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IDENTIFICATION AND *IN-SILICO* ANALYSIS OF ANTI-CANCER COMPOUNDS FROM HERBAL MIX OF NORTH-EAST INDIA

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ABSTRACT: A successful study was undertaken in the already practiced traditional herbal medicine and we identified two potential anti-cancer compounds for the first time, namely, Ethyl p-Methoxycinnamate and Pinostrobin chalcone from the herbal mix CAN-1 and CAN-2, respectively. These herbal mix CAN-1 & CAN-2 are practiced for the treatment of cancer patients of Nongstoin area of Meghalaya [1-3]. These herbal medicinal mix CAN-1 & CAN-2 have been analyzed using HPLC and we identified the major part of phytochemicals that are responsible for the cancer curing actions. The characterization analysis has been carried out using GCMS, LCMS, LCMSMS and ^{1}H & ^{13}C for the prep-HPLC isolated lead compounds with the purity more than 95% under area normalization method. Further, the isolated pure compounds have been subjected to cell-line studies and molecular docking studies.

KEYWORDS: Anti-cancer compound; North-east India; Medicinal Plant; Phytochemical identification; Herbal medicine.

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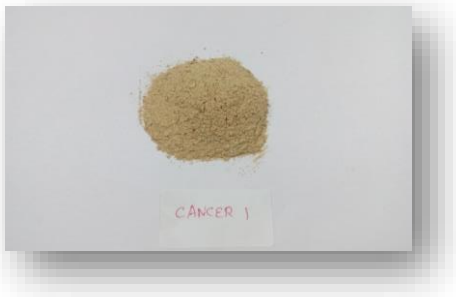
1. INTRODUCTION

It is well known that, in recent days the usage of traditional medicine is tremendously increased in all the areas of healthcare, due to its fewer side effects. The medicinal properties of the plants are due to their active secondary metabolites present and the plants possess anti-microbial, anti-inflammatory, anti-diabetes, anti-oxidant and anti-cancer properties. Therefore, herbal plants are used in the Ayurveda, Homeopathy and Siddha. The healthcare applications of the herbal plants are well attracted by the Bio-Medical area of research. In this context, a study was undertaken in the already practiced herbal medicine of Dr. Marthong Boss Clinic, West Khasi Hills, Nongstoin in Meghalaya. This Herbal Clinic uses combinations of medicinal plants to treat various types of cancers. Among them, two successful preparations of mixture of herbal plants namely, CAN-1 and CAN-2 were taken for the study towards the identification of lead molecules, which are responsible for the curing actions of cancer. The pictures of CAN-1 and CAN-2 formulations are shown in Fig 1.



A picture of CAN-1 herbal mix

A picture of CAN-2 herbal mix

Fig 1: The picture of CAN-1 and CAN-2 formulations

2. MATERIALS AND METHODS

The herbal mix CAN-1 and CAN-2 have been subjected to three different polar solvent extractions [4], namely, polar (Ethanol), mid-polar (Ethyl acetate) and non-polar (Dichloromethane) solvents to extract all the polarity range of phytochemical compounds that are present. The extracted fractions were analysed using HPLC [5-6] to know the composition of phytochemicals using the PDA detection. The HPLC Chromatograms are shown in Fig 2.

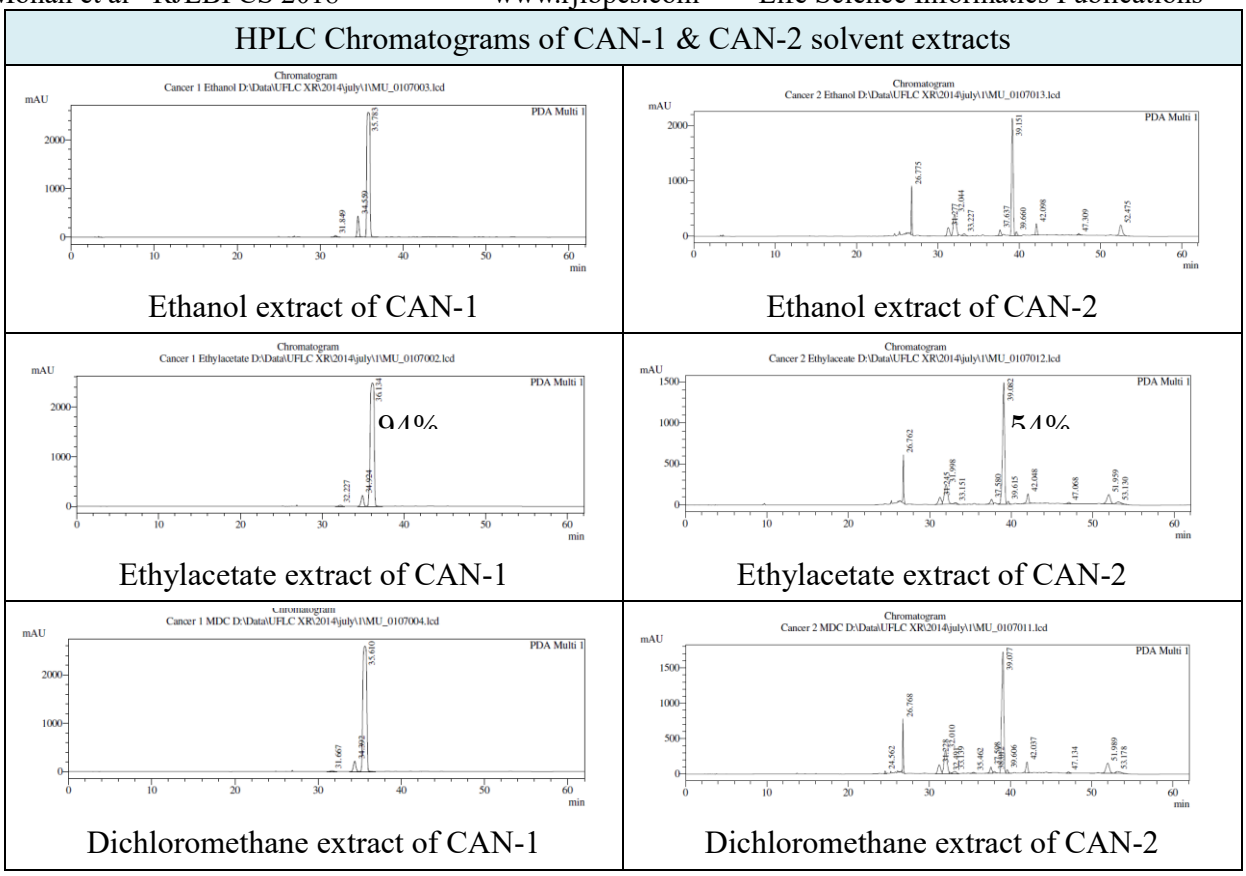


Fig 2: HPLC Chromatogram of extracts of CAN-1 and CAN-2

The major phytochemicals were extracted from ethylacetate in both CAN-1 and CAN-2. The ethylacetate (EA) concentrated extracts were subjected to preparative isolation and the pure compounds were isolated with HPLC purity of more than 95% for both CAN-1 and CAN-2. The solvents Ethanol, Ethylacetate and Dichloromethane Merck make have been used. The Shimadzu HPLC with PDA detector, Shimadzu Preparative HPLC with UV detector make: Japan, were used for the purity analysis and isolation of the phytochemicals. The mobile phase of 0.05% Formic acid (make: Merck) and Acetonitrile with 7.0mL/min flow rate were used with the isocratic elution to isolate the major phytochemicals from CAN-1 and CAN-2 ethylacetate extracts. The 'Enable' make Semi-prep column with the dimension 250mm x 10mm, 10µm was used. The purity analysis has been done using the mobile phase-A of 0.01M Ammonium acetate pH 5.0 adjusted with acetic acid and mobile phase-B of a mixture of acetonitrile:methanol (30:70 ratio) using the gradient analysis using the PDA detector at 310nm. The HPLC column of Phenomenex Gemini NX (250 x 4.6mm, 5µm) was used for the final purity analysis; later the same volatile HPLC method has been used for the molecular mass identification analysis in the LCMS (Liquid Chromatography Mass Spectrometer) [7]. A typical preparative chromatogram from CAN-2 during isolation and the purity chromatograms are shown in Fig 3.

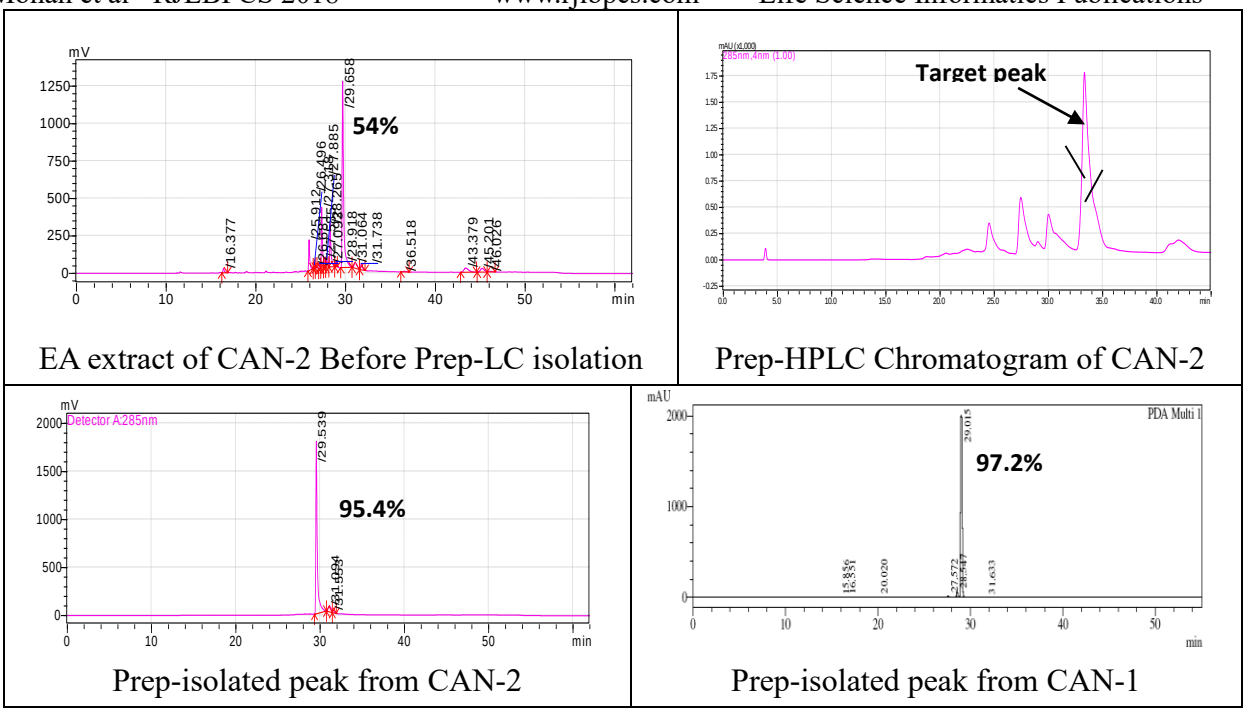


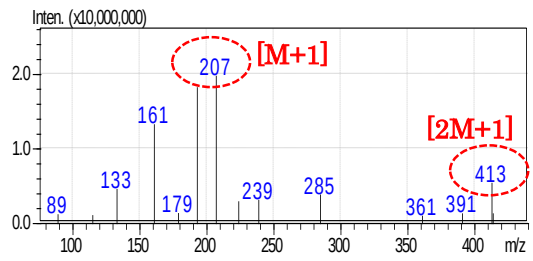
Fig 3: The HPLC Chromatogram of preparative and purity analysis

The isolated peaks had been subjected to LCMS analysis and the molecular masses of the compounds were identified. The identified molecular mass of the peak from CAN-1 is 206 Da and that from CAN-2 is 270Da. The Shimadzu LCMS-8040 Triple Quadrupole Mass Spectrometer (Make: Japan; Courtesy: Spincotech Pvt Ltd, Chennai) with ESI interface was used for the molecular mass identification and MSMS fragmentation analysis. The isolated compounds were further subjected to NMR (Bruker, 400MHz) and GCMS analysis to understand and characterize the structure of the compounds as Ethyl-p-methoxycinnamate from CAN-1 and Pinostrobin Chalcone from CAN-2. The cancer cell-line experiments using HCT-116 and MCF-7 for the above two compounds were carried out. Further, *in-silico* docking studies were carried out using the Schrodinger suite, Glide version 6.2, New York. 2014 (Schrodinger is a leader in computational chemistry, providing software solutions and services for life sciences and materials research) with the inflammatory targets of COX-1 & COX-2 and the results are discussed. The induced fit docking studies have been carried out for the isolated Ethyl-p-methoxycinnamate and Pinostrobin Chalcone and compared with the other anti-cancer compounds with the targets Factor VIII, TNF-α, GSK 3β kinase, MMP2, mTOR, Transglutaminase2 and VEGFR2. It was found that, both the compounds bind with the active sites of all the targets. Bio-energetic pathway for the identified compounds with regard to proliferation of cells can be controlled by blocking two pathways, namely, Glycolysis and ATP production. This work is being carried out at the laboratory of our collaborator, Dr. Atanu Bhattacharjee, using *"SeaHorse"* Analyser.

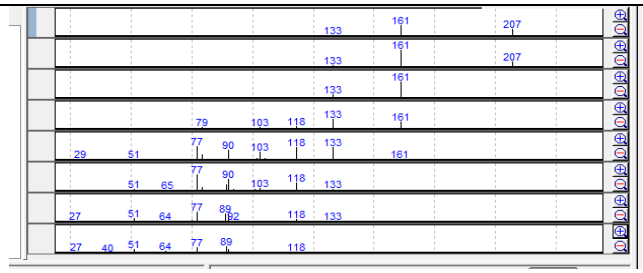
3. RESULTS AND DISCUSSION

3.1 Characterization of compound isolated from CAN-1

The prep-HPLC isolated compounds from CAN-1 and CAN-2 were separately subjected to LCMS analysis and the molecular masses were identified. The identified quasi molecular ion for the CAN-1 isolated compound is m/z 207 [M+1] with further confirmation of its dimer ion m/z 413 [2M+1]. The identified quasi molecular ion m/z 207 is further subjected to MSMS fragment analysis in the Triple Quadrupole Mass Spectrometer using eight different collision energies (5V, 10V, 15V, 25V, 35V, 40V, 50V & 60V) under ESI positive ionization mode to understand the structural fragments of its chemical structure. The lower collision energies impart the major fragments of the structure and the higher collision energies impart the complete small fragments of the structure and hence the different collision energies were chosen in the collision cell of mass spectrometer. This is called nMS^2 technique in the triple quadrupole mass spectrometric technique. This will ease the MSMS data interpretation in the form of true and consistent ions from higher fragments to lower fragments. Based on the MS and MSMS data with the help of other complementary techniques NMR and GCMS analysis, the structure of the compound was identified and confirmed as Ethyl-p-methoxycinnamate. The mass spectrum and its MSMS spectral data with the proposed MSMS fragmentation are shown in Fig 4a and Fig 4b.



The ESI+ Mass Spectrum of isolated compound from CAN-1



The MSMS spectra under different collision energies (5V (upper spectrum) & 60V (lower spectrum)

Fig 4a: The MS and MSMS spectra of compound isolated from CAN-1

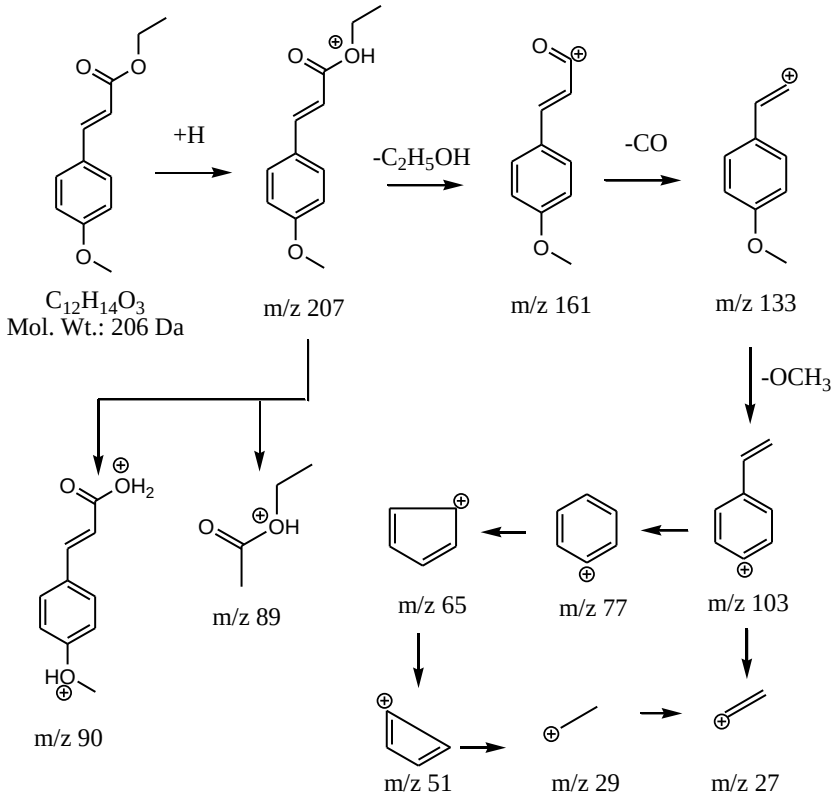
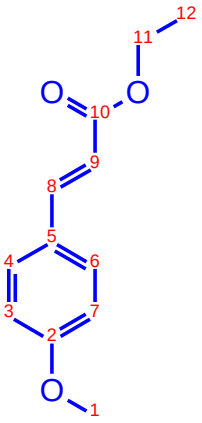


Fig 4b: The proposed MSMS fragmentation pathway for the identified Ethyl-p-methoxycinnamate

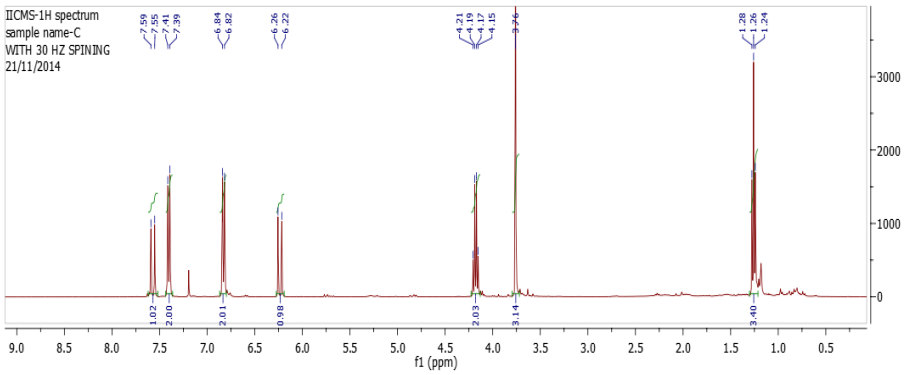
The protonated ethoxy group of Ethyl-p-methoxycinnamate produced the quasi molecular ion of m/z 207 in ESI positive ionization mode. The loss of ethanol from the molecular ion produces the ion of m/z 161 and further loss of CO from m/z 161 yields the ion of m/z 133. These two major stable fragment ions are observed in the lower collision energies. Upon the collision energy 60V, these fragments are completely fragmented into pieces of ions m/z 103, m/z 89, m/z 77, m/z 65, m/z 51 & m/z 29. The ion m/z 90 is proposed to be a di-charged ion.

The CAN-1 isolated compound has been subjected to ^{1}H and ^{13}C NMR analysis and the structure of ethyl-p-methoxycinnamate was confirmed. The NMR spectrum and its interpretation details are provided in Fig 5 and Table-1.

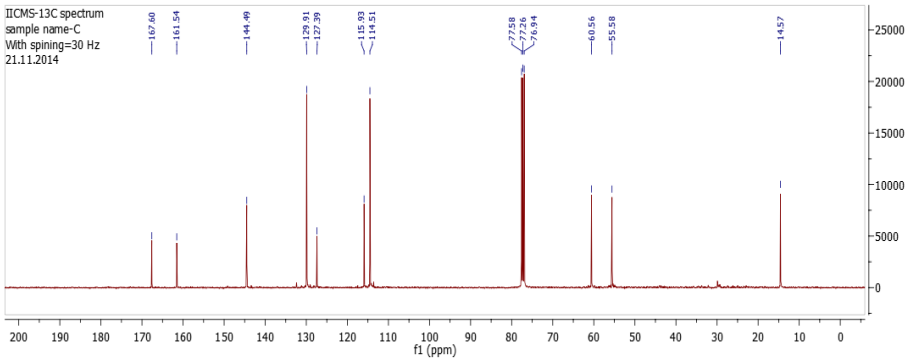


$C_{12}H_{14}O_3$

Mol.Wt.: 206 Da



The ^{1}H NMR Spectrum of prep-isolated compound from CAN-1



The ^{13}C NMR Spectrum of prep-isolated compound from CAN-1

Fig 5: ^{1}H & ^{13}C NMR Spectra of prep-isolated compound from CAN-1

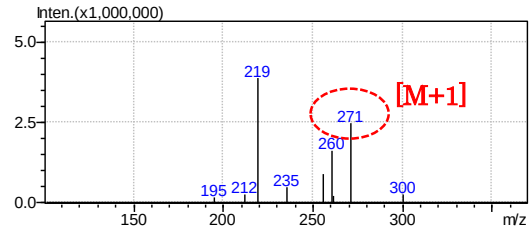
Position	^{1}H	^{1}H δ(ppm)	Multiplicity	^{13}C δ(ppm)	^{13}C
1	3H	3.76	s	55.58	CH_3
2	--	--	--	161.54	C
3	1H	6.84 – 6.82	d	114.51	CH
4	1H	7.41 – 7.39	d	129.91	CH
5	--	--	--	127.39	C
6	1H	7.41 – 7.39	d	129.91	CH
7	1H	6.84 – 6.82	d	114.51	CH
8	1H	7.59 – 7.55	d	144.49	CH
9	1H	6.26 – 6.22	d	115.93	CH
10	--	--	--	167.60	C
11	2H	4.21 – 4.15	q	60.56	CH_2
12	3H	1.28 – 1.24	t	14.57	CH_3

Table-1: Assignment of ^{1}H and ^{13}C NMR signals

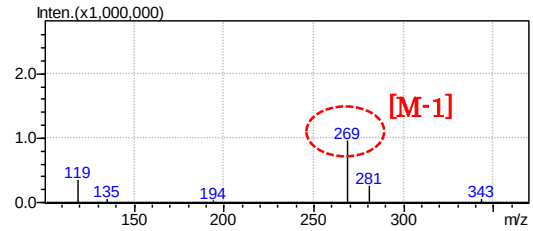
(Carbon numbering is for NMR data interpretation only)

3.2 Characterization of compound isolated from CAN-2

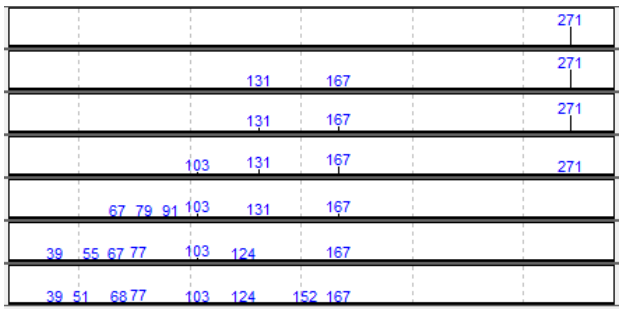
The prep-HPLC isolated compound from CAN-2 was subjected to LCMS analysis and the molecular mass was identified. The identified quasi molecular ion for the CAN-2 isolated compound has m/z as 271 [M+1] under ESI positive ionization mode. The molecular ion was further confirmed with its negative ion m/z 269 [M-1] in the simultaneous detection of ESI negative ionization mode. The identified quasi molecular ion m/z 271 was further subjected to MSMS fragment analysis in the Triple Quadrupole Mass Spectrometer using seven different collision energies (5V, 10V, 25V, 35V, 40V, 50V & 60V) under ESI positive ionization mode to understand the nMS^2 structural fragments of its chemical structure. Based on the MS and MSMS data with the help of other complementary technique GCMS analysis, the structure of the compound was identified and confirmed as Pinostrobin Chalcone. The mass spectrum and its MSMS spectral data with the proposed MSMS fragmentation are shown in Fig 6a and Fig 6b.



The ESI positive Mass Spectrum of isolated compound from CAN-2



The ESI negative Mass Spectrum of isolated compound from CAN-2



The MSMS spectra under seven different collision energies (5V (upper spectrum) & 60V (lower spectrum)

Fig 6a: The MS and MSMS spectra of the compound isolated from CAN-2

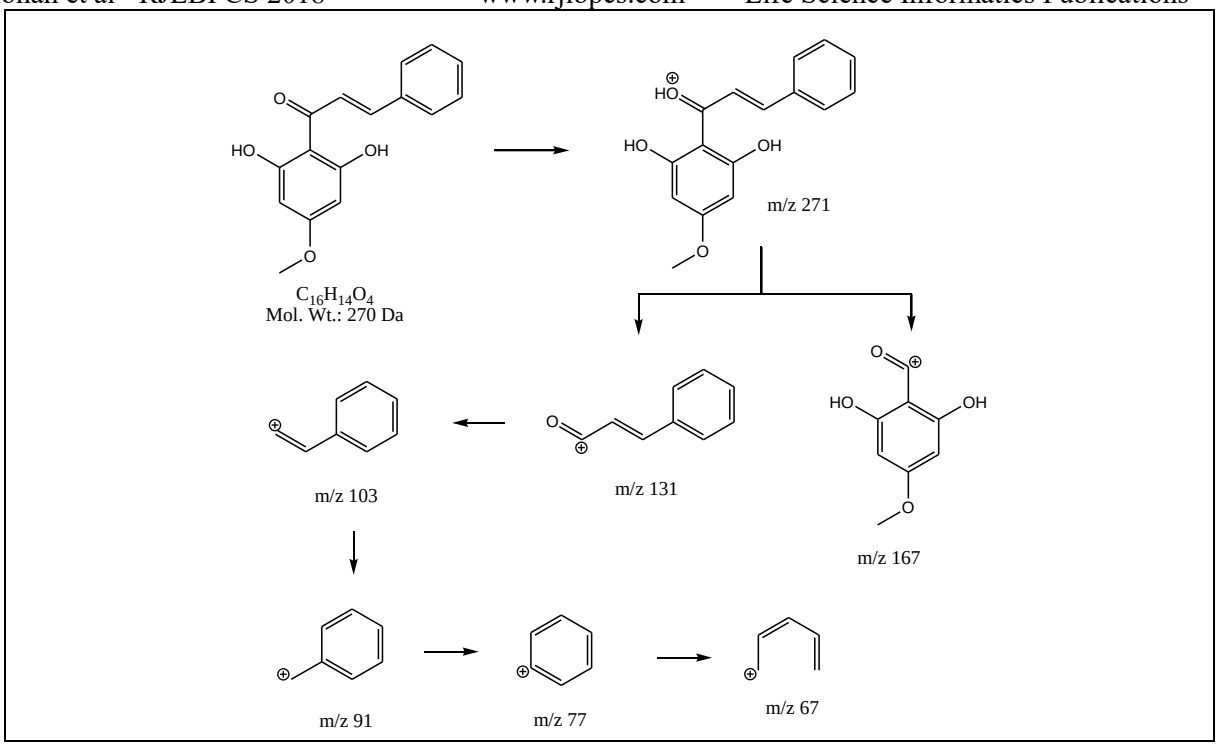


Fig 6b: The proposed MSMS fragmentation pathway for the identified Pinostrobin Chalcone

The protonated ketone group (due to the conjugation effect) produces a stable quasi molecular ion of m/z 271[M+1] and thus has fragmented into the ion of m/z 167 and m/z 131. The loss of CO from the ion m/z 131 produces the ion m/z 103 and further yields the ion m/z 91, m/z 77 and m/z 67.

The GCMS analysis of the extracts of the CAN-2 medicinal mix has shown (based on the finger print mass spectral match with the Wiley Library) the compound with the CAS No. 18956-15-5 meant for Pinostrobin Chalcone. The GCMS library data in comparison with the LCMS, LCMSMS data interpretation confirms the molecule Pinostrobin chalcone isolated from the CAN-2 medicinal mix.

3.3 Cell line studies of the isolated compounds

The cell line studies for the isolated compounds p-methoxy cinnamate and Pinostrobin Chalcone from the ethylacetate extract of CAN-2 have been carried out at Dept of Pharmaceutical Biotechnology, JSS College of Pharmacy, Ootacamund using the cell lines HTC-116 (Colon Carcinoma, Human) and MCF-7 (Breast Carcinoma, Human).

Table 2: Cancer cell-line study data

Sample Description	HCT-116 (Colon Carcinoma, Human) (IC_{50} µg/mL)	MCF-7 (Breast Carcinoma, Human) (IC_{50} µg/mL)
Ethyl-p-methoxy cinnamate (Isolated from CAN-1)	30	33
Pinostrobin chalcone (Isolated from CAN-2)	43	46

Ethyl-p-methoxy cinnamate showed the IC_{50} value of 30µg/mL for HCT-116, Colon Carcinoma, Human and 33µg/mL for MCF-7, Breast Carcinoma, Human. Pinostrobin chalcone isolated from CAN-2 showed the IC_{50} value of 43µg/mL for HCT-116, Colon Carcinoma, Human and 46µg/mL for MCF-7, Breast Carcinoma, Human. The above table shows that both the compounds have reasonable IC_{50} values in the control of colon and breast cancer.

3.4 *In-silico* model – anti-inflammatory macromolecule target analysis

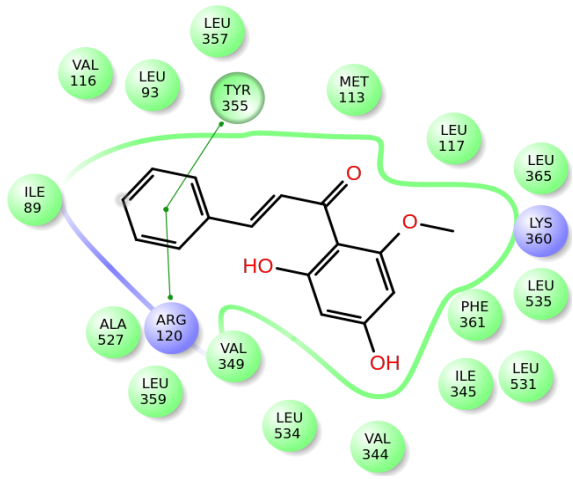
The isolated compounds Ethyl-p-methoxy cinnamate and Pinostrobin chalcone have been subjected to molecular docking [8-9] with the macromolecular targets Cox-1 PDB ID:3N8X and Cox-2, PDB ID:4PH9 to understand the anti-inflammatory behavior of the isolated compounds. The same have been assessed with the Cardamonin [10-15] (a known compound which inhibits metastasis of Lewis Lung Carcinoma cells by decreasing mTOR activity), an isomer of Pinostrobin chalcone and also with the co-crystal for its anti-inflammatory activities. The values of docking scores and Glide energies are tabulated in Table-3.

Table 3: *In-silico* model study data with Cox-1 and Cox-2 targets

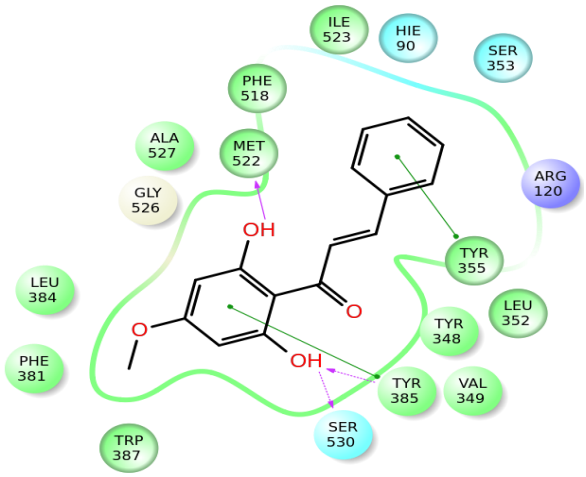
Sample Description	Cox1(PDB ID:3N8X)		Cox2 (PDB ID:4PH9)	
	Docking score (kcal/mole)	Glide energy (kcal/mole)	Docking score (kcal/mole)	Glide energy (kcal/mole)
Cardamonin	-9.00	-41.01	-7.41	-43.32
Pinostrobin chalcone	-10.48	**-44.75**	-10.01	**-47.03**
Ethyl-p-methoxy cinnamate	-6.98	**-37.04**	-7.74	**-36.61**
Co-crystal	-7.68	-37.04	-8.63	-36.35

The Glide energy value of Pinostrobin chalcone complex is comparatively better than that of its isomer Cardamonin as well as the co-crystal complexes. The Glide energy of ethyl-p-methoxy cinnamate is comparable with the co-crystal. The lig-plot [16-18] of Cox-1 and Cox-2 are shown in Fig 7.

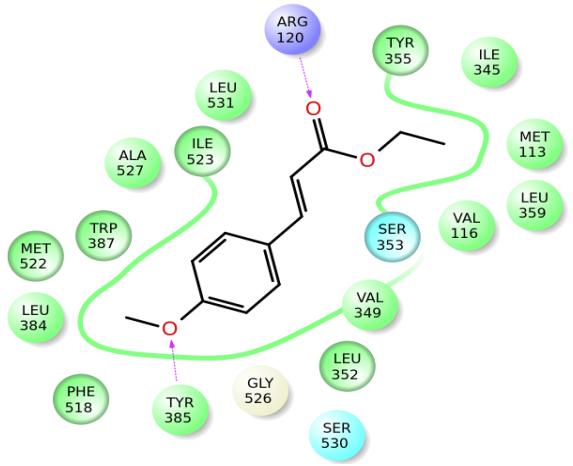
Cox-1 Ligplot (ligand interaction diagram)



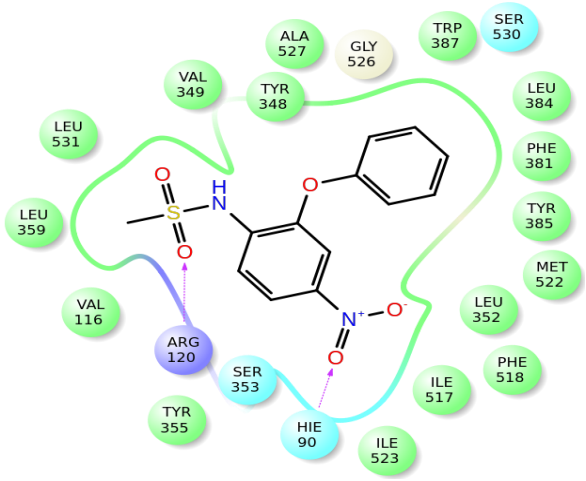
Cardamonin



Pinostrobin Chalcone

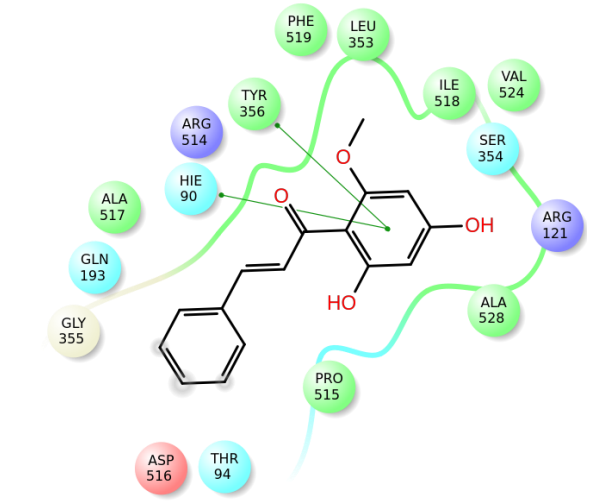


Ethyl-p-methoxycinnamate

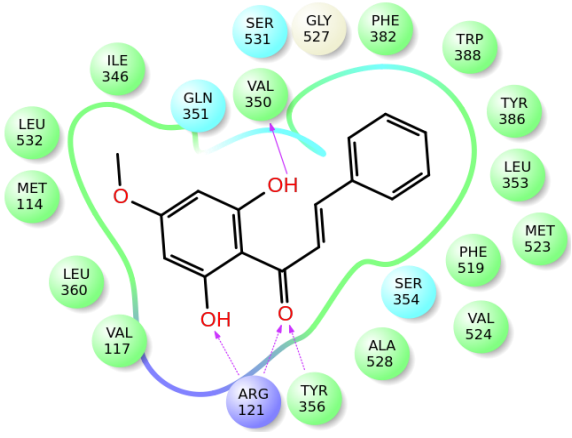


Co-crystal Inhibitor

Cox-2 Ligplot (ligand interaction diagram)



Cardamonin



Pinostrobin Chalcone

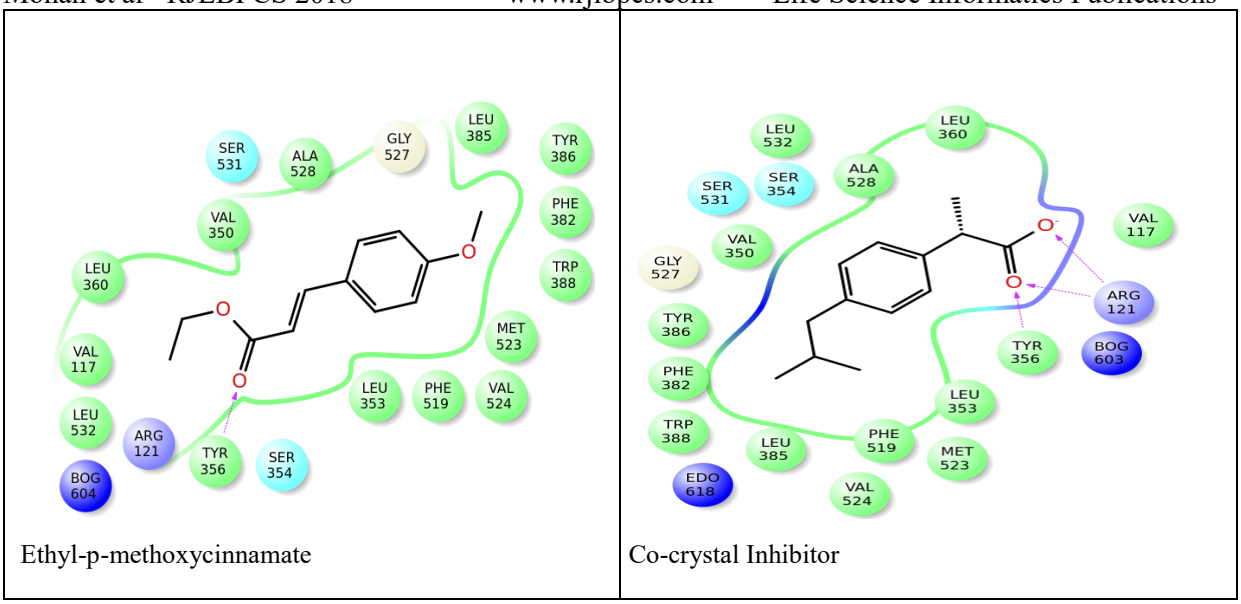


Fig 7: The ligplot (ligand interaction diagram) of cox-1 and cox-2

The literature evidences that, both the compounds interfere in the TNF-α and NF-κB pathways [14-15 and 19-23].

3.5 Induced fit docking studies of other cancer targets analysis

The induced fit docking studies have also been carried out for Ethyl-p-methoxycinnamate and Pinostrobin Chalcone and compared with other anti-cancer compounds with the following targets Factor VIII, TNF-α, GSK 3β kinase, MMP2, mTOR, Transglutaminase2 and VEGFR2. These two compounds bind at the active sites of the above targets [24-26].

4. CONCLUSION

This research article confirms that the already practiced traditional herbal medicines have the potential anti-cancer leads, which may be responsible for the curing action. The identified potential anti-cancer leads were evidentially proven by isolation (by Prep-HPLC), characterization (through LCMS, LCMSMS & NMR) and the data obtained from Human cancer cell-line studies. The cell-line studies show that the identified leads Ethyl p-Methoxycinnamate and Pinostrobin chalcone in the herbal medicinal mix have better IC_{50} values for the colorectal and breast cancer.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research study.

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