Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com
JIPBS
ISSN: 2349-2759

Research Article

Determination of 1, 2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester from the twigs of *Thevetia peruviana* as a Colwell Biomarker

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Abstract

Thevetia peruviana is a tropical plant of the Apocynaecae family and is primarily cultivated in India. A wide range of secondary metabolites, such as enolides, flavanones, flavones, thevetosides, theveside, and glycosides, have been isolated from the roots, kernel, seeds, flowers, and leaves of the plant and are of diverse medicinal value. In this study, 1, 2-Benzenedicarboxylic acid, *bis*(2-ethylhexyl) ester was isolated from the *T. peruviana* twigs extract using preparative TLC technique in a 5% methanol in ethyl acetate solvent system. The structure of this compound was subsequently confirmed using NMR techniques such as LC–MS, FTIR, and HRMS. Literature survey in PUBMed, Scifinder, and Dictionary Natural Products (DNP) revealed that this compound was present in 13 other medicinal plants, including Indian Ginseng (leaves of *Panax Pseudoginseng* subsp *himalaicus)*. Thereafter, bioactivity experiments revealed positive anti-cancer activity of this compound on PC3, MCF, HCT-116, A549, and MIAPACA cell lines, and this compound was proved to be a strong immunomodulatory B-cell stimulant. We propose that this compound is a potential biomarker.

Identification of new biomarkers and their commercialization is crucial for the treatment of diseases. Currently, no or very few biomarkers (standard or otherwise) are available that are prohibitively invasive or highly expensive. Therefore, it is critical to develop and quantify high-impact biomarkers to expedite therapeutic development and patient care.

Key words: *Thevetia peruviana* twigs; Colwell; plant biomarker; 1, 2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester; medicinal plants; *Panax Pseudoginseng.*

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1. Introduction

Thevetia peruviana is a tropical native plant of North America and Mexico and is cultivated in Southeast Asia, particularly in India and Srilanka [1]. The plant has been studied extensively for its cardiac glycosidic effects as well as antimicrobial,

antifungal, [2] and antioxidative properties. Furthermore, the plant compounds exhibit antineoplastic, cytotoxic [3], antimycobacterial [4], and antidiarrhoeal activities and exert cardio tonic and feeding deterrent effects. In addition, the plant inhibits HIV-1 reverse transcriptase and HIV-1 integrase [5]. In this paper, we analyzed *T. peruviana* for its chemical composition [6] and antiinflammatory, immunomodulatory, and anticancer activities [7].

For this, 500 g of *T. peruviana* twigs (Figure 1) were soaked in 5 L of 1:1 water: MeOH solvent system for 48 h for extraction. Thereafter, the extract was freeze dried, generating 125 g of brown crystals (Figure 2). This extract was analyzed for its chemical composition and bioactivity [8].

In our study, we used column chromatography, and the extract was analyzed using the following 11 solvent systems: pure Petroleum ether (PET), pure Chloroform ($CHCl_3$), pure Ethyl Acetate (EtOAc), 5% Methanol (MeOH) in EtOAc, 10% MeOH in EtOAc, 20% MeOH in EtOAc, 30% MeOH in EtOAc, 40% MeOH in EtOAc, 100% Dichloro Methane (DCM), 100% MeOH, and 2% water in MeOH.

The fraction obtained using 5% MeOH in EtOAc was further purified using preparative thin layer chromatography (TLC) for isolating the compounds. One of the isolated compounds was further analyzed using NMR, Fourier transform infrared (FTIR), LC–MS, and HRMS spectroscopy and was determined to be 1, 2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (Figure 3). Although this compound is well known and has been reported in literature, it has not been reported as a constituent of *T. peruviana*.

We propose this compound as a potential biomarker and wish to name it COLWELL Biomarker—a humble gesture and recognition towards my mentor Prof. Rita Colwell, Professor Emeritus University of Maryland.

Figure 1. Thevetia *peruviana* twigs

Figure 2. Dark Brown crystals crude extract

1,2-Benzenedicarboxylic acid; Bis(2-ethylhexyl) ester

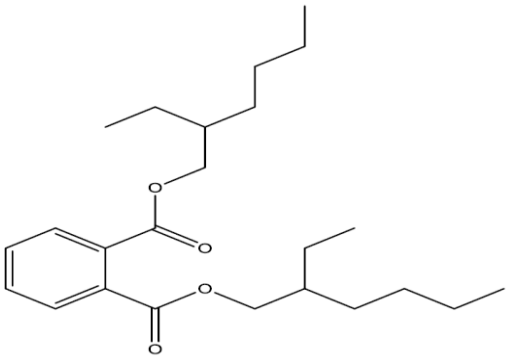

Figure 3. 1, 2-Benzenedicarboxylic acid *Bis* (2-ethylhexyl) ester

2. Materials and Methods

Reagents

All reagents and solvents used in conducting the experiments were procured from E Merck (India) Ltd.

Collection of twigs and extraction of compounds

Twigs – the section between the nodes of leaves and that of bark were collected from *Thevetia peruviana* plants. Five hundred gm of twigs were collected and air dried indoors for two weeks. They were later pulverized using a high speed mixer. These pulverized dried twigs were consecutively extracted by soaking in 1:1 hydro alcoholic solvent system i.e. 1.5 litre methanol and 1.5 litre water over a period of four days using an industrial Soxhlet apparatus. The lower layer of the 250 ml extract was aspirated and lyophilized at -25^{o}C for 72 hrs. This procedure yielded a total of 125 gms of dried extract, which was dark brown crystalline in nature.

Isolation of Tp5E compound using Column chromatography technique

56 gms of extract was mixed with 30 gms of silica gel (chromatography grade, mesh size 100 – 200, Merck). This mixture was made into a slurry with 25 ml methanol and air dried for 24 hrs. This dried mixture was then filled into a 4 feet tall borosil glass chromatography column, and was then subjected to column chromatography using the following 11 solvent systems that were of increasing polarity.

This compound was extracted from 5% methanol in ethyl acetate solvent system. The purity of this fraction was monitored by TLC using two solvent systems namely, 4:1 Chloroform: MeOH, and 1:1 PET: DCM. This fraction was rota evaporated and studied further for isolation of compounds. Rota evaporated fraction was dissolved in the effective 4:1 Chloroform: MeOH solvent system (as mentioned above) and was subjected to an industrial size preparative TLC. TLC plates were developed using iodine vapors and the compound band was scraped.

Experimentation

Using preparative TLC in a 4:1 $CHCl_3$: MeOH solvent system, we extracted 2 g of the compound. We purified this compound using column chromatography using the same solvent system. After applying analytical techniques to elucidate the structure, we subsequently conducted bioactivity experiments that revealed anticancer and immunomodulatory properties of the compound (strong B-cell stimulant). Because the compound is present in 13 other plants and exhibits bioactivity, we propose this compound as a potential biomarker.

Investment in biomarker-related research

One of the objectives of our research was to identify potential biomarkers isolated from the twig extracts that could contribute to disease cure or drug development. There are two types of biomarkers: biophysical biomarkers (isolated from plants, fruits, and leaves), and biochemical biomarkers (isolated from genes, tissues, and cells). Our compound was a biophysical biomarker.

Identification of new biomarkers and their commercialization is crucial for the treatment of diseases. Currently, no or few biomarkers (standard biomarkers and otherwise) are available that are prohibitively invasive or highly expensive. Therefore, it is critical to develop and quantify high-impact biomarkers to

expedite therapeutic development and patient care.

Structural analysis-determination of the structure

To further confirm the structure, the isolated compound was elucidated using various NMR spectroscopy techniques, such as ^{1}H, ^{13}C, 2D, HMBC, and COSY, followed by FTIR, LC–MS, and subsequently HRMS. The compound was identified to be a symmetrical dimer on the basis of the spectra. FTIR indicated the presence of phthalate and carboxylic acid functional groups. LC–MS indicated that the molecular weight was 413.2760. The molecular formula was determined as $C_{24}H_{38}O_4$ using HRMS and the compound was confirmed as a dimer. We used Scifinder, PUBMed, and the Dictionary of Natural Products (DNP) to configure the structure, and the search results revealed that the compound was present in 13 other medicinal value plants. We concluded that the structure was 1, 2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (Figure 3).

This compound was first isolated by researchers in 1989 and subsequently from the following different medicinal plants *Ehretia laevis* [9], *Piper longum L. Piperaceae* [10], *Catharanthus roseus* [11], leaves of Korean *Perilla frutescens* Korea [12], *Gorgonian Melithaea sp* from the South China Sea [13], leaf and bark extracts of *Salix subserrata* [14], essential oil of *Cirsium japonicum DC*., *Dryopteris ryo-itoana Kurata*, *Aspidium* of the Dryopteridaceae family, and the aerial part of *Dryopteris sublaeta*, leaves of *Panax Pseudoginseng* subsp *himalaicus* and its varieties, sunflower varieties var. *Angustifolius* and var. *Bipinnatifidus*. Furthermore, this compound was isolated from the seahorse *Hippocampus kuda* [17] [18].

Analytical interpretation of the data

The NMR analysis of the isolated compound revealed a clean spectrum of all the interactions of the molecule. The ^{13}C investigation identified 12 types of C atoms in the molecule. The double bond equivalent (DBE) equation confirmed that the molecule had 1 ring or 3 pi bonds, and 2D analysis confirmed that the compound was a dimer.

Interpretation of the ^{1}H–NMR spectral data

^{1}H–NMR (400 MHz, $CDCl_3$) δ 7.72 (d, J = 0.01 × 400 Hz = 4 Hz, 2H, aromatic), δ 7.54 (d, J = 0.01 × 400 Hz = 4 Hz, 2H, aromatic), δ 4.26–4.18 (m, 3H, heptate), δ 1.70–1.65 (m, 2H, quartet), δ 1.44–1.25 (m, 20H), and δ 0.94–0.88 (m, 9H) (Figure 4).

Interpretation of the ^{13}C–NMR spectral data

The following values were obtained, thus indicating the following different functional groups as seen in figure 5. ^{13}C–NMR ($CDCl_3$, 100MHz): δ167.7 (C=O, acids and esters), δ132.4 (C=C, aromatic ring), δ130.8 (C=C, aromatic ring), δ128.8 (C=C, aromatic ring), δ68.1 (C–O, R-CH_2-OH), δ38.7 (C–C, R_3-CH), δ30.3 (C–C, R_3-CH), δ28.9 (C–C, R_3-CH/CH_3CO-), δ23.7 (C–C, R_2CH_2/CH_3CO-), δ23.0 (C–C, R_2CH_2/CH_3CO-), δ14.0 (C–C, RCH_3), and δ10.9 (C–C, RCH_3)

The ^{13}C–NMR spectra indicated that the compound had 12 C atoms (primary, tertiary, and secondary) in different electronic environments that are present in aromatic rings, acids, and esters. In-depth analysis using ^{13}C (Figure 6) indicated that 2 CH and 4 CH_3 molecules were present above 6 C atoms, and CH_2 molecules were located on top of the remaining 6 C atoms. 2D spectra (Figure

7) confirmed the presence of 2 CH_3 molecules at δ10 and δ15; 5 CH_2 molecules at δ20, δ21, δ28, δ30, and δ70; 1 CH molecule at δ35, CHx2 at δ130; and CHx2 at δ132. Results of 2d COSY (Figure 8), 2d COSY integration (Figure 9), and 2d HMBC (Figure 10) revealed and confirmed that the compound was 1, 2-Benzene dicarboxylic acid, bis(2-ethylhexyl) ester.

LC–MS data interpretation confirmed that the molecular weight was 413.2760. HR–MS predicted the molecular formula to be $C_{24}H_{38}O_4$. The number of DBEs in this molecule was six, determined using the following equation: DBE = 24 – (38/2) + (0/2) + 1 = 6. The equation confirmed that the molecule had 1 ring or 3 pi bonds. Thus, the results of ^1H (Table 1), LC–MS, and HR–MS (Table 2a) revealed that the molecule had one aromatic ring and was a symmetric dimer.

The FTIR technique was used for molecular fingerprinting (Table 3) by quantifying the energy absorbed by the chemical bonds and molecules of 1, 2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (Figure 11), thus facilitating the analysis and determination of the functional groups of the molecule. Our results are consistent with those of other studies, and the values lie within experimental error.

The experiment was performed using a Perkin Elmer Nicolet FTIR spectrometer. The resolution of the spectrometer was set at 1 cm^{-1} for all spectra. A nitrogen-cooled cadmium-telluride detector was used. A KBr pellet was used with chloroform as the solvent.

The functional IR group data of the molecule as observed in spectra are shown in Figure 11 and the interpreted values are indicated in Table 1. HRMS confirmed the compound to be a dimer at 803.5462, $(2M+Na)^+$, as shown in Figure 12.

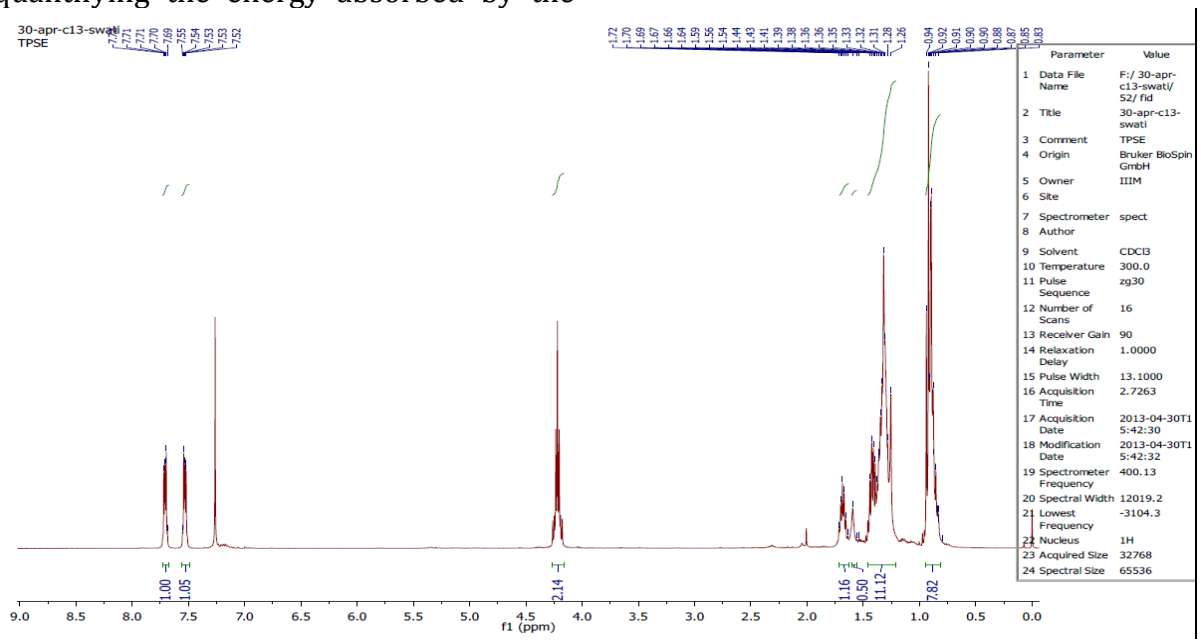

Figure 4. ^1H NMR for the extract

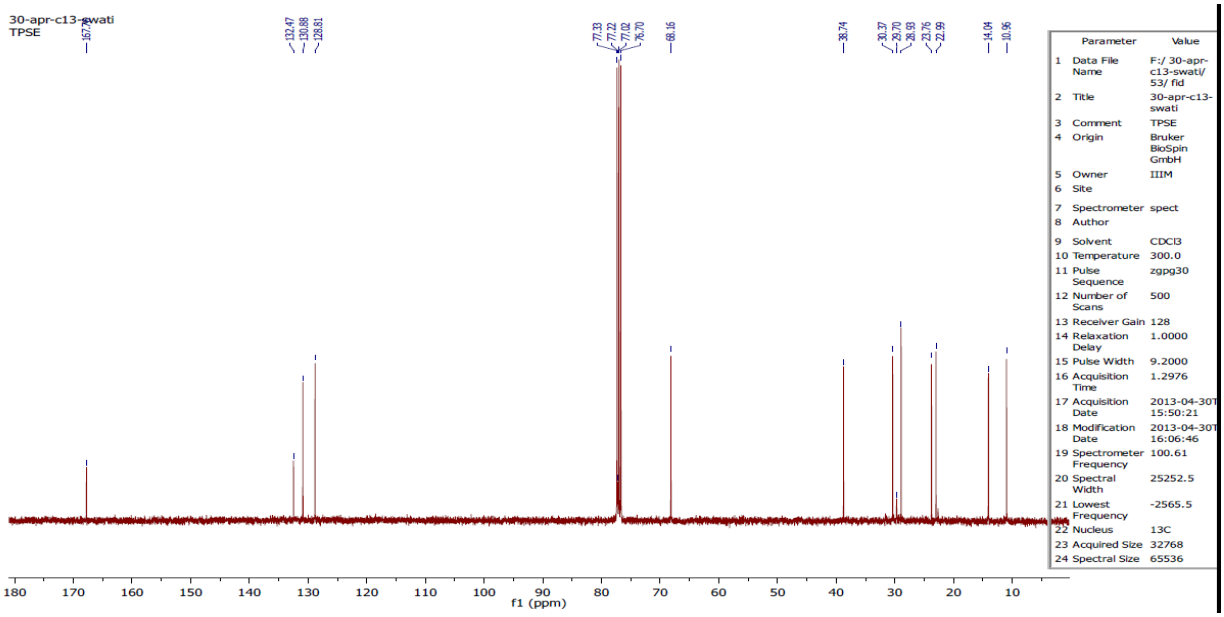

Figure 5. ^{13}C NMR Spectra

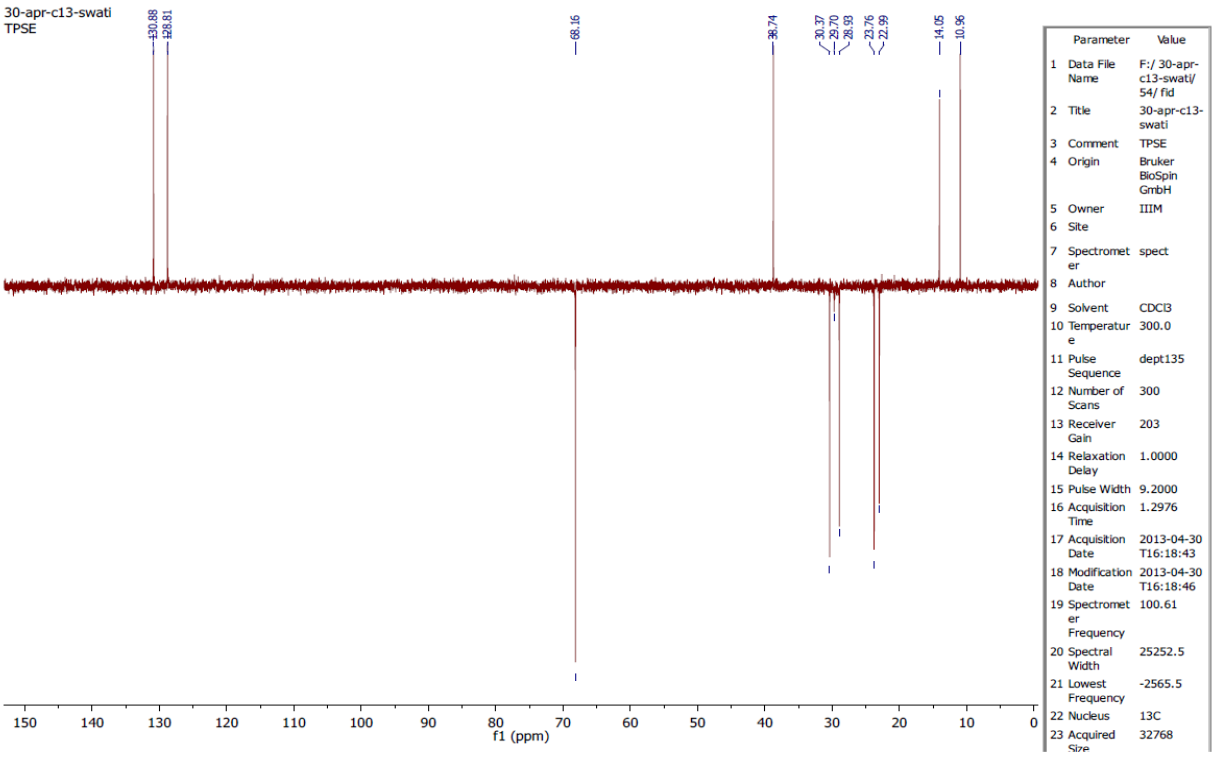

Figure 6. ^{13}C Depth analysis

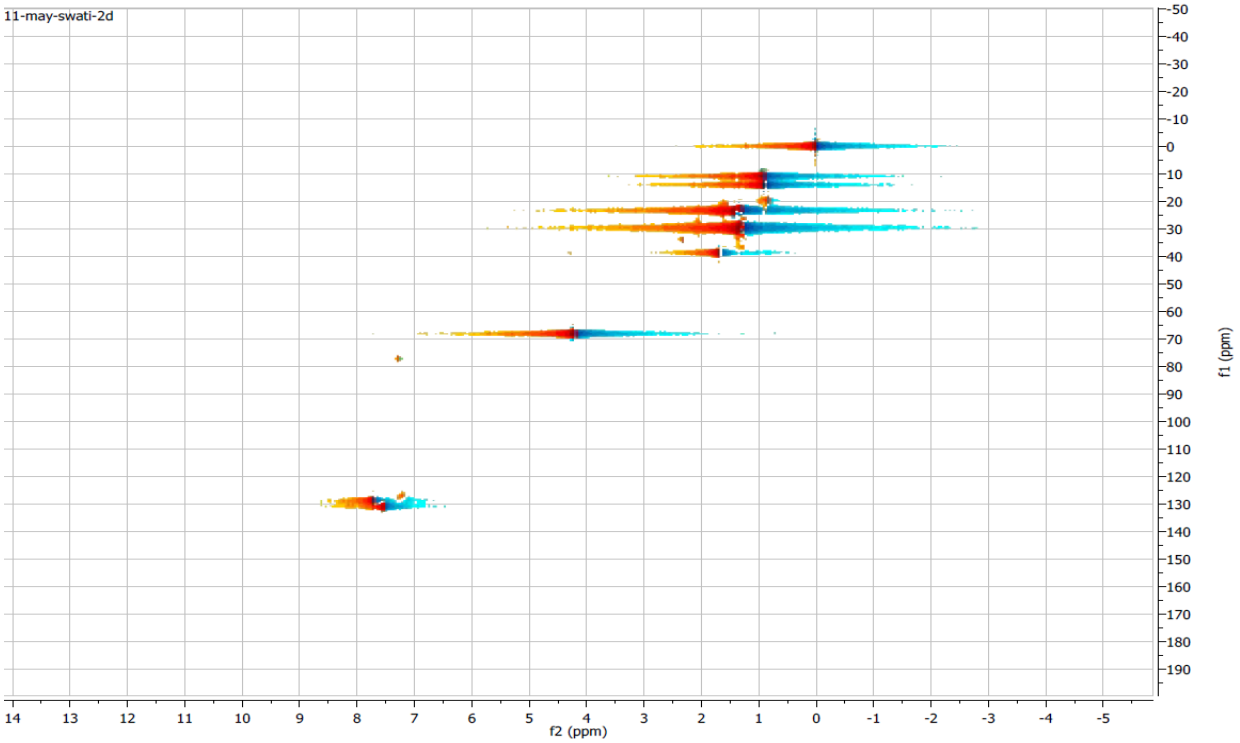

Figure 7. ^{13}C 2D Spectra

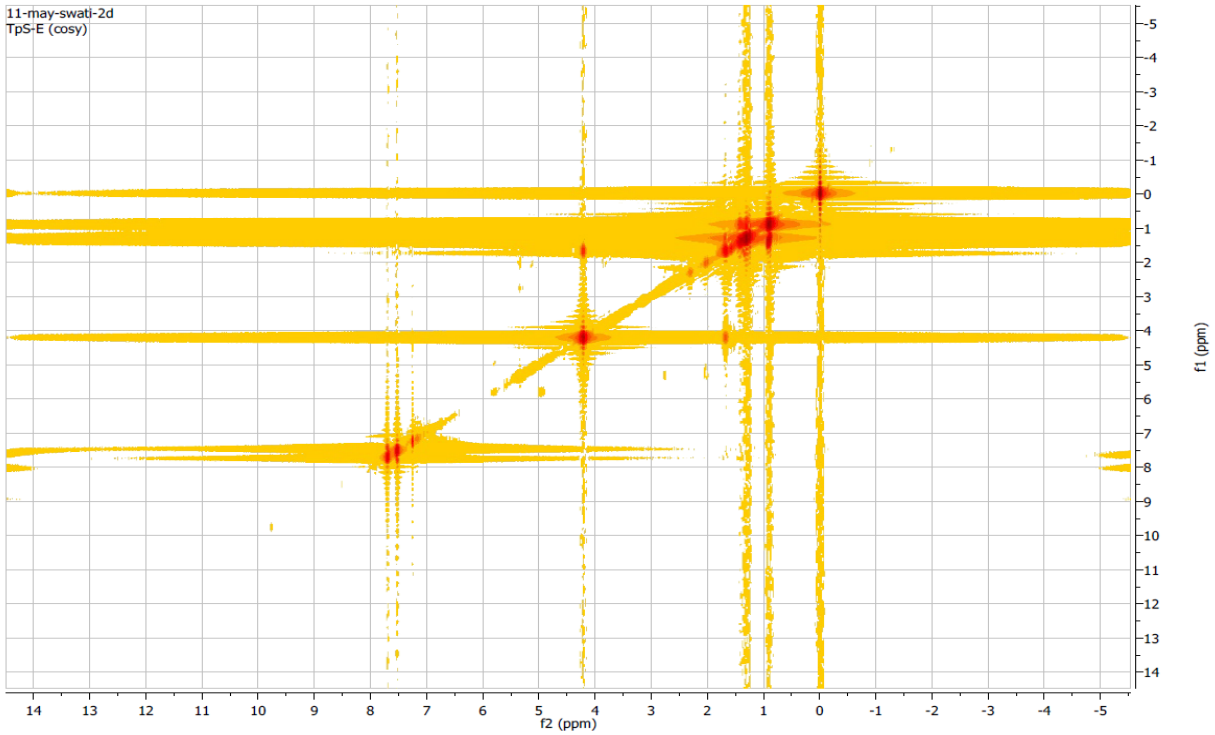

Figure 8. 2D NMR COSY

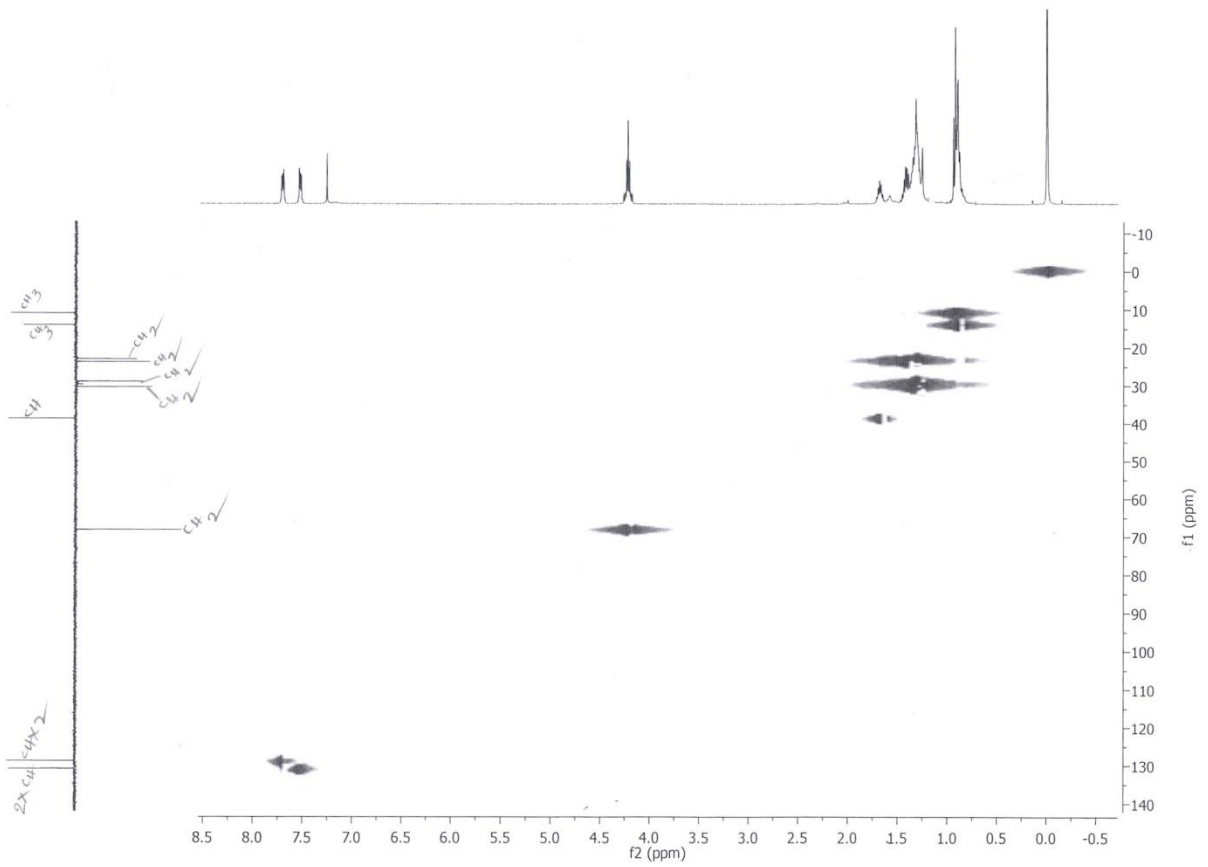

Figure 9. 2D spectra integration

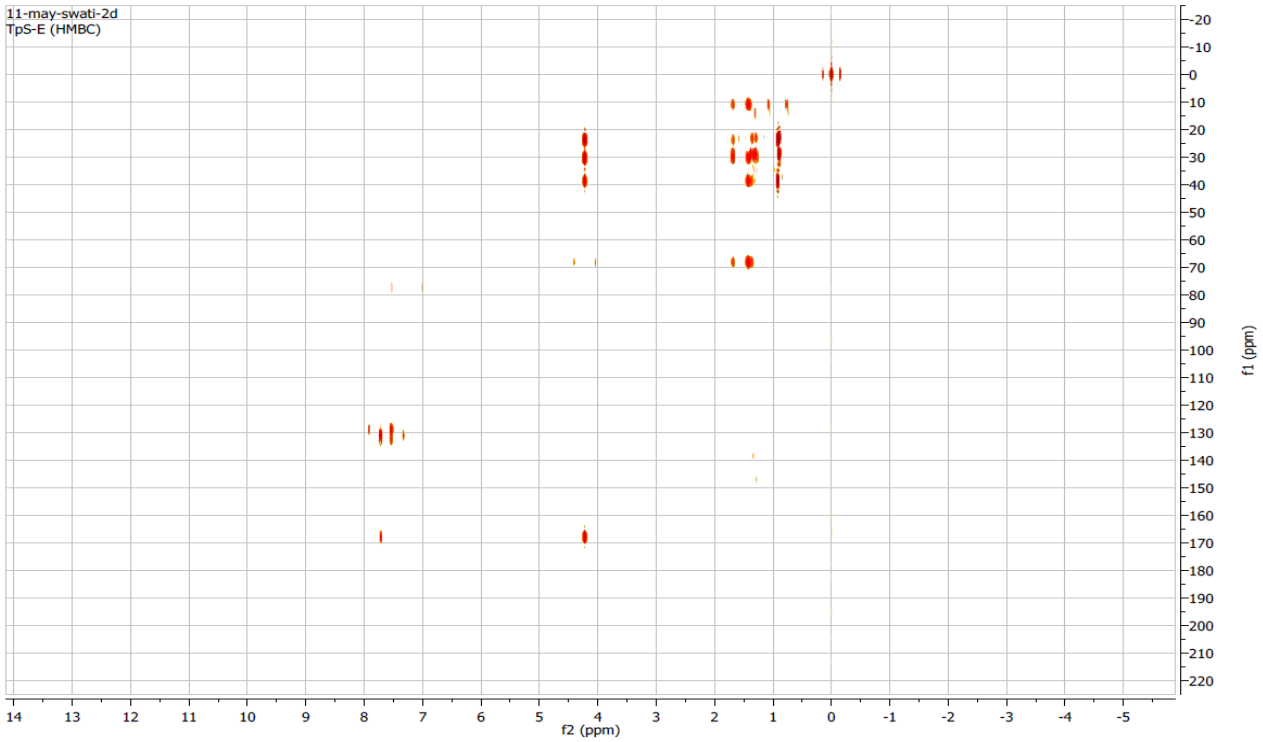

Figure 10. 2D HMBC Spectra

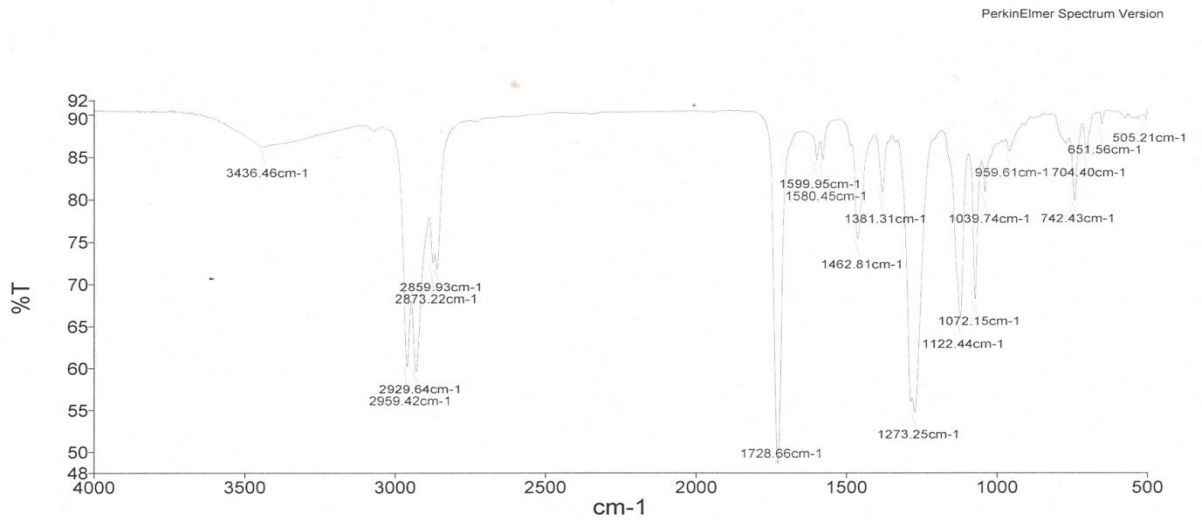

Figure 11. FTIR spectra

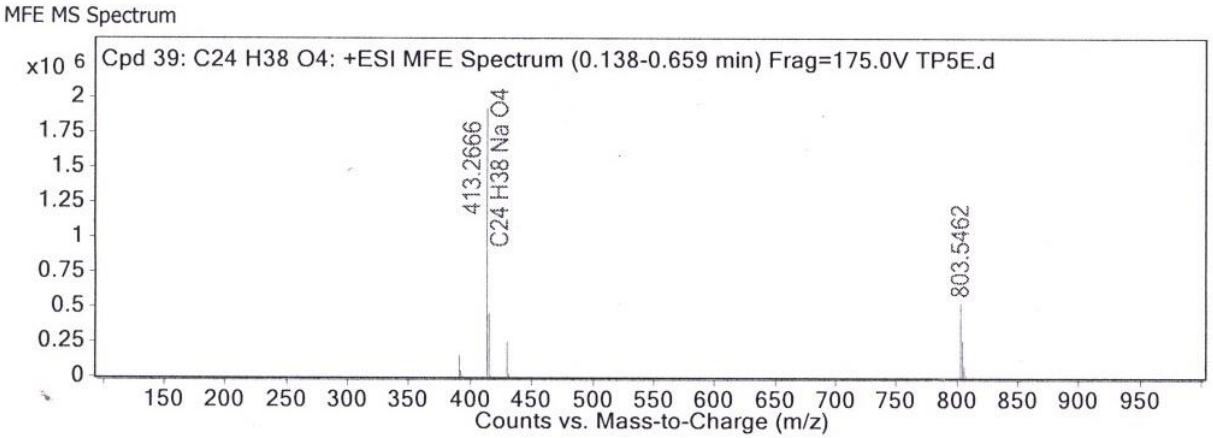

Figure 12. HRMS Spectra

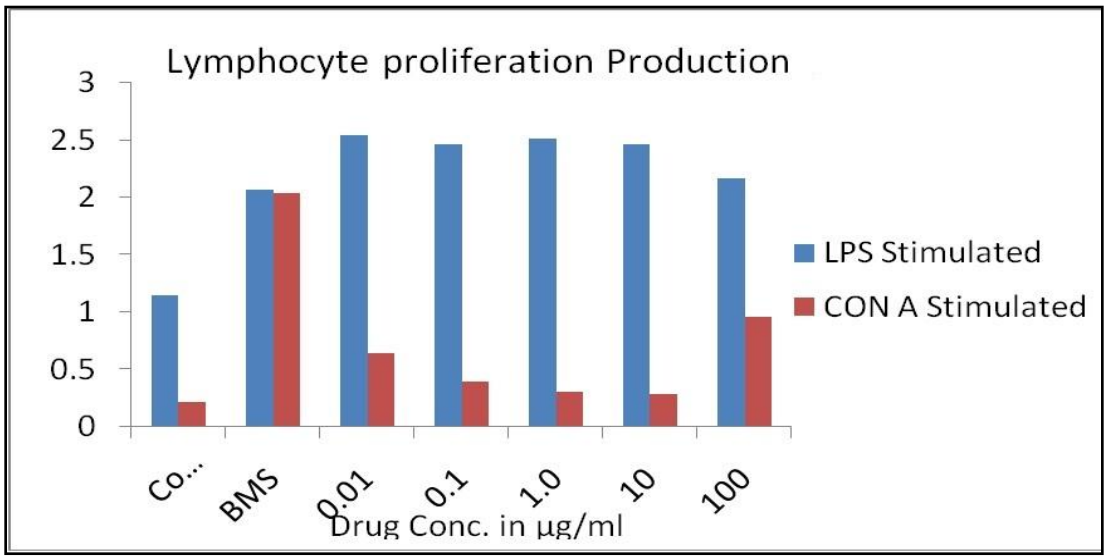

Figure 13. Lymphocyte proliferation assay of 5% methanol in ethyl acetate extract

Table 1. ^{1}H-NMR Analysis spectral interpretation data

Chemical Shift	Splitting	Integral	# of H	Implications
7.72	d	1	2	(CH) ~C_6H_6
7.54	d	1	2	(CH) ~C_6H_6
4.26 – 4.18	m	2	3	2 x (OCH_2)
1.70 – 1.65	q	1	2	2 (CH_2)
1.44 - 1.25	m	10	20	(CH)
0.94 – 0.88	m	7	9	CH

Table 2. FTIR Values of the compound

Functional Group	Type of Vibrations	Characteristics Absorption	Intensity
O-H, Carboxylic Acid	Stretch	3436.46	Weak, Broad
C-H, Aromatic	Bending	3069.09	Weak
C-H, Alkane	Stretch	2959.42, 2929.64	Strong, Sharp, multiple bands
=C-H,	Bending	2873.22, 2859.93	Medium,Sharp,multiple bands
C=O, cyclic ketone	Stretch	1728.66	Strong, Sharp
C=C, aromatic	Stretch	1599.95, 1580.45	Weak, Sharp, multiple bands
C=C, aromatic	Bending	1462.81	Medium, Sharp
C-O, acid	Stretch	1286.70, 1273.25	Strong, Sharp
C-O, ester	Stretch	1072.15, 1122.44	Strong, Sharp, multiple bands
C-H, Aromatic	Bending	959.61 – 530.87	Weak, Sharp

Table 3: Anti-cancer experiment results

CELL LINE TYPE			PC3	MCF	HCT-116	A549	MIAPACA
TISSUE TYPE			PROSTATE	BREAST	COLON	LUNG	PANCREATIC
Extract	CODE	CONC	PERCENTAGE INHIBITION				
Original	Tp	50ug/ml	86	50	71	78	83
5% M:EtOAc	Tp5E	50ug/ml	88	41	75	83	81
Pure 5%M:EtOAc	Pure Tp5E	50ug/ml	0	0	77	0	6
Standard	5FU	20uM	32	53	41	66	50

Table 4: Lymphocyte Proliferation Assay of *Thevetia peruviana* 5% Methanol in Ethyl Acetate (Tp5E) extract

Methanol in Ethyl Acetate	Acts upon T cells?	Acts upon B cells?
Concanavalin A (**C**onA)	Strong	medium
Lipopolysaccharide (LPS)	Strong	Strong

3. Results

Bioactivity studies on Tp5E extracts revealed anticancer (Table 3) and immunomodulatory activities (Tables 4a and 4b, Table 5). Tables 4a and 4b list the positive immunomodulatory stimulatory activities. The results (Fig. 13) indicated the impact of antigens ConA and LPS on both T and B cells. LPS antigens activated B cells (Table 4a) indicating that the Tp5E extract is a promising treatment option for anemia. Antigen-induced B cell proliferation was comparable or higher than that of BMF. Conversely, the Tp5E extract moderately induced T cell (Table 4b) proliferation.

Results of anticancer experiments (Table 3) indicated that the crude and purified Tp5E extracts exerted positive anticancer effects on the prostrate, breasts, colon, lungs, and pancreatic human cancer cell lines by inducing the loss of activity in most cancer cell lines.

4. Discussion

Pulverization and extraction of twigs using the Soxhlet apparatus yielded a dark brown, crystalline, crude extract with a phenolic smell. To assess the general chemical composition of the crude extract, the extract was eluted on a C-18 HPLC column with an increasing methanol gradient. The HPLC chromatograph indicated the presence of 17 compounds. TLC was performed on one of the crude extracts of Tp5E using two different solvent systems, and the results indicated that the crude extract primarily contained a phenolic secondary metabolite. This compound was further purified using preparative TLC in 4:1 $ChCl_3$:MeOH solvent system. Figure 12 illustrates the HRMS spectrum that revealed the compound as a dimer and a phthalate ester. Tables 6a–6d indicate the m/z values of the peaks. Tables 6c and 6d indicate the isotope values of the compound.

NMR spectroscopy techniques, 1H, 2D, HMQC, HMBC, LCMS, FTIR, and HRMS, and the HPLC technique were used for determining the structure, and the results of instrumentation experiments confirmed that the compound was a phenolic dimer. After this, we conducted two bioactivity experiments on the extract to determine whether it exhibited anticancer and immunomodulatory activities, thus evaluating its potential as a biomarker and understanding its unique properties by comparing it with similar compounds found in 13 other medicinal plants.

Table. 4a. B cell proliferation assay of Tp5E

Stimulating agent	Effect on B cells proliferation
Phytohaemaglutinin (PHA)	No
Concanavalin A (conA)	No
Lipopolysaccharide (LPS)	Strong
Tp5E + LPS	Strong

Table. 4b. T cell proliferation assay of Tp5E

Stimulating agent	Effect on T cell proliferation
Phytohaemaglutinin (PHA)	Strong
Concanavalin A (conA)	Strong
Lipopolysaccharide (LPS)	No
Tp5E	Strong

Table 5. Immunomodulatory activity experimental details of the extract 5% MeOH in EtOAc

	LPS STIMULATED	CON A STIMULATED
CONTROL	1.142333	0.213
BMS	2.065	2.034
0.01	2.540333	0.637
0.1	2.459667	0.339
1.0	2.505667	0.305333
10	2.453333	0.284667
100	2.164333	0.958

Table 6a. HRMS Compound formula data

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cmpd 39	0.189	390.2775	$C_{24}H_{38}O_4$	$C_{24}H_{38}O_4$	-1.18	$C_{24}H_{38}O_4$

Table 6b. HRMS compound m/z data

Compound Label	m/z	RT	Algorithm	Mass
Cmpd 39	413.2666	0.189	Find by Molecular Feature	390.2775

Table 6c. HRMS Compound formula and abundance data

m/z	Z	Abundance	Formula	Ion
391.2845	1	149174.42	$C_{24}H_{39}O_4$	(M+H)+
392.2873	1	41460.79	$C_{24}H_{39}O_4$	(M+H)+
413.2666	1	1917410.38	$C_{24}H_{38}NaO_4$	(M+Na)+
414.2704	1	454337.38	$C_{24}H_{38}NaO_4$	(M+Na)+
415.2726	1	66227.59	$C_{24}H_{38}NaO_4$	(M+Na)+
429.2405	1	24494.94	$C_{24}H_{38}KO_4$	(M+Na)+
430.2436	1	62820.62	$C_{24}H_{38}KO_4$	(M+Na)+
803.5462	1	531786.56		(2M+Na)+
804.5486	1	264718		(2M+Na)+
805.5506	1	73211.27		(2M+Na)+

Table 6d. HRMS abundance calculation

Isotope	m/z	Calc m/z	Diff. (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	391.2845	391.2843	-0.57	100	100	75.58	76.47
2	392.2873	392.2877	0.93	27.79	26.56	21.01	20.31
3	393.291	393.2906	-1.1	4.51	4.21	3.41	3.22

Conclusion

This study confirms that the Tp5E extract from the twigs of *T. peruviana* can be considered a novel candidate for bioprospection and drug development in the treatment of cancer, arthritis, and microbial allergies and may act as an immune booster.

The economic rationale behind studying the extraction and isolation of the bioactive constituents present in herbs, spices, and edible fungi is that the isolated compounds possess medicinal properties and are of therapeutic value. These compounds facilitate organic chemists in synthesizing and isolating compounds that are potent analogs, thus fueling the discovery and use of new and cheap drugs in therapy.

We propose that these novel, isolated compounds might bind to newer targets or known targets and facilitate the understanding of the bioactivity of the enzymes or antibodies that exhibit anticancer or immunomodulatory activities. Furthermore, the promotion of low-cost, non-invasive, and effective biomarker commercialization is essential.

Acknowledgment

Conflict of Interest statement and disclosure

This study bears no accountability to any donor agency, as this research was not funded and was undertaken as part of the Ph.D. research of the author.

Data integrity: The authors had full access to all data in this study and take complete responsibility for the integrity and accuracy of data analysis.

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