible in the IR spectrum of the compound are: the C-H stretch aliphatic at 2926 cm^{-1} and the C-C stretch band at 1204 cm^{-1}. In the ^{1}H-NMR, the signal at 5.81 ppm can once again be assigned to the methine proton at position 8 of the artemisinin nucleus while the aromatic protons on the phenyl ring gave signals as multiplets around the characteristic region of 7.18 to 7.31 ppm and these also indicate that the product was formed. In the ^{13}C NMR, a signal at 173.69 ppm belonging to C=O group proved the formation of compound. The liquid

chromatogram of the compound showed one main peak with retention time of 1.491 minutes while the mass spectrum of the compound displayed a peak with m/z of 431.6 ($M^+ + H$) which corresponds to the molecular ion peak (M) with addition of hydrogen and this agrees with the molecular formula $C_{25}H_{34}O_6$.

Antimalarial Activity of the Conjugate

4-Phenylbutyric acid is a low molecular weight, short chain aromatic fatty acid that has been approved by the United States FDA to treat urea cycle disorders [40]. A number of studies have expanded the therapeutic applications of the drug to diseases such as Cancer [41], Alzheimer's disease [42], homozygous β-thalassemia [43], spinal muscular atrophy [44], type-2 diabetes [45 - 46], cystic fibrosis [47] and Parkinson's disease [48]. The therapeutic actions of Phenylbutyric acid in these diseases is believed to be via two primary mechanisms: a chemical chaperone (improves protein folding and reduce endoplasmic reticulum stress) and histone deacetylase inhibition (HDACi) [49–52]. Interestingly, the drug possesses a good safety profile and shows minimal toxicity in most of the clinical conditions it is presently used to manage [53].

Histone deacetylase enzyme has been validated as a promising target for development of antimalarial drugs [6]. It has also been found that HDAC inhibitors can target multiple Plasmodium life cycle stages (asexual, gametocyte erythrocytic and exoerythrocytic liver stage forms of the parasite) and this observation has potentially important implications for the future development of this class of compounds as antimalarial drugs [54]. After an extensive literature search, it was observed that there is presently no report on the investigation of the antimalarial activity of 4-Phenylbutyric acid alone but it is likely to possess antimalarial activity due to its ability to inhibit histone deacetylase enzyme as has been shown for other HDACi's. However, due to its modest pharmacological activity when used alone in treating various conditions, most investigators advocate that Phenylbutyric acid should be combined with other agents that act in a synergistic manner [55] and this formed the basis for its conjugation with Dihydroartemisinin as a potential antimalarial agent.

When the antimalarial activity of the dihydroartemisinin–4-phenylbutyric acid conjugate was evaluated against chloroquine-sensitive 3D7 *P. falciparum*, the conjugate was found to have better activity than the control drugs Chloroquine and Dihydroartemisinin as its IC_{50} value was about 46 % better than that of DHA alone and about 58 % higher than the activity of Chloroquine alone. The values also showed statistically significant difference when compared by ANOVA. It can be inferred that the parasiticidal action of the artemisinin component of the conjugate was augmented by the simultaneous interaction of the conjugate with the histone deacetylase enzyme in the parasite leading to enhanced antimalarial activity. Comparatively, the antimalarial activity of this conjugate in the present study is better than that obtained for a closely related conjugate in a previous study [56]. In that study, the investigators synthesized dihydroartemisinin conjugates of Phenylbutyric acid in which the DHA was linked to the Phenylbutyric acid via ether linkages on the aliphatic chain with the aim of improving water solubility of the conjugates and not with the aim of preparing a dual acting conjugate. In addition, another study was carried out in which several structural analogues of 4-Phenyl butyric acid were synthesized but were all found to have less histone deacetylase inhibitory activity compared to the original molecule thus implying that structural modifications of 4-Phenylbutyric acid on the straight chain may lead to attenuation of its histone deacetylase inhibitory activity [57] as reflected in the lower antimalarial activity as compared to the conjugate prepared in this study where care was taken not to tamper with that portion of the molecule. The hybrid also displayed minimal cytotoxicity (LC_{50} value: 139.25 ± 5.46 µg/mL) as compared to the control drug Cyclophosphamide (LC_{50} value: 1.08 ± 0.20 µg/mL) as shown in Table 3 and it is therefore considered sufficiently safe to warrant further drug development.

Conclusion

In this study, a new dihydroartemisinin conjugate of a histone deacetylase enzyme inhibitor has been synthesized in good yield. The conjugate was found to have good antimalarial activity with minimal cytotoxicity. The conjugate therefore shows promise as a new approach for the treatment of malaria.

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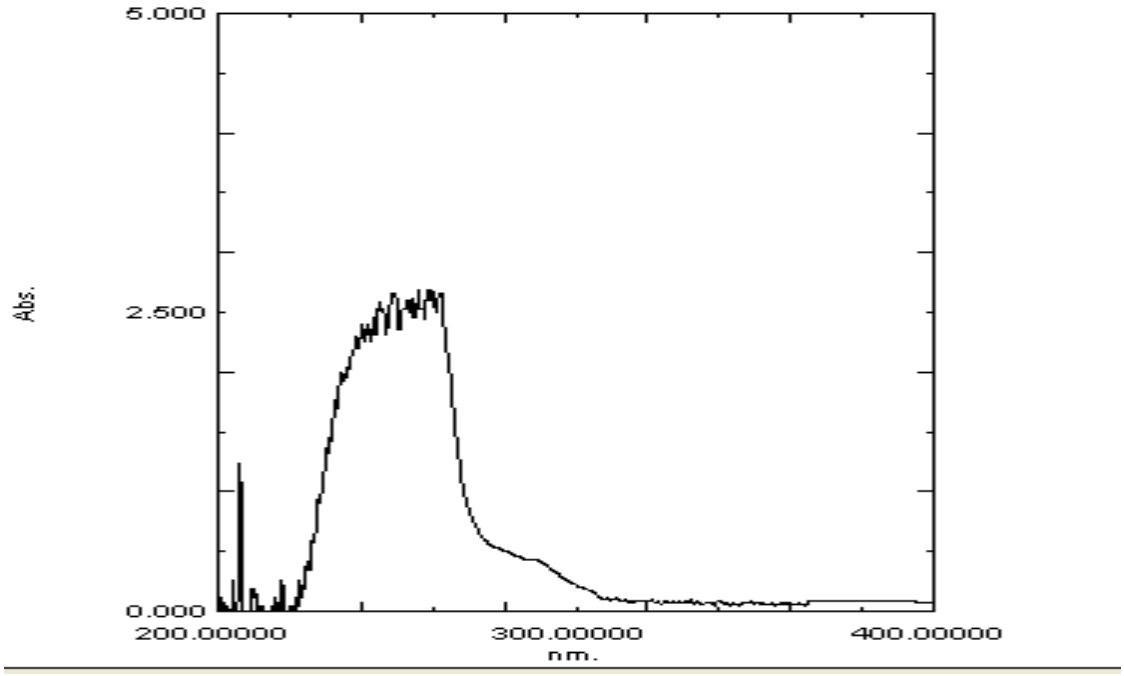
Supplementary Materials

UV Spectrum of PBEC



FTIR Spectrum of PBEC Conjugate

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Background Scans:16
Resolution:8
System Status:Good
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User:Admin
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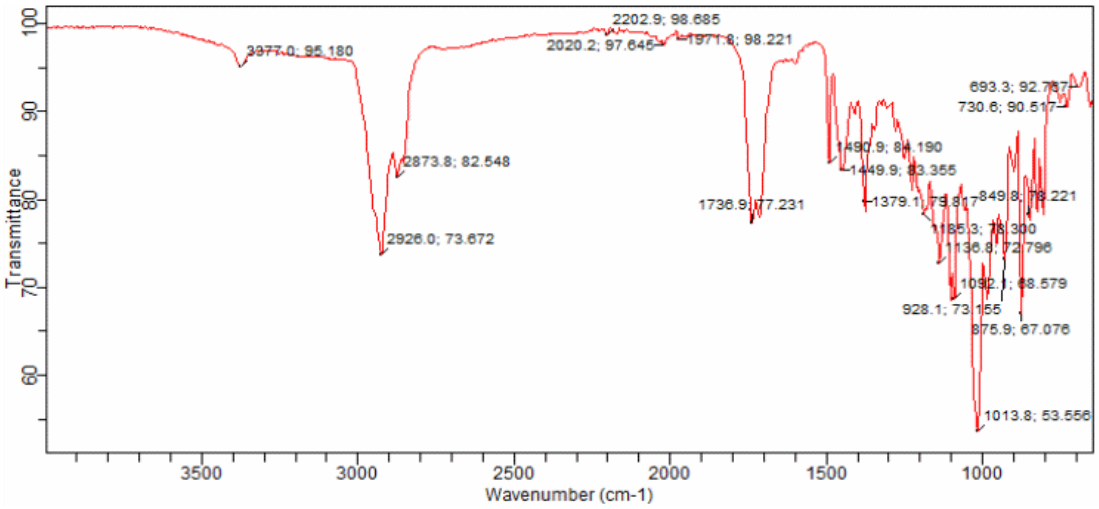


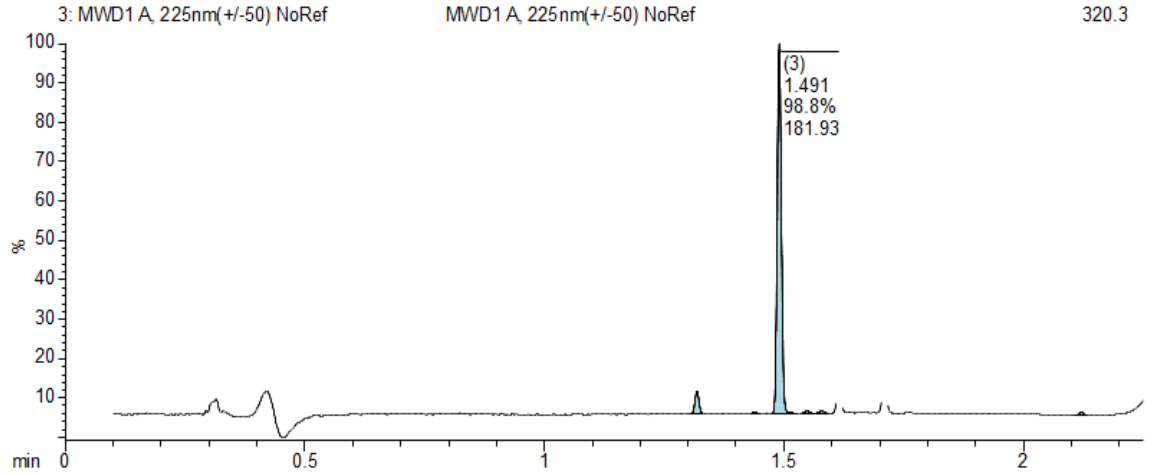
LCMS Chromatogram and Spectrum for PBEC

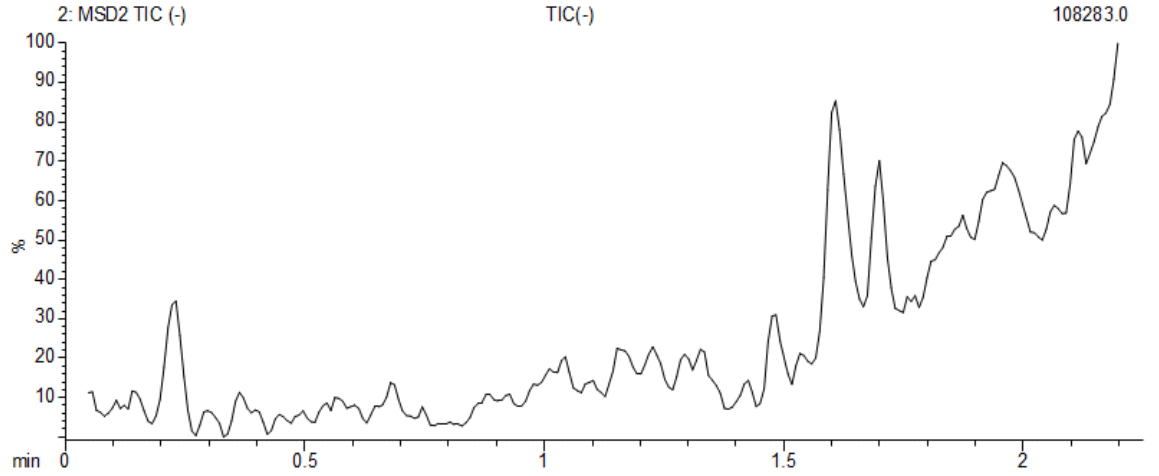
Sample Name: PBEC
Location: 1,1:C,10
Acquired: 10/10/2018 16:13

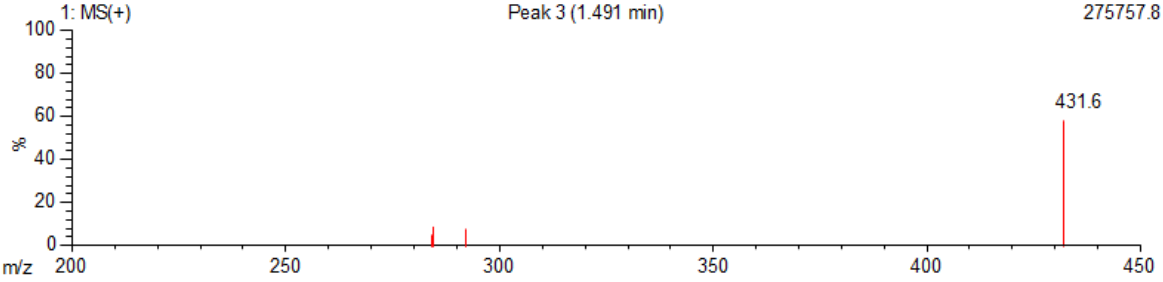
Filename: PBEC.D
Instrument: INSTRUMENT 1
User:

Submitter:
Job Code:

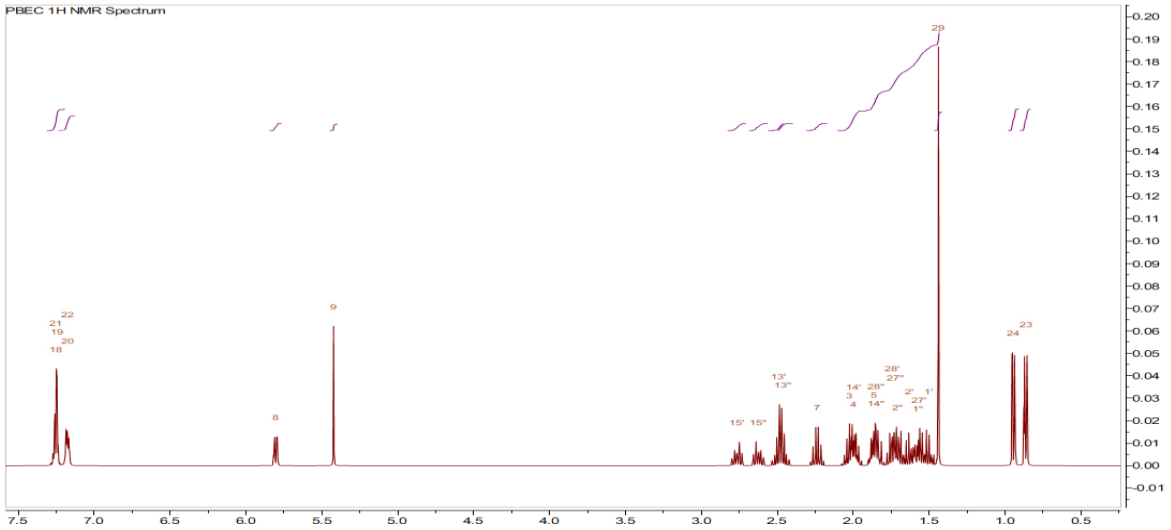
Peak #	Target …	Time	Area Abs MWD1 A, …	Area Abs TIC(-)	Area Abs TIC(+)	Area % MWD1 …	Area % TIC(-)	Area % TIC(+)	Width MWD1 A,…	Width TIC(-)
1	NA	1.317	10.89	0	0	4.7	0.0	0.0	0.027	0.000
2	NA	1.440	0.91	0	0	0.4	0.0	0.0	0.019	0.000
3	NA	1.491	181.93	0	0	98.8	0.0	0.0	0.048	0.000
4	NA	1.549	1.49	0	0	0.7	0.0	0.0	0.027	0.000
5	NA	1.580	1.56	0	0	0.7	0.0	0.0	0.029	0.000
6	NA	1.614	14.82	0	0	6.4	0.0	0.0	0.036	0.000
7	NA	1.709	17.84	0	0	7.7	0.0	0.0	0.039	0.000
8	NA	2.121	1.37	0	0	0.6	0.0	0.0	0.026	0.000







^{1}H NMR Spectrum of PBEC



^{13}C NMR Spectrum of PBEC