Isolation and identification of β-sitosterol from *Lantana camara* L.

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ABSTRACT

Objective: The present studies explore the anticancer activity of the fractions along with isolation and identification of β-sitosterol from *Lantana camara* L.
Methods: Phytochemicals screening of the fractions obtained from the n-hexane extract of the whole plant of *L. camara* indicated the presence of various secondary metabolites. Alkaloids, flavonoids, steroids, saponins, and terpenoids are the major phytoconstituents obtained from the plant to produce various activities. Through SRB assay the fractions of the extract shows anticancer activity and Columnchromatography was employed for isolation of phytoconstituent and identified by spectroscopic methods.
Results: Chromatographicandspectroscopic analysis revealedthe existence ofβ-sitosterolfrom the plant *L. camara.*
Conclusion: After Isolationand followed by purification gives whitecrystallinepowderwhichwas put through chemicalandspectral analysis thecompound was pinpoint asβ-sitosterol.
Keywords: *Lantana camara*,SRB assay, Column chromatography, isolation,identification, β-Sitosterol.
DOINumber:10.14704/nq.2022.20.8.NQ44737 **NeuroQuantology2022;20(8):7147-7154**

1. INTRODUCTION

For early decades, traditional medicines are one of the most important sources for the treatment of various human diseases. Plants can be used directly or in the form of extracts are useful for the management of various ailments. The different plant derived secondary metabolites are used by human being for their betterment [1]. Now a day's traditional system of medicine is more valuable for their pharmacological activities with fewer side effects. Last five to ten years

so many researchers carried out their research with thousand numbers of plantsand submit lots of evidence regarding the potential of traditional system of medicines [2].

As per World Health Organization (WHO) definition Phytomedicine as a herbal preparation are those plant materials into various forms like extraction, maceration, percolation, or other physical or biological processes are used for their biological activity by human being for their betterments [3].In India near about 80% population are living in rural and they are depending on traditional system medicine due to expensive treatments, more side effects. Overall world's population majority has dependable in phytomedicine based on plant derived traditional medicine for their primary treatments [4].

The family Verbenaceae containing thirty two genera with eight hundred species [5]. *Lantana. camara* L. is a class of this family originates from the American tropics [6]. The plant has the capacity to grow in various diversity of environmental conditions [7]. As per the research activity of L. camara the plant has numerous ranges of phytoconstituents which are producing multiple biological activities, like antitumor, antioxidant, anti-inflammatory, antimicrobial and cardiovascular effects [8].

In our research work, we have studied the whole plant of *L. camara* in various aspects and isolate a phytosterols which is an important source of phyto components used as various diseases. Through various experimental analysis we conform that the plant contains a pentacyclic triterpenoid that is β-sitosterol a class of phytosterols [9]. Column chromatography and Gas chromatography-tandem mass spectrometry are the major analytical technique for Phytochemical research which helpful for isolating and identifying novel entities like β-Sitosterol in our study [10]. In various studies it is consider that β-Sitosterol is a very good biomarker for mammalian system for its biological activity. In major literature, it was considered that it is a better source as traditional medicine for anticancer, antioxidant, anti-inflammatory, antimicrobial and cardiovascular effects etc [11].

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All the chemicals used were of synthetic grade and sourced from Merck specialties Pvt. Ltd. (Mumbai, India) and Himedia Laboratories (Nashik, India).

2.2. Collection and identification of plant materials.

Lantana camara L. was collected in the month of March, 2019 from University Department of Pharmaceutical Science (20.306° Latitude, and 85.840° Altitude) located at Bhubaneswar, Khordha district of Odisha. The plant was authenticated taxonomically by visualidentification by Dr. K.B. Satapathy, Professor, Centurian University of Technology and Management, Bhubaneswar, Odisha, India. Plant name was checked with the www.theplantlist.org website accessed on 11 April 2022.

2.2 Preparation of Plant Extract

The whole plant material (1500 gm) was collected and washed under running tap water, dried under shade and made to coarse powder. The coarse powder was passed through sieve no.22 and used for extract preparation. The n-hexane extract was prepared by using solute to solvent 1:5 ratio in a Soxhlet for 4 hrs. The supernatant was collected by filtration through a muslin cloth, concentrated using rotary evaporator (IKA Model RV 10D S96) under reduced pressure and stored in deep freezer for further use. The extract was fractionated by column chromatography techniques.Through modern qualitative analytical technique like Thin-layer chromatography (TLC), FTIR, and Mass spectroscopy gives idea about the presence of various Phytoconstituents.

2.3 Preparation of fractions

A 100 gm of plant extract was dissolved in chloroform and us ed for spotting on TLC plate. The sample solution was spotted on aluminium TLC plates precoated with silica gel 60 F254.

Then the TLC plate was developed by a suitable solvent system and viewed under UVlight. The developed plate was derivatised with 5% v/v of sulphuric acid in methanol. The TLC plate shows that the compounds of unsaponifiable fract ion were separated by the solvent system of chloroform and n-hexane in the proportion of 9.5:0.5. On exposed to iodine chamber yellowish brown soon turns to green or violet spots were identified with approximate Rf value of 0.38, 0.5, 0.55, 0.6, 0.76, 0.81, 0.89. Based on this data we proceed further for isolation of phytoconstituents.

Column chromatography was performed by using the prepared plant extracts and silica gel (Mesh size 100~200) by wet packing method in ethylacetate. The colum n was run with various ratio of ethyleacetate, chloroform and methanol by gradient eluti on technique. Further with help of TLC techniques the eluted fractions were tracked and total of 120 elutes were collec ted. The fractions having same R_f value were mixed together to make 15 fractions. The fractions are concentrated under reduced pressure and dried over steam bath, to remove the solvents.

2.4 Cell Culture of the Fractions

A-549 (lung cancer) cells were maintained at 1×10^6 no cells were cultured in T-25 corning cell culture flask maintained in RPMI 1640 medium containing 10% fetalbovine serum (FBS), 2mM L-glutamine,100 Units/ml penicillin and 100µg/ml streptomycine. The cells were incubated at 37°C, 5% $CO_2$and 100% relative humidity for 24 hours prior to the addition of experimental drugs.

2.5 Cytotoxicity Assay

The obtained five fractions from the plant was first carried out for cytotoxicity assay. As the n-Hexane extract of *L. camara* exhibited cytotoxic activity on A-549. 90 µl of A-549 cell suspensionwere seeded onto 96 well culture plates. All the dried fractions were solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1 mg/ml using water and stored at -20°C until further use. Prior to sample addition in the cell suspension, the frozen sample solution was diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml. 10 µl of each of the dilutions were added to the microtiter plate resulting in the final drug concentrations to 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml followed by incubation of cells for 48 hours. 50 µl of ice cold 10% TCA was added for 1hr at 4°C to terminate the assay. The plate was washed five times with distilled water and was dried in air. 50 µl Sulphorhodamine (SRB) solution (0.4% w/v in 1% acetic acid) was added to each of the 96 well plates and incubated for 20 minutes at room temperature. After staining, the plates were washed five times with 1% acetic acid to remove the unbound dye.The plates were then dried and 100 µl of 10mM trizma base (pH 10.5) was added to solubilize the stain. Finally, the absorbance was measured in a multimode reader at a wavelength of 540nm.

2.6 GCMS Analysis of the fraction

The GC-MS analysis of the second fraction of n-hexane extract of *L. camara* was carried out as per the method followed by Muthukumaran Pakkirisamy et al. 2017 [12]. The sample was acquired using a Scion 436-GC (Bruker) coupled with a triple quadrupole mass spectrometer (TRACE 1310/ISQ–LT; Thermo Scientific) equipped with a BR-5 MS (5% Diphenyl/95% Dimethyl poly siloxane) capillary column (30m ×0.25 mm, 0.25 µm particle size), at a constant flow of 1.0 mL/min and injection volume of 2µl was employed at a split ratio 10:1. The oven temperature was maintained at 110 °C for 3.5 min, ramped to 200 °C at a rate of 10 °C/min , and then ramped to 280 °C at a rate of 5 °C/min held for 12 min. Mass spectra collected in the positive electron ion mode with 70 eV ionization energy were recorded at 0.50 second scan interval, at a scanning range of 50 to 500 amu. The source temperature and the inlet line temperature were 250 °C and 290 °C respectively.

2.7 Purification and Characterization

Out of all fractions, the fraction 02 (100% Chloroform) contains the Phytoconstituents β-sitosterol [$C_{29}H_{50}O$]which was confirmed by GCMS analysis. Purification carried out through preparative TLC and colored spots were point out. Finally a single spot was detected after using several solvent systems including chloroform: n-hexane (9.75:0.25), chloroform:ethyl acetate (9.75: 0.25), n-hexane: ethyl acetate (4:1) and exhibit a homogenous compound with sharp melting point 138 °C.

2.9 Purity by RP-HPLC

Test for chromatographic purity was performed with reverse phase high performance liquid chromatography (RP-HPLC) (Pharma Spec. UV-1700, Shimadzu) assembled with Zorbax Eclipse XDB column of 150mm of length, 4.6 mm of diameter, 5µm of particle size and a Photo diode array (PDA) detector. 100µg/mL of dried fraction of n-Hexane extract was prepared with DMSO and analyzed with an injection volume of 10µl. Gradient elution technique of mobile phase-A (15 mM Ammonium Acetate in water and pH 3 adjusted with Orthophosphoric acid solution) and mobile phase – B (Acetonitrile) was used (Table No.1).

Time (Min)	MP-A (%v/v)	MP-B (%v/v)
0	80	20
45	20	80
55	80	20
60	80	20

Table 1: Gradient programme of RP - HPLC

2.10 Measurement of Spectra

FTIR spectrum was recorded on a Perkin Elmer 1330 spectrometer with KBr pellets.

2.11 Molecular weight determination

High-resolution electrospray ionization mass spectrometry (HR–ESI–MS) measurement was carried out on a Q-Tof Micro YA263 spectrometer in the positive ion mode.

3. Results

3.1 *In vitro* cytotoxic activity

The cytotoxicity assay is mainly done to screen potentially toxic compounds that affect basic cellular functions. This assay includes both cytocidal and cytostatic effect of molecules. The in vitro cytotoxicity study (SRB assay) has been carried out as per recommendations of National Cancer Institute (NCI, USA).

From the current activity study it is observed that the second fraction of the n-Hexane extract of *L. camara* showed good activity at 80ug/ml (Figure 1) on A-549 lung cancer cell lines as comparedto the Adriamycinstandard.

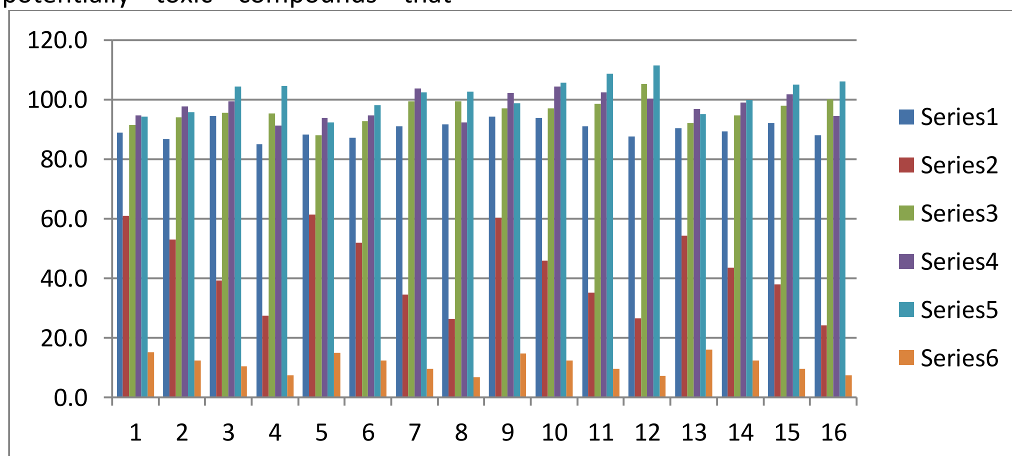

Figure 1: In vitro cytotoxic activity of the fractions of L. camara againsthuman lung cancer cell line A-549.

3.2 Identification of plant metabolites by GC-MS:

The fraction of n-Hexane extract of *L. camara* which showed cytotoxic effect, were analysed by GCMS for characterization of different phytoconstituents. The retention time (RT) and molecular formula of the compounds separated and identified are presented (Table 2).

Pk. No.	Compound Name	Formula	RT
1	Cholestan-3-ol, 2-methylene-, (3β,5α)	$C_{28}H_{48}O$	09.63
2	Caryophyllene	$C_{15}H_{24}O$	12.30
3	2-Methylenecholestan-3-ol	$C_{28}H_{48}O$	12.88
4	5α-Cholestan-3β-ol, 2-methylene	$C_{28}H_{48}O$	14.21
5	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	14.86
6	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.40
7	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	17.22
8	tert-Hexadecanethiol	$C_{16}H_{34}S$	17.71
9	β-Sitosterol	$C_{29}H_{50}O$	27.52
10	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	32.06
11	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	36.53
12	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	40.00

Table 2: Compounds identified in the fraction of n-Hexane extract of Lantana camara

10 compounds were identified in fraction of n-hexane extract which are Cholestan-3-ol, 2-methylene-, (3β,5α), Caryophyllene, 2-Methylenecholestan-3-ol, 5α-Cholestan-3β-ol, 2-methylene, Oleic acid, 3-(octadecyloxy)propyl ester, n-Hexadecanoic acid, 7-Methyl-Z-tetradecen-1-ol acetate, tert-Hexadecanethiol, β-Sitosterol and 1-Monolinoleoylglycerol trimethylsilyl ether. The GCMS chromatogram is furnished (Fig. 2).

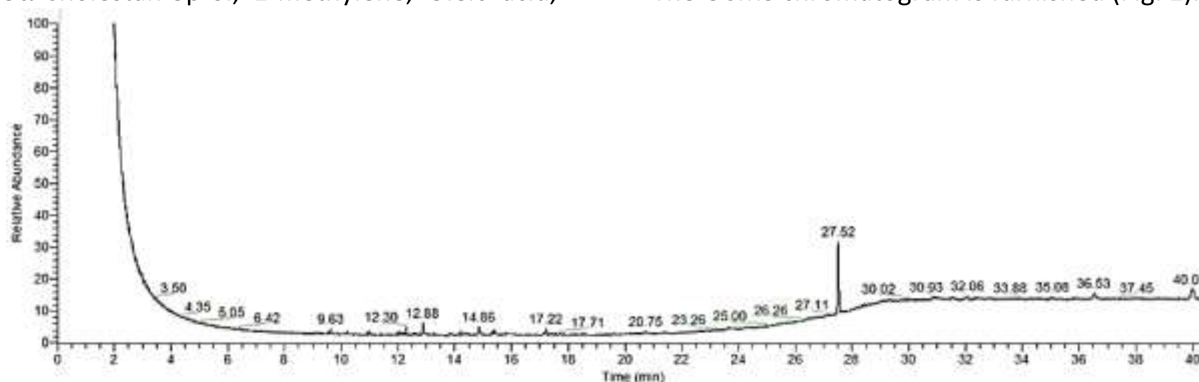

Figure 02: GC-MS chromatogram of Lantana camara of n-Hexane extract

3.3 Purity of compound by HPLC

From Reverse Phase HPLC the chromatographic purity of the isolated compound present in the fraction of n-Hexane extract of the plant *L. camara* is found to be 96.51 % area (Table 3).

Sl. No.	Name	RT	Area	% Area	Purity 1 angle	Purity 1 Threshold
1	Peak 1	4.674	61923	0.35	6.980	0.246
2	Peak 2	5.446	243094	1.38	0.249	0.196
3	Peak 3	5.806	50819	0.29	3.168	0.281
4	Peak 4	7.347	20303	0.12	0.516	0.331
5	Compound 1	10.841	17008426	96.51	5.246	15.669
6	Peak 1	18.123	51916	0.29	0.129	0.205
7	Peak 1	34.437	186804	1.06	0.046	0.221

Table 3: Peak Reasults

3.4 Analysis of Spectra

The FTIR spectrum (Fig. 3) showed absorptions band (cm^{-1}) for OH at 3426.45, CH_3 at 2958.63 and 2947.23, CH_2 at 2854.85 and 2853.64, unconjugated olefinic (C=C) at 1640.65, cyclic methylene groups $(CH_2)n$ at 1465.32, gem-dimethyl ($–CH(CH_3)_2$) group at 1385.26 and C–OH of secondary alcohol at 1053.68.

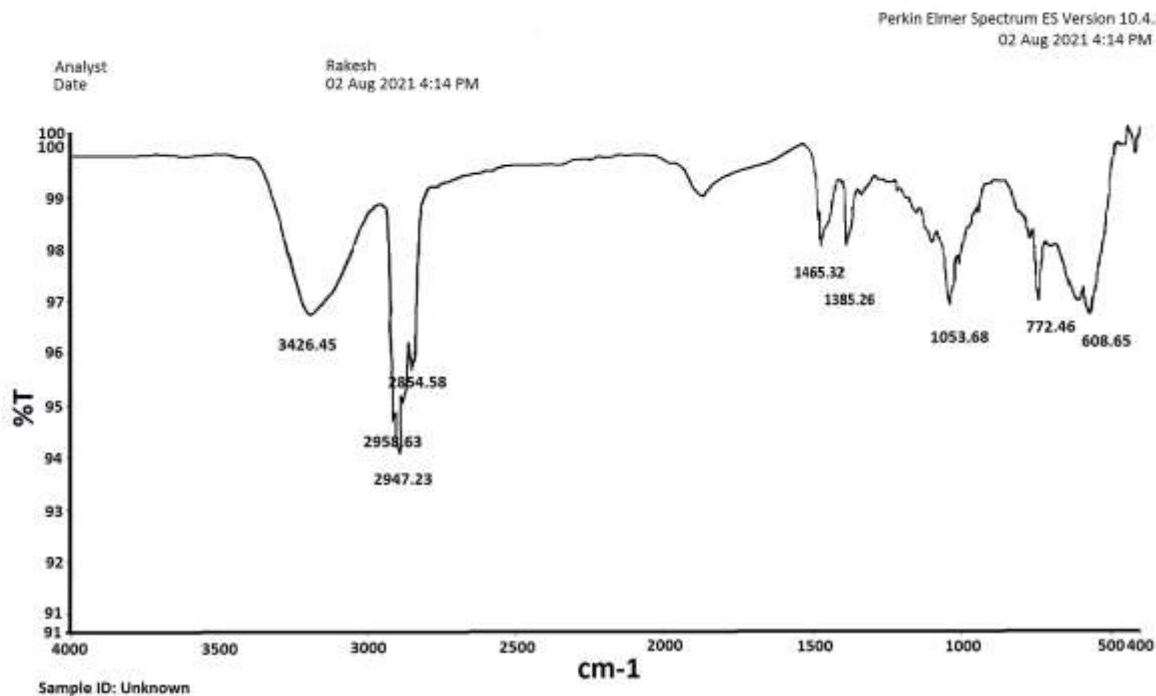

Figure 03: IR Spectra of the compound

The mass molecular ion of the compound appeared in HR–ESI–MS spectrum (Fig. 4) at m/z 437.3521 which is approximately 23 higher than the expected because the compound was ionized under positive mode HR–ESI by addition of Na atom. This indicated that the isolated compound with molecular weight of 414.3521, in good agreement with the theoretical value (calculated for $C_{29}H_{50}O$, 414.7066). The characteristic peak was given at m/z 413.39 that corresponds to (M-H) or loss of H.

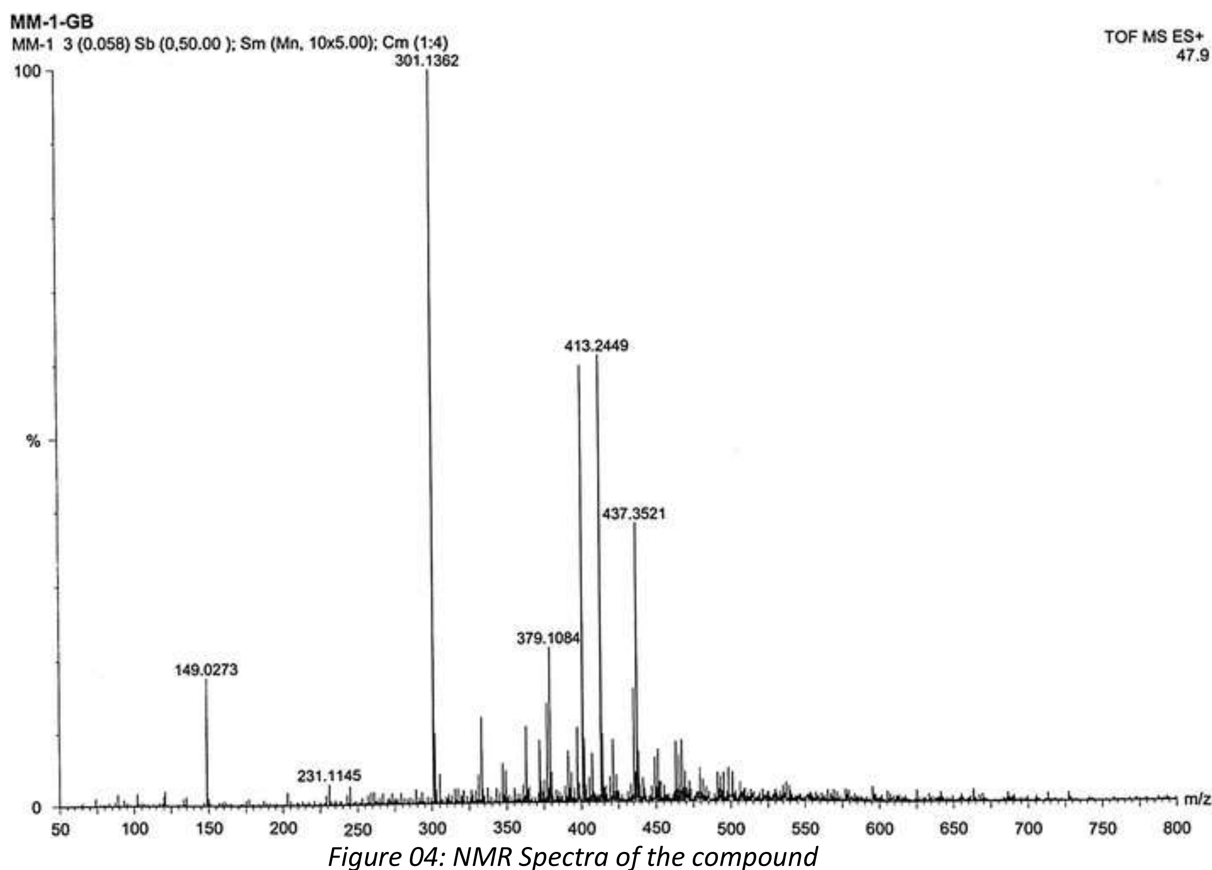

Figure 04: NMR Spectra of the compound

4. Discussion

Dried coarse plant material of *Lantana camara* L. was extracted initially with n-Hexane and carried out for Column chromatography, then after preliminary cytotoxicity study of all the fractions against A-549 (lung cancer) cell line only one fraction [Fraction - 02 (100 % Chloroform)] gives activity against A-549 cell line as compared with the positive control Adriamycin.

On GCMS analysis of Fraction - 02 (100 % Chloroform) gives massage regarding the presence of 10 different phytoconstituents. The analysis revealed the presence of 5α-Cholestan-3β-ol, β-Sitosterol and Caryophyllene which have been reported to be anti-cancerous in nature [13].

In Reverse Phase HPLC study for the purity of the isolated compound it is confirmed that the fraction found to be 96.51 % area. Through FTIR and mass spectrum analysis it is conformed as the presence of β-Sitosterol in the fraction [14].

From FTIRthe characteristic absorption bands followed by its functional groups it is confirmed that the compound may be β-Sitosterol andit is further conformed through Mass spectroscopy.

Conclusion:

The present study indicates the presence of the β-sitosterol as major phytochemical constituents in the fraction of n-Hexane extract of the plant *Lantana camara* L.The anticancer activity showed by the fraction is may be due to presence of β-sitosterol. The in vitro cell line studies shows partial inhibition of cancer cell growth. In column chromatographic method β-sitosterol was isolated and molecular weight, chromatographic purity were checked. Further, chromatographic study can be performed in the *L. camara* whole plant to isolated new phytochemical constituents.

Authors' Contribution

Sribatsa Lanchhana Dash: Conceptualization, Writing - original draft, Data Curation, Analysis and Interpretation of Data**. Arpit Katiyar:** Data Curation, Analysis

and Interpretation of Data. **Ranjit Mohapatra:** .Co-supervision, Analysis and Interpretation of Data and Writing-Review **Sagar Kumar Mishra:** Conceptualization, Supervision, Writing-Review and editing

Acknowledgment

SLD, AK, RM, SKM acknowledge University Department of Pharmaceutical Sciences, Utkal University, Vani Vihar, Bhubaneswar- 751004, Odisha, India for carried out the research work, Advanced centre for treatment, research & education in cancer for cytotoxic activity study against different human cell lines. and State pollution control board, Bhubaneswar, Odisha for GCMS analysis.

Reference:

1. Akhtar P, Ali M, Sharma MP, Farooqi H, Khan HN (2010). Phytochemical investigation of fruits of *Corylus colurna*Linn. *J. Phytol;*2(3): 89-100.
2. Mulukuri NVLS, Mondal NB, Prasad MR, Renuka S, Ramakrishna K (2011). Isolation of diterpenoid lactones from the leaves of *Andrographis Paniculata*and its anticancer activity. Int J PharmacogPhytochem Res; 3(3): 39-42.
3. Sivananthan M (2013). Antibacterial activity of 50 medicinal plants used in folk medicine. Int J Biosci b; 3(4): 104-121.
4. Sivananthan M, Elamaran M (2013). *In vitro* evaluation of antibacterial activity of chloroform extract *Andrographis paniculata*leaves and roots, *Duriozibethinus*wood bark and *Psidium guajava* leaves against selected bacterial strains. Int J Biomol Biomed a; 3(1): 12-19.
5. Mohammed FI, Al-Essa MK, Shafagoj YA, Afifi FU (2006). Investigation of the direct effects of the alcoholic extract of Elaeagnus angustifolia L. (Elaeagnaceae) on dispersed intestinal smooth muscle cells of guinea pig. Sci Pharm; 74:21-30.
6. Cansev A, Sahan Y, Celik G, Taskesen S, Ozbey H (2011). Chemical properties and antioxidant capacity of Elaeagnus angustifolia L fruits. Asian J Chem; 23:2661-5.
7. Farzaei MH, Bahramsoltani R, Abbasabadi Z, Rahimi R (2015). Acomprehensive review on phytochemical and pharmacological aspects of Elaeagnus angustifolia L. J Pharm Pharmacol; 67:1467-80.
8. Tehranizadeh ZA, Baratian A, Hosseinzadeh H (2016). Russian olive (Elaeagnus angustifolia) as a herbal healer. BioImpacts: BI; 6:155-67.
9. Trautwein EA, Demonty I (2007). Phytosterols: Natural compounds with established and emerging health benefits. Oléagineux Corps Gras Lipides; 14:259-66.
10. Ahmed OH, Hamad MN, Jaafar NS (2017). Phytochemical investigation of Chenopodium murale (Family: Chenopodiaceae) cultivated in Iraq, isolation and identification of scopoletin and gallic acid. Asian J Pharm Clin Res; 10:70-7.
11. Shriniwas P. Patil, Subhash T.Kumbhar (2018). Evaluation of terpene-rich extract of *Lantana camara* L. leaves for antimicrobial activity against mycobacteria using Resazurin Microtiter Assay (REMA). Beni-Suef University Journal of Basic and Applied Sciences; 7(4):511-515
12. MuthukumaranPakkirisamy, Suresh Kumar Kalakandan* and Karthikeyen Ravichandran (2017). Phytochemical Screening, GC-MS, FT-IR Analysis of Methanolic Extract of Curcuma caesiaRoxb (Black Turmeric). Pharmacogn J.; 9(6):952-956
13. Torwane NA, Hongal S, Goel P, Chandrashekar BR. Role of Ayurveda in management of oral health. Pharmacogn Rev. 2014 Jan;8(15):16-21. doi: 10.4103/0973-7847.125518. PMID: 24600192; PMCID: PMC3931197.
14. Wasan KM, Peteherych KD, Najafi S, Zamfir C, Pritchard PH. Assessing the plasma pharmacokinetics, tissue distribution, excretion and effects on cholesterol pharmacokinetics of a novel hydrophilic compound, FM-VP4, following administration to rats. J Pharm Pharm Sci. 2001 Sep-Dec;4(3):207-16. PMID: 11737986.