TUMKUR UNIVERSITY
VISHWAVIDYANILAYA KARYALAYA, B.H.ROAD, TUMAKURU - 572 103
KARNATAKA, INDIA

Prof. B.S. Gunjal
Registrar

No.TU:DEV-02:2017-18/4084 **Dated: 17.03.2018**

To,

Under Secretary (FD III)
University Grants Commission
Bahadurshah Zafar Marg
New Delhi 110 002.

Respected Sir,

Sub: Forwarding the proceedings of the final report assessment /Evaluation Certificate of the UGC Major Research Project
Ref: UGC letter No. 42-179/2013(SR) Dated:25.03.2013

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With reference to the subject cited above, I am forwarding the proceedings of the final report assessment/Evaluation Certificate of the UGC Major Research Project entitled **"Phytochemical Investigation and antiviral Properties of some Potential Medicinal Plants by Proteomic approaches"** submitted by **Dr. Raja Naika H., Assistant Professor, Department of Biotechnology, Tumkur University, Tumkur** for your kind perusal and needful action.

Thanking you,

Yours faithfully,

Registrar 17/03/18
Registrar
Tumkur University
Tumkur

Phone No: 0816-2254546, 2271924 Ext : 202, 207 Fax : 0816: 2255597 email: registrar.tut@gmail.com www.tumkuruniversity.ac.in

Title of the Project:

"Phytochemical investigation and antiviral properties of some potential medicinal plants by proteomic approaches".

Annexure-IX

UNIVERSITY GRANTS COMMISSION
BAHADURSHAH ZAFAR MARG
NEW DELHI-110002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT DONE ON THE PROJECT

1.	**TITLE OF THE ROJECT:**	**"Phytochemical investigation and antiviral properties of some potential medicinal plants by proteomic approaches"**
2.	**NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR**	Dr. Raja Naika H Assistant Professor, DOS&R in Biotechnology, Tumkur University, Tumkur-572103
3.	**NAME AND ADDRESS OF THE INSTITUTION**	Tumkur University, Tumkur-572103.
4.	**UGC APPROVAL LETTER NO.AND DATE**	UGC No.42-179/2013(SR), Dated: 25 -03-2013
5.	**DATE OF IMPLEMENTATION**	01st April, 2013
6.	**TENURE OF THE PROJECT**	31st March 2017
7.	**TOTAL GRANT ALLOCATED**	14,06,800/-
8.	**TOTAL GRANT RECEIVED**	1st installment: 9,47,800/- 2nd installment: **Not Received**
9.	**FINAL EXPENDITURE**	
10.	**TITLE OF THE PROJECT:**	**"Phytochemical investigation and antiviral properties of some potential medicinal plants by proteomic approaches"**
11.	**OBJECTIVES OF THE PROJECT**	➢ Isolation and Characterization of Bioactive constituents from medicinal plants ➢ Establishment of cell cultures; application of herbal extract preparations and synthetic drug agents in dose dependent manner. ➢ Proteomic analysis of human liver carcinoma cells harboring Hepatitis virus sub genomic replicon. ➢ Establishment of mice/rat model and validation some objective in the model
12.	**WHETHER OBJECTIVES WERE ACHIEVED(GIVE DETAILS)**	In this project, main objects are to Isolation and Characterization of Bioactive constituents from medicinal plants.

		➢ Isolation and preparation of herbal extracts with different solvent system. ➢ Qualitative and quantitative analysis of phytoconstiuents. ➢ Identification and isolated phyto constituents by IR,NMR and Mass spectroscopic studies. Establishment of cell cultures; application of herbal extract preparations and synthetic drug agents in dose dependent manner. ➢ Culture establishment of Huh 7 Cells having HCV Genome ➢ MTT assay for cell cytotoxicity. ➢ Screening of antiviral agents (Herbal extracts and phytoconstituents) applications. Proteomic analysis of human liver carcinoma cells harboring Hepatitis virus sub genomic replicon. ➢ Establishment of Huh 7 cells ➢ Huh 7 cells were transfected with RNA transcribed from linearized plasmid DNA containing sub genomic replicon of particular genome. ➢ Protein sample subjected 2-Dimensional gel Electrophoresis to differentiate up regulation, down regulation, differentially expressed and un altered protein spots. ➢ Image acquisition and analysis of 2D gels ➢ Protein identification by MALDI-TOF mass spectrometry (MS) and a database search.
13.	**ACHIVEMENTS OF THE PROJECT**	In this project, to achieve the identified the antiviral activities on HCV Protease and proteomic approaches of viral proteins studies.Although a number of novel antivirals against HCV for clinical use are being tested, it is still of importance to develop complementary and/or alternative drugs for treatment of HCV infection from clinical and economical points of view. In this regard, antiviral substances obtained from natural products, including medicinal plants, are potentially good targets to study. It is well known that certain medicinal plants possess antiviral activities. A wide variety of active phytochemicals, such as flavonoids, alakaloids, carbohydrates, sterols, tannins, quinines, glycosides, saponins etc .have been

		identified to inhibiting HCV NS3/NS5B proteases. Plant extracts of *Clematis gouriana, Euphorbia geniculata, Gynandropis pentaphylla, Aegle marmelos, Leucas aspera, Naravelia zeylanica and Phyllanthus amarus.* were shown to inhibit HCV protease activities *in vitro.* Finally, the potential medicinal effect shows on two plants such as *Clematis gouriana* Roxb. and *Naravelia zeylanica* (L.) DC. The crude extracts of *C. gouriana* and *N. zeylanica* viz., petroleum ether, chloroform, methanol extracts from leaves were subjected to the qualitative tests to detect the Bio-active chemical constituents such as Ursolic acid from methanolic extract of *C. gouriana* and Berberine from methanolic extract *N. zeylanica,* petroleum ether extract of *N.* zeylanica is isolated compounds such as terpenoidTaraxerol and sterol β- sitosterol. The above isolated compounds for screening *invitro* and *in silico* activity against NS3/NS5B Protease Domain of Hepatitis C Virus. The isolated compounds such as UA and BB showed moderate inhibitory effect, while the remaining compounds such as BS and TA did not exhibit significant inhibitory effect on HCV NS3 protease activity, while isolated compounds such as UA, BB, BS and TA. The isolated compounds of BB, BS and TA showed moderate inhibitory effect, while the remaining compound UA did not exhibit significant inhibitory effect on HCV NS5B protease activity. The isolated compounds of *Insilico* studies of inhibitor of NS3protein, screened compounds against wild-type protein and three different mutations (R155K, A156V, and A156T of NS3 protease with ligand. The compound Berberine and its charged counterpart displayed best scores against the wild type protein and mutant proteins -10.678 and-11.321, -11.532 and -10.782kcal/ mol, respectively.They also have moderately good scores against different mutations compared to Ursolic acid, β-sitosterol, and Taraxerol compound. In *insilico* studies of inhibitor of NS5B protein, screened compounds against wild-type protein and three different mutations. The rank of each compound was determined on the basis of the binding free

		energy of the lowest energy cluster. The tested compounds showed a binding energy ranging from -21.756 kcal/mol to -6.474 kcal/mol. The top three compounds with low binding energies Berberine, Taraxerol and β -sitosterol, whereas the compound with the highest binding energy (--21.756 kcal/mol) Berberine. Viral infections are the major ailment inflicting the human population. Conventional treatment with interferon-alpha is very expensive and has many serious side effects. Alternative medicine using different routes has been reported to be effective against viral infections. Viral infection is a major health problem worldwide developing effective antiviral therapy is the need of the hour. The viral enzymes are essential for polyprotein processing in viral multiplications thus can be potential targets for screening and identification of viral proteins. The comparative protein expression of host cells in response to viral infection. In this study, cellular protein response to viral infection in Huh cells was analyzed, using the proteomic method of two-dimensional gel electrophoresis (2D) coupled with MALDI-TOF-MS identification. The total of 17 altered cellular proteins that differentially expressed in replicon infection was identified in this study. Most of these proteins were involved in transcription and translation processes, vesicle transport, signal transduction, and alteration of the cytoskeleton networks.
14.	**SUMMARY OF THE FINDINGS (IN 500 WORDS)**	Naturally occurring, the bio-constituents are derived from medicinal plants have been extensively studied in terms of their antiviral activity. A number of inhibitors have been designed based on the cleavage of native substrates shows inhibition of HCV NS3/NS5B protease, many of which are large peptidomimetic compounds with less pharmacokinetic properties.*In vitro* and *insilico* studies revealed that the 181-residue N-terminal protease domain of protein formed a hetero dimer with the small 54- residue peptide cofactor, leading to the subsequent downstream cleavage of the HCV polyprotein at the junctions between viral proteins. Therefore, drug design targeting the NS3/NS5B to block viral replication

		and restore hepatocyte innate immune control of HCV replication has been significantly investigated. Varying in their all over the world prevalence and response to treatments in majority of phytoconstituents against HCV Protease enzyme. Finally, the potential medicinal effect shows on two plants such as *Clematis gouriana* Roxb. and *Naravelia zeylanica* (L.) DC. The crude extracts of *C. gouriana* and *N. zeylanica* viz., petroleum ether, chloroform, methanol extracts from leaves were subjected to the qualitative tests to detect the Bio-active chemical constituents such as Ursolic acid from methanolic extract of *C. gouriana* and Berberine from methanolic extract *N. zeylanica,* petroleum ether extract of *N.* zeylanica is isolated compounds such as terpenoid Taraxerol and sterol β - sitosterol. The above isolated compounds for screening *invitro* and *in silico* activity against NS3/NS5B Protease Domain of Hepatitis C Virus. Inhibition of HCV NS3 protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of UA and BB showed moderate inhibitory effect, while the remaining compounds such as BS and TA did not exhibit significant inhibitory effect on HCV NS3 protease activity. *Insilico* studies of inhibitor of NS3protein, screened compounds against wild-type protein and three different mutations (R155K, A156V, and A156T of NS3 protease with ligand. The compound Berberine and its charged counterpart displayed best scores against the wild type protein and mutant proteins -10.678 and-11.321, -11.532 and -10.782kcal/mol, respectively. They also have moderately good scores against different mutations compared to Ursolic acid, β-sitosterol, and Taraxerol compounds.Further, the inhibition of HCV NS5B protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of BB, BS and TA showed moderate inhibitory effect, while the remaining compound UA did not exhibit significant inhibitory effect on HCV NS5B protease activity. *In silico* studies of inhibitor of NS5B protein, screened compounds against wild-type protein

		and three different mutations. The rank of each compound was determined on the basis of the binding free energy of the lowest energy cluster. The tested compounds showed a binding energy ranging from -21.756 kcal/mol to -6.474 kcal/mol. The top three compounds with low binding energies Berberine, Taraxerol and β -sitosterol, whereas the compound with the highest binding energy (--21.756 kcal/mol) Berberine. Viral infections are the major ailment inflicting the human population. Conventional treatment with interferon-alpha is very expensive and has many serious side effects. Alternative medicine using different routes has been reported to be effective against viral infections. Viral infection is a major health problem worldwide developing effective antiviral therapy is the need of the hour. The viral enzymes are essential for polyprotein processing in viral multiplications thus can be potential targets for screening and identification of viral proteins. The comparative protein expression of host cells in response to viral infection. In this study, cellular protein response to viral infection in Huh cells was analyzed, using the proteomic method of two-dimensional gel electrophoresis (2D) coupled with MALDI-TOF-MS identification. The total of 17 altered cellular proteins that differentially expressed in replicon infection was identified in this study. Most of these proteins were involved in transcription and translation processes, vesicle transport, signal transduction, and alteration of the cytoskeleton networks. The study provides large scale protein-related information that should be useful for understanding the pathogenesis of viral infection. The purpose of this study is to determine the proteins which are responsible for viral multiplications.
15.	**CONTRIBUTION TO THE SOCIETY (GIVE DETAILS)**	Nature is a unique source of structures of high phytochemical diversity, many of them possessing interesting biological activities and medicinal properties. Medicinal plants contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments such as AIDS, chronic diseases and a variety of

		cancers, an intensive search for new lead compounds for the development of novel pharmacological therapeutics is extremely important. Phytoconstituents are the natural bioactive compounds found in plants and photochemistry is a distinct discipline somewhere in between organic chemistry, plant biochemistry and closely related to natural products. It deals with a variety of organic substances accumulated in plants. At present, the major concern with the use of phytomedicines regards the maintenance of consistent medicinal quality in botanical medicines. The modern drugs have saved many lives and have prolonged life span of many patients during the last few decades. But the use of modern drugs is like using of double edged weapon resulting in a variety of physical and drug-induced diseases. The synthetic drugs being pure synthetic chemicals may induce cellular changes, act as foreign substance to the body system and cause several side or toxic effect. To get relief from such tragedies of the use of modern drugs, modern medical scientists think of better alternatives and the use of medicines made from traditional and folklore drugs of plant origin is found to be the best alternative. Therefore, renovation of old ideas of herbal medicine with modern techniques and technologies is the need of the day and therefore, ethno medico botanical investigations have emerged as a promising branch for research. Whereas the focus has tended to be on quality control in herbal manufacturing practices, variation in phytomedicinal content due to environmental effects upon secondary plant metabolism in the plant material could represent a significant factor. It is clear that understanding how environmental factors affect phytomedicinal production will be of great importance toward optimizing field growth conditions for maximal recovery of phytomedicinal chemicals. Moreover, the phytochemicals isolated so far, nature must still have many more in store. In most cases, it is also unknown as to the extent to which levels of phytomedicinal chemical production by

		medicinal plants are determined by genetic potential versus environmental modulation. Medicinal plants have shown potential against viral infections and investigation of their active compounds has taken antiviral research to a new horizon. The studied plants extracts or compounds isolated from these extracts might not be used as unique anti HCV treatment but in combination with the therapeutic arsenal to reduce the cost of the actual therapy and increase the number of people with access to this therapy. The present study was aimed at making a sustained search for antiviral compounds and studies their therapeutic potential as anti-HCV drug. To this end, in-vitro bioassay was developed for screening antiviral activity of medicinal phytoconstituents. Resultantly three active fractions against HCV were identified and combination of these active fractions with interferon may open new avenues of future HCV therapies.
16	**WHETHER ANY PH.D.ENROLLED/PRODUCED OUT OF THE PROJECT**	**NIL**
17	**NO.OF PUBLICATIONS OUT OF THE PROJECT (PLEASEATTACH)**	**03**

(PRINCIPAL INVESTIGATOR)

Dr. RAJA NAIKA. H. M.Sc., Ph.D
Assistant Professor in Bio Technology
Principal Investigator,
UGC - Major Research Project,
Tumkur University,
TUMKUR-572103.(Karnataka)

17/3/18

(REGISTRAR/PRINCIPAL)
(Seal)

Registrar
Tumkur University
Tumkur

I. INTRODUCTION

Hepatitis C virus (HCV) is a dreadful viral disease and has always been a global health issue. It can be found in liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It is one of the major health problems that infect approximately 170 million people all over the world, and the majority of HCV-exposed individuals become persistently infected with the highest infection rates in Africa and Asia [1-3].

HCV belongs to the Flaviviridae family and has a positive single stranded RNA genome of 9.6 Kb. The genome of HCV has 5' untranslated region which works as an internal ribosomal entry site. The 5' UTR is 324-341 in length and the IRES is considered important for Cap-independent translation of viral RNA [4-8]. This entry site leads to the translation of an open reading frame that encodes a 3010 amino acid poly protein precursor whichis ultimately cleaved by host and viral proteases into 10 viral proteins in the order of NH (2) -Core-E1-E2-p7- NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH [9].

According to past research, the structural proteins (Core, E and E2) and the nonstructural proteins (NS3 protease and NS5B RNA dependent RNA polymerase) have been considered the best targets to develop novel molecular inhibitors. Among these proteins, NS3 in association with NS4A has been hugely investigated due to its protease and helicase domains that are important in viral replication [10-12].

HCV has six major genotypes with a series of subtypes[13]. To date, many medicinal plants have been tested against HCV and have proved beneficial as antiviral mediators. The reasons to prefer medicinal plants over traditionalmedicines are their fewer side effects, low cost and multiple target activities[14]. The phytochemicals of the medicinal plants, such as limonoids, alkaloids, lignana, organosulfur, furyl, thiophenes, polylines, terpenoids, flavonoids, polyphenolics, sulphides, saponins, coumarins, chlorophyllins, are considered important due to their efficiency at hampering viral entry, blocking/limiting the RNA/DNA genome replication and their anti-oxidant activity[15].

In past decade, there are few antiviral drugs that can efficiently work against HCV as most of the antiviral drugs show side effects and many of the viruses acquire resistance against them; thus, there is a strong need to develop antiviral compounds that can suppress HCV without side effects. Therefore, medicinal plants due to their magical powers are being

investigated to discover antiviral agents that can efficiently target the entry or replication of HCV virus and are believed to be our future inhibitors for this dreadful disease[16-17].

The viral infections with high mortality and morbidity rate are the leading cause of human deaths worldwide. Viruses start their life cycle through attachment and entry into the host cell and then increase their progeny by transcription and replication of the genome. The RNA viruses such as influenza, HIV have become a matter of concern as these are highly variable and lack an RNA dependent RNA polymerase proofreading mechanism [18]. Development of vaccines against viral diseases such as polio, mumps and smallpox has controlled these diseases but infections like HCV have been hard to target because of variation in genotypes. Infectious diseases have widely been treated using the medicinal plants and about 25% of current medicines have compounds from medicinal plants.

There are plenty of plants that are known for their magical medicinal properties and these plants can serve as an important reservoir for drug discovery against infectious diseases. Current separation techniques have enabled researchers to find active compounds of plants as antiviral agents and to overcome the challenge of emerging infectious disease in human population. There is a wide range of medicinal plants which are being used to extract natural compounds that are being used for their antiviral activity. Liver diseases have been treated around the world using numerous medicinal plants and their formulations and this has given confidence to researchers to investigate the effect of these medicinal plants against HCV in more depth [19].

HCV infection is a leading cause of deaths among patients. To date, many drugs have been tested against HCV and many of them have successfully completed clinical trials but the problem of viral resistance against these drugs and side effects caused by these drugs have marked a question of developing better therapeutics against HCV [20]. The novel drug discovery is being focused on medicinal herbs for HCV due to the lack of appropriate standard therapy. Acetonic and methanolic extracts of *Acacia nilotica* (AN) have shown novel inhibition of HCV titer *in-vitro* confirmed by real-time PCR[21]. Preclinical evaluation of the lyophilized juice of ginger and aqueous extracts of *Milk thistle* (MSE) has demonstrated anti HCV effects in the HepG2 cell line. Both of these plants have shown effective antiviral activity [22].

Recently, the potential medicinal plants from Indonesia have been tested for their antiviral activity against HCV. Ethanolic extracts of Indonesian plants were analyzed in the Huh 7.5 cell line and HCV strains of 9 different genotypes namely 1a-71, 1b and 2b. Among the tested plants, *Toona sureni* leaves (TSL) showed IC_{50} value of 13.9, *Melicope latifolia* leaves (MLL) showed IC_{50} of 3.5, *Melanolepis multiglandulosa* stem (MMS) exhibited IC_{50} of 17.1 and *Ficus fistulosa* leaves (FFL) showed IC_{50} of 15.0. Among all of these, MLL, TSL, FFL and MMS exhibited antiviral activity against all genotypes of HCV and thus, it is suggested that these plants may prove good candidates to develop novel inhibitory drugs against HCV though there is a need to further investigate these plants to develop drugs for the effective inhibition of HCV[23].

During the recent decades, advancements in drug discovery have revealed NS3/4A protease as an important drug target in overcoming HCV infection. Many inhibitors against NS3 have successfully entered clinical trials but there is still need to improve their functionality and efficiency [24-25].

Various medicinal plants are also being investigated to develop anti HCV drugs that are not only efficient but are also easily available to developing countries due to their low cost[26]. Methanolic and water extracts of medicinal plants used in Sudanese traditional medicine such as *Boswellia carterii, Acacia nilotica*, *Quercus infectoria, Embelia schimperi*, *Trachyspermum ammi*, *Piper cubeba* and *Syzygium aromaticum* have been tested against HCV protease [27].

An *in-silico* approach has been used to test *Acacia nilotica* phytochemicals against NS3/4A protease and found that they may serve as a potential drug candidate with relatively simple structural changes against HCV NS3/4A protease[28]. *Solanum nigrum* (SN) has also been tested against HCV and its methanolic and chloroform extracts exhibited significant inhibition against HCV protease in liver infected cells[29].

Recently, *Viola yedoensis* has been investigated to find an anti HCV compound targeting protease. Using the various chromatographic procedures, 3 coumarins have been isolated and characterized from *Viola yedoensis*. Among the isolated compounds, a dimeric coumarin 5, 5'-bi (6, 7-dihydroxycoumarin) has significantly inhibited NS3/4A protease with IC_{50} value of 0.5 μg/ml. Thus, this dicoumarin can serve as an important molecular template to design novel anti HCV drugs[30].

Proteomics is a novel methodology to detect components of cellular protein interactions as well as host cellular pathophysiological processes that occur during virus infection [31]. Viruses rarely on the cellular translation machinery to translate their own proteins, which facilitates the rapid production of viral proteins and renders an inhibitory effect on the production of host proteins, including host anti-viral proteins [32]. Translation factors have been well documented as playing crucial roles in viral RNA and protein synthesis [33].

In herpes simplex virus type 1 (HSV-1) infected HeLa cells, the synthesis of several ribosomal proteins and their assembly into ribosomes continue in spite of a general inhibition of cellular protein synthesis [34]. Acidic ribosomal protein is located in the active part of the ribosome particle, at which mRNAs, tRNAs and translation factors interact during protein synthesis [35]. Viruses may inhibit host protein synthesis by targeting multiple steps in the gene expression process via various pathways, for instance, the vesicular stomatitis virus (VSV) M protein inhibits the initiation of the transcription of host genes [36] and the SARS-CoV spike protein inhibits host cell translation by interaction with eIF3f [37].

Protein phosphatase 2A (PP2A) is an evolutionarily conserved enzyme that represents a major portion of serine/threonine phosphatase activity in cell extracts [38]. PP2A enzymes have been clearly involved in regulation of cell transcription, cell cycle and viral transformation [39]. Up-regulation of PP2A scaffolds subunit A and subsequent dephosphorylation of Tyr-307 in the catalytic subunit was found, suggesting PP2A activation in Huh7 infected cells [40, 41]. Activation of serine-threonine PP2A was found in Huh7 cells upon HSV-1 infection, and PP2A activation paralleled dephosphorylation and inactivation of the downstream mitogen-activated protein (MAP) kinase pathway [42].

The actin and microtubule cytoskeleton play important roles in the life cycle of viruses [43]. Numerous viral proteins interact with actin-binding proteins or directly with actin [44]. Microtubules and microtubule-associated proteins are known to play important roles in intracellular trafficking of viral components as well as virons in the infected host cell [45]. Several proteomics analysis about coronavirus had been done including SARS-CoV [46], IBV [47, 48] and MHV [49].

Currently research and development of new drugs from natural resources in a systematic strategic manner has become the global trend. In the past decade, the alternative to conventional chemical agents, large number of phytochemicals has been recognized as a way to control infections caused by viruses. So far several potential medicinal plant extracts have been screened for their antiviral effect against HCV Virus namely *Clematisgouriana, Euphirbia geniculata, Gnandropis pentaphylla, Aeglemarmelos, Leucas aspera, Naravelia zeylanica* and *Phyllanthus amarus.* Finally, the molecular studies on antiviral potentially significant in the plants extract namely *Clematisgouriana* and *Naravelia zeylanica* of HCV on NS3 and NS5B Protease in vitro. Based on above results, to isolate the bioconstiuents from medicinal plants such as *C.gouriana* and *N.zeylanica* against HCV on NS3 and NS5B Protease *in vitro* and *insilco* studies. Further, the identification of cellular proteome using two-dimensional gel electrophoresis, and identification of differentially expressed, upregulated, down regulated and unaltered proteins by isoelectric focusing and with the help of different software, some of the unique protein spot was trypsin digested and subjected for MALDI-TOF Mass spectroscopy.

II. REVIEW OF LITERATURE

Hepatitis C is a major healthcare problem worldwide caused by a viral infection with a high tendency to become chronic. Chronic hepatitis C is linked to the development of cirrhosis and hepatocellular carcinoma. The virus responsible for this disease was discovered more than 20 years ago [50]. Since hepatitis C virus (HCV) discovery, blood screening diagnostics have greatly reduced the blood-borne transmission of the virus. However, the transmission still occurs through other modes of contamination and the slow development of the disease results in many persons not knowing their infected status. It is estimated that about 160 million persons (2.35% of the world population) are infected with HCV [51].

Currently, there is no vaccine against HCV and the high diversity of viral isolates will probably make it very difficult to develop a vaccine. On the other hand, we know that, in contrast to hepatitis B and human immunodeficiency viruses, HCV can be eradicated from chronically infected patients with antiviral treatments. However, the standard therapy, which is based on a combination of pegylated interferon alpha (IFN-α) and ribavirin [52], results in highly variable outcomes [53], is very expensive and has severe side effects that are difficult to endure for the patients.

Nevertheless, it is currently thought that efficient anti-HCV therapies will be achieved with direct acting antivirals (DAAs) [54]. The protease inhibitors to the standard anti-HCV therapy have already improved sustained virological response rates in patients infected with genotype- 1 HCV. New drugs targeting other viral proteins are in clinical trials and will probably also help improving response to HCV therapy [55-56].

A combination of DAAs will reduce the risk of selecting viral escape mutants. DAAs combinations in the absence of interferon will probably enable to greatly reduce side effects of the therapy, which are mainly associated with the use of interferon and contribute to the failure of the treatment.

Moreover, to have a chance of eradicating HCV, the therapy should be cheap so as to be able to cure infected patients from low-income countries and stop the transmission of the virus [57]. Its single-stranded genomic RNA contains a single open reading frame surrounded by two untranslated regions (UTR) that are necessary for the translation and the replication of the viral genome [58-60].

The translation of the open reading frame is under the control of an internal ribosome entry site (IRES), located in the 5'UTR. It gives rise to a polyprotein precursor, which is cleaved by host- and viral-encoded proteases into ten polypeptides. The N-terminal part of the polyprotein contains structural proteins: the core protein C, a component of the viral capsid, and the two envelope glycoproteins E1 and E2.

The C-terminal part of the polyprotein contains non-structural proteins required for RNA replication: NS3, which has protease and helicase activities; NS4A, a co-factor of NS3 protease; NS4B, a polytopic membrane protein; NS5A, a phosphoprotein; and NS5B, the viral RNA-dependent RNA polymerase. Between the structural proteins and the non-structural proteins involved in RNA replication, the polyprotein also contains two additional polypeptides required for viral assembly, which are dispensable for RNA replication: the viroporin p7 and the NS2 protein, which has an autoprotease activity during the maturation of the polyprotein precursor.

The structure of the viral particle is still unknown. In patients, circulating HCV particles are associated with apolipoproteins (Apo) B and E, and have highly variable buoyant densities, the lighter ones being the most infectious [61]. It is currently thought that infectious HCV particles are initially secreted as very low-density lipoprotein (VLDL)-like particles by infected hepatocytes and then potentially undergo lipolysis in the bloodstream, which progressively converts them into intermediate density lipoprotein (IDL)and LDL-like particles.

However, it is not yet clear what in this process reduces the specific infectivity of HCV viral particles. HCV life cycle has greatly improved in recent years, following the finding of a viral strain (JFH-1) able to replicate in cell culture [62-64]. The JFH-1-based cell culture model hasbeen named HCVcc.

The reasons that are still unknown, other viral isolates do not efficiently replicate in cell culture. Before the HCVcc model was established, specific steps of the HCV life cycle had been studied with other experimental systems recapitulating RNA replication, with the sub genomic replicon model [65-66], or viral entry, with the HCV pseudoparticles model [67-69].

The replicon model is based on a modified HCV genome, in which the coding region of the structural proteins is replaced by a selection marker. In vitro synthesized

subgenomic replicon RNA is introduced in cells by electroporation and the cells replicating it express the selection marker and can thus be selected.

There is no release of viral particles, and this model only allows studying cellular and molecular mechanisms involved in viral RNA replication. Hepatitis C virus pseudoparticles are retroviral particles pseudotyped with HCV envelope glycoproteins E1E2. Hepatitis C virus entry is a very complex process, which involves a series of host entry factors [70].

On the viral particle, envelope glycoproteins E1E2 play a major role during entry. The viral particle probably initially binds to glycosaminoglycans (GAG) on the surface of the target cell. It has been proposed that interactions between the LDL receptor (LDL-R) and apolipoproteins of the viral particle might also participate in the initial binding to the cell surface.

Following these rather non-specific initial binding events, several host entry factors are specifically involved in the entry process [71]. The tetraspanin CD81 [72], the scavenger receptor class B type I (SR-BI) [73], and the tight junction proteins claudin-1 (CLDN1) [74] and occludin (OCLN) [75-76] are mandatory for HCV entry. Epidermal growth factor receptor, ephrin receptor A2 [77] and the cholesterol transporter Niemann-Pick C1-like 1 also participate to the entry process [78].

The particle is internalized by clathrin-mediated endocytosis [79] and the viral genome is released into the cytosol of the cell following the fusion of the viral envelope and the endosomal membrane.Once in the cytosol, the viral genome is translated. Non-structural viral proteins NS3/4A, NS4B, NS5A, and NS5B assemble into replication complexes [80] that generate new viral genomic RNA molecules through the prior synthesis of negative RNA strands, complementary to the genomic RNA. Much like for many positive stranded RNA viruses, HCV replication occurs in host cell cytoplasm in association with rearranged membranes, named ‘membranous webs’ [81].

A large number of host factors probably participate to the formation and the functioning of HCV replication complexes, which are recruited through interactions with viral proteins. A major host cell factor regulating HCV replication recently identified is the class III phosphatidylinositol 4-kinase alpha [82-83]. The protease NS3/4A and the RNA

polymerase NS5B are the two major druggable viral factors involved in HCV replication, which have been used in antiviral screens.

Medicinal plants have shown potential against viral infections and investigation of their active compounds has taken antiviral research to a new horizon. The medicinal plant derivatives being tested against HCV and some of them have shown significant inhibition in entry, replication on viral life cycle. Diosgenin (3β-hydroxy-5-spirostene), which is a plant-derived sapogenin, has effectively blocked the replication of the HCV subgenomic replicon system at both the mRNA and the protein level [84].

Silymarin, which is isolated from *Silybum marianum* has been tested against HCV and is found to be effective in inhibiting the viral activity of HCV. Silymarin has been tested against the HCV core protein of genotype 3a and was found to be effective in inhibiting the core expression. Silibinin, which is a combination of two diastereoisomers, is the major component of silymarin responsible for anti HCV activity [85-86].

HCV entry has been blocked using different iridoids from Lamium album using HCV pp. The aqueous extract of Lamium album containing lamiridosins and iridoids aglycone epimers have reduced HCV Pseudoparticles entry by disturbing the contact of HCV envelope 2 proteins (E2) with the CD81 receptor [87]. The replication of HCV RNA has been blocked by 5-fluoro derivative of Isatin in Huh 5-2 cells [88].

Naringenin is a predominant flavanone found in the grapefruit and has been tested on the HCV particles[89-90]. Epigallocatechin-3-gallate (EGCG) is found in green tea extract and recently this compound has been found significant in inhibiting HCV entry.

EGCG had no effect on HCV assembly, replication and release but it efficiently inhibited cell-culture-derived HCV (HCVcc) entry into hepatocellular cell lines and this effect was independent of the HCV genotypes [91]. The effect of EGCG on HCV entry blockage has also been confirmed by another research group using a new anti HCV molecule screening assay [92].

Quercetin, present in vegetables, fruits, grains and leaves has been investigated as an anti HCV agent. Quercetin not only reduced IRES activity but also its augmentation by NS5A [93]. Similarly, in another study quercetin suppressed RNA replication in a subgenomic RNA replicon and also inhibited replication in a model containing NS3

substrates suggesting that it may be related to NS3 protein of HCV and blocks replication by targeting the NS3 protease[94].

In combination with cyclosporine, it showed interesting synergetic results against all the genotypes of HCV [95]. Luteolin and apigenin have also been identified as anti HCV agents respectively. Among both compounds, luteolin showed persuasive inhibition of NS5B polymerase activity respectively.

III. MATERIALS AND METHODS

1. Collection of plant materials

Different medicinal plants such as *Clematis gouriana, Euphorbia geniculata, Gynandropis pentaphylla, Aeglemarmelos, Leucas aspera, Naravelia zeylanica* and *Phyllanthus amarus* used in this study were identified and collected from Lakeville reserve forest range of Western Ghats region of Karnataka, India. Plants were identified by Prof. V. Krishna, bycomparing with the authenticated specimens.

2. Preparation of Plant extracts

The plant materials (Fig.1 and Table.1) were washed with running tap water followed by distilled water for 3-4 times and then shaded dried at room temperature for 15-20 days. After shaded dried plant material then powdered mechanically using Electric mixture and sieved (sieve No.10/44).The shade dried, powdered materials of both the plants were subjected to successive solvent extraction [96-97] as described below.

The powdered materials of *Clematis gouriana* Roxb*, Euphorbia geniculata* (L.)*, Gynandropis pentaphylla*(L.)*, Aegle marmelos*(L.)*, Leucas aspera*(L.)*, Naravelia zeylanica*(L.) and *Phyllanthus amarus* (L.) leaves were taken separately in one liter capacity thimble of soxhlet apparatus and extracted successively with Petroleum ether(40-60°C),Chloroform (50-70°C) and methanol (50-80°C) for 72 hrs in four batches of 250g each.Every time, before extracting with the next solvent the mark was dried at room temperature. All the extracts were filtered and concentrated in vacuum using rotary flash evaporator. Left over solvent was completely removed on water bath and finally dried in the desiccators. The crude extracts obtained from each of the solvents were labeled and stored in refrigerator for further uses.

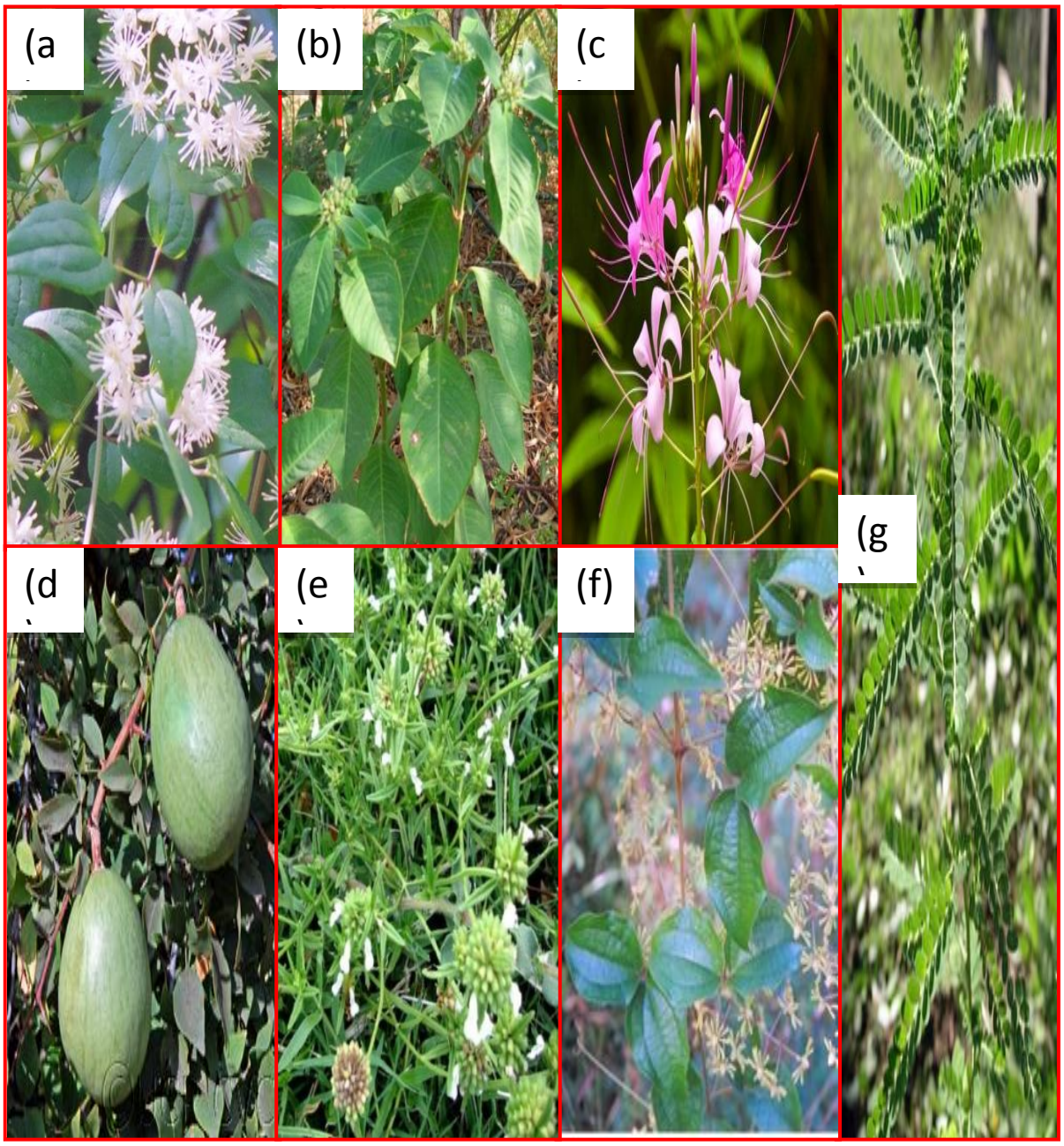


Fig.1. Shows as (a) ***Clematis gouriana,*** **(b)** ***Euphorbia geniculata,*** **(c)** ***Gynandropispentaphylla*** **(d)** ***Aegle marmelos (*****e) *Leucas aspera,*** **(f)** ***Naravelia zeylanica and*** **(g)** ***Phyllanthusamarus.***

Table 1: Plants and their parts used in this study

No	Plant name	Code	Part used
1	*Clematis gouriana*	CG	Leaf
2	*Euphorbia geniculata*	EG	Leaf
3	*Gynandropis pentaphylla*	GP	Leaf
4	*Aegle marmelos*	AM	Leaf
5	*Leucas aspera*	LA	Aerial part
6	*Naravelia zeylanica*	NZ	Leaf
7	*Phyllanthus amarus*	PA	Aerial part

3. Phytochemical investigation.

3.1. Qualitative phytochemical analysis

The crude extracts of *Clematis gouriana, Euphirbia geniculata, Gynandropis pentaphylla, Aeglemarmelos, Leucasaspera, Naravelia zeylanica and Phyllanthusamarus*viz., petroleum ether, chloroform, methanol extracts and extracts from calli were subjected to the following qualitative tests to detect the active group of chemical constituents [99].

a) Test for alkaloids

Mayer's test: Test solution + Mayer's reagent (potassium mercuric iodide) gives cream colored precipitate.

Wagner's test: The acidic test solution with Wagner's reagent (iodine in potassium iodide) gives brown precipitate.

Hager's test: The acidic test solution with Hager's reagent (saturated picric acid solution) gives yellow precipitate.

Dragendorff's test: The acidic test solution with Dragendorff's reagent (potassium bismuth iodide) shows reddish brown precipitate.

b) Test for sterols

Salkowaski test: Test solution + concentrated sulphuric acid, shaken and allowed to stand. The lower layer turns red indicating the presence of sterols.

Liebermann-Burchard test: Test solution + few drops of acetic anhydride + concentrated sulphuric acid along the sides of the test tube. Brown ring forms at the junction of the two liquids and the upper layer turn green.

*Sulphur test:*Sulphur when added to the test solution, it sinks to the bottom indicating the presence of sterols.

c) Test for flavonoids

Ferric chloride test: Test solution + few drops of ferric chloride solution give intense green color.

Shinoda test: Test solution + few fragments of magnesium ribbon + concentrated hydrochloric acid, shows pink to magenta red color.

Zinc-Hydrochloric acid reduction test: Test solution + zinc dust + few drops of hydrochloric acid shows magenta red color.

Alkaline reagent test: Test solution + sodium hydroxide solution shows increase in the intensity of yellow color which becomes colorless on addition of few drops of dilute acid.

Lead acetate solution test: Test solution + few drops of lead acetate (10%) solution gives yellow precipitate.

d) Test for glycosides

Baljet test: Test solution + sodium picrate gives yellow to orange color.

Keller-Killiani test: Test solution + few drops of ferric chloride solution, mixed. When concentrated sulphuric acid containing ferric chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.

*Raymond test:*Test solution + dinitrobenzene in hot methanolic alkali gives violet color.

Bromine water test: Test solution + bromine water gives yellow precipitate.

*Legals test:*Test solution + pyridine (made alkaline by adding sodium nitroprusside solution) give pink to red color.

e) Test for triterpenoids

*Salkowaski test:*Test solution + few drops of concentrated sulphuric acid, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

*Liebermann-Burchard test:*Test solution + few drops of acetic anhydride. + Concentrated sulphuric acid along the sides of the test tube. Development of deep red color indicates the presence of triterpenoids.

f) Test for tannins

*Ferric chloride test:*Test solution + few drops of ferric chloride solution give dark red color.

*Gelatin test:*Test solution + gelatin solution give white precipitate.

g) Test for quinines

Test with potassium iodide: Potassium iodide gives hydrogen iodide on reaction with dilute sulphuric acid. The liberated hydrogen iodide reacts with quinones producing iodine, which can be tested with starch paper, which turns into blue color.

h) Test for Saponins

Foam test: Saponins when mixed with water and shaken shows the formation of froth, which is stable at least for 15 min.

Haemolysis test: 2 ml each of 18% sodium chloride solution was taken in two test tubes. To one test tube 2 ml of distilled water and to another test tube 2 ml of test sample was added. A few drops of blood was added to both the test tubes, mixed and observed for hemolysis under microscope. Hemolysis of blood cells indicates the presence of saponin.

i) Test for carbohydrates

Molisch's test: Test solution + few drops of Molisch's reagent + 2ml of concentrated sulphuric acid along the sides of the test tube. A purple ring formed at the junction of two liquids indicates the presence of carbohydrates.

Barfoed's test: Test solution + Barfoed's reagent, boiled on water bath. Brick red precipitate indicates the presence of carbohydrates.

*Benedict's test:*Test solution + Benedict's reagent, boiled on water bath. Reddish brown precipitate indicates the presence of carbohydrates.

*Fehling's test:*To 2 ml of aqueous extract, 1 ml mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of red or brick red colored precipitate indicated the presence of reducing sugar.

j) Test for proteins

*Millon's test:*Test solution + Millon's reagent, heated on a water bath. Yellow coloration indicates the presence of protein.

Xanthoproteic test: Test solution + concentrated nitric acid, on boiling gives yellow precipitate.

*Biuret test:*Test solution + 40% sodium hydroxide + dilute copper sulphate solution. Blue color indicates the presence of protein.

Ninhydrin test: Test solution + Ninhydrin reagent gives blue color.

3.2. Isolation and Characterization of the active constituent from significanteffect of HCV on crude extract of selected plants.

Isolation and characterization of the active constituents from methanol extract of the leaves of *Clematis gouriana* and methanol extract and petroleum ether extract of the leaves of *Naraveliazeylanica.*

(a) Isolation and Characterization of the active constituents from methanol extract of the leaves of *clematis gouriana*

Methanol extract

Activated (in hot air oven at 110^0C for one hour) Silica gel H (s.d.fine chem. Ltd, Mumbai) column chromatography grade was taken. Different solvent systems were used for the elution of constituents of methanol extract of the leaves of *C. gouriana*. The mobile phase consists of methanol: chloroform in the ratio of 8.5:1.5 was used for the separation of compounds

Adsorbent	:	Silica gel, 60-120 mesh size
Length of the column	:	75 cm
Length of the adsorbent	:	50 cm
Diameter of the column	:	2.5 cm
Wt. of silica gel used for column	:	175 g
Wt. of extract used for column	:	10 g
Solvent used for elution	:	Methanol: Chloroform (8.5:1.5)
Rate of elution	:	5-6 drops/min
Volume of elute collected	:	5 ml each

Thin layer chromatography of eluted fractions of column:

Adsorbent	:	Silica gel G (activated)
Plate size	:	20cm x 8cm

Plate thickness	:	3 mm
Activation temperature	:	110^0C for 1 h
Mobile Phase	:	Methanol: Chloroform (8.5:1.5)
Developing chamber	:	Iodine fumes.

The compound isolated was named as CGM and the yield was 0.516 grams (2.58%).

(b) Isolation and Characterization of the active constituents from the leaves of *Naraveliazeylanica*

Petroleum ether extract

Activated silica gel slurry was made by using mobile phase solvent system Benzene and ethyl acetate in the ratio 9:1 and charged into the column. Glass wool was plugged at the bottom of the column. The air bubbles in the column were removed by gentle tapping and by the addition of excess of solvent, in order to ensure uniform packing of the adsorbent. The tap of the column is then opened to remove the excess solvent and small quantity of the solvent system was allowed to remain on the top of the column (about 2 cm). The silica gel bed was allowed to stabilize overnight. Care was taken to prevent the drying of the column by plugging the top end with the aluminum foil.

The crude extract obtained from the petroleum ether extract, was dissolved in small quantity of petroleum ether solvent and carefully introduced into the stabilized column. The top at the bottom was operated to run out very small amount of solvent in the column in order to bring the sample just below the top of the medium. The eluting solvent was added gradually to maintain a steady flow. The rate of flow of the elution was adjusted to 5 to 6 drops/min and allowed the separation to continue. The eluted fractions were collected at the intervals of 5 ml each. The suitability of the eluted samples was tested by TLC and grouped into fractions. The eluted fractions were collected separately and dried. The details of the column and TLC of the eluted fractions are given below.

Adsorbent	:	Silica gel, 60-120 mesh size
Length of the column	:	75 cm
Length of the adsorbent	:	50 cm
Diameter of the column	:	2.5 cm
Wt. of silica gel used for column	:	175 g
Wt. of extract used for column	:	10 g
Solvent used for elution	:	benzene: ethyl acetate (9:1)
Rate of elution	:	5-6 drops/min
Volume of elute collected	:	5 ml each

The fractions collected was subjected TLC which showed two spots. The fraction was recrystallized with benzene, white crystalline compound was precipitated out during solvent evaporation, which was filtered under vacuum, and the filtrate was concentrated and purified with benzene. These compounds were named as NZP-1 and NZP-2 were collected and tested qualitatively for the presence of phytochemical groups. The fractions NZP-1 showed positive test for triterpenoids and NZP-2 showed positive chemical test for steroids**.** The purity of all the eluted fractions was monitored by TLC. The yield was 0.516 grams (2.58%) and 0.216 grams (1.98%) respectively.

Methanol extract

The crude extract of methanol was dissolved in methanol subjected for Preparative TLC with different solvent systems and varied concentrations and polarity were tried. The solvent system used for the elution of methanolextract by preparative TLC of fractions is given below.

Preparative TLC method for elution

Adsorbent	:	Silica gel G (activated)
Plate size	:	20cm x 8cm

Plate thickness : 5 mm

Activation temperature : 110^0C for 1hour

Mobile Phase : Methanol: Water: Ammonium hydroxide 8:1:1

Visualization : UV chamber

The compounds isolated was named as NZM the yield was 0.343gms (2.29%)

(c) Characterization of the isolated constituents

The characterization requires the identification of molecular framework, the nature of the functional group, which are present, their location within the skeletal structure and finally the establishment of any stereo chemical relationship, which might exist. The characterization of the compounds has been revolutionized by the progressive adoption of the wide range of spectroscopic techniques, which are now available. These have been applied extensively in the preparative section to confirm the structure of the expected compounds. The same was applied extensively in the preparative section to confirm the structure of newly isolated compounds.

The melting points of these isolated constituents; CGM, NZP-1, NZP-2 and NZM were determined by using the melting point apparatus (Jindal, New Delhi). The UV spectroscopy of the compounds was done by using UV spectrophotometer (Shimadzu, Kyoto, Japan). The IR Spectra were recorded with KBr pellets on a Perkin- Elmer 1710 FT-IR spectrophotometer. ^{1}HNMR spectra of the compound were taken on Bresker AMX (400 MHz) NMR spectrometer using $CDCl_3$ and methanol as solvents and Mass spectra were recorded on a MAT 312 spectrophotometer and FAB–MS (positive) data on JEOL SX 102/DA–600 at Central Drug Research Institute (CDRI) Lucknow. All these spectral data were incorporated in the result section. Based on the spectral data structure of the compounds were characterized.

4. Evaluation of different plant extracts and bio-constituents of *Clematis gouriana* and *Naravelia zeylanica* against NS3 –NS5B protease.

All chemicals were purchased from Sigma-Aldrich and Merck such as Dimethyl sulfoxide (DMSO) Cyclosporine A (CsA) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] Dulbecco's modified Eagle's medium (DMEM), Tris NaCl etc,.

pYB-43 plasmid contains the single-chain protease NS4A-NS3 (scNS3) and pYB-44 vector contains NS3 cleavage site at middle flanked by Enhanced Green Fluorescent Protein (EGFP) and cellulose binding domain (CBD) (both are kind gift from Dr. ItaiBenhar, Tel Aviv University, Israel). A cell culture adapted infectious H77scDNA construct [99] was kindly provided by Stanley Lemon, University of Texas, and Galveston and was used to screen inhibitory effect of plant extracts against HCV replication. The HCV replicon construct pSGRLuc JFHI [100] was linearized with Xba I and the HCV-Luc replicon RNA was generated using T7RNApolymerase.

4.1.HCV NS3 protease activity assay

The inhibitory effect of different solvents crude plant extracts namely*Clematis gouriana, Euphorbia geniculata, Gynandropis pentaphylla, Aeglemarmelos, Leucasaspera, Naravelia zeylanica and Phyllanthusamarus and* isolated bio constituentssuch as Ursolic acid (UA), Berberine (BB), Taraxerol (TA) and β - sitosterol (BS) from *Clematis gouriana* and *Naravelia zeylanica*against HCV NS3 protease was assessed earlier by high through put fluorescent based assay. The cleavage of recombinant substrate by recombinant HCV NS3 protease was performed as described earlier by [101] with little modification. In brief 0.01–0.1μM of recombinant protease and different dilutions of the isolated compounds were pre-incubated in an eppendorf for 15 min at 37 ◦C in reaction buffer (50mM Tris–HCl pH 7.5, 150mM NaCl, 0.05% Tween, 20% Glycerol, and 1mM DTT (0.25–1.0 mM). The recombinant substrate is added to the above reaction mix and further incubated for 1 h at 37 ◦C, followed by the addition of cellulose slurry, pre-equilibrium with reaction buffer). The reaction tube was rocked for 10 min at 4^{o}C followed by centrifuged for 2min at 12,000 rpm. The supernatant containing released EGFP was transferred to a black 96-well plated and fluorescence was measured using excitation filter 485nm and emission filter 538nm in a fluorometer (Modulus TM Micro plate Multimode reader). The difference between the

fluorescence obtained in the presence and absence of the compounds were taken as the measure of the inhibitory activity. As a results represent the average of three independent experiments.

4.2. HCV NS5B protease activity assay

The inhibitory effect of different solvents crude plant extracts namely*C.gouriana, E.geniculata, G.pentaphylla, A.marmelos, L.aspera, N.zeylanica and P.amarus and* isolated bio constituentssuch as UA, BB, TA, & BS from *Clematis gouriana* and *Naravelia zeylanica*. The NS5B gene of HCV was inserted into Nde1/Not1 restriction sites of pET29a expression vector to obtain recombinant NS5B/pET29a clone. NS5B enzyme was over expressed in Escherichia coli BL21 (DE3) cells and purified using published protocol [102]. The HCV-NS5B activity was measured using radio labeled α-P32 UTP in 30 µl assay buffer which includes 20mM Tris (pH 7.0), 100mM NaCl, 0.5mM DTT, 0.01% Tween-20, 5% glycerol (Merck), and 0.5mMMnCl2. In this assay, the enzymatic reaction mixture comprising of NS5B and polyA-dT were incubated on ice for 5min. Reactions were started by addition of radio labeled UTP mixture (BRIT) and were incubated for 1 h at 30 ◦C. The reactions were terminated by the addition of ice-cold 5% (v/v) trichloro acetic acid (TCA) containing 0.5mM sodium pyrophosphate (Sigma–Aldrich) and were left overnight for precipitation at 4 ◦C. The quenched reaction mixtures were then transferred to GF-C filters, washed with chilled 5% TCA buffer to remove unincorporated UTP followed by washing with water and ethanol before vacuum drying. The amount of radioactive UMP incorporated into RNA products was quantified on a LKB wallac 1209 Rack Beta liquid scintillation counter. To measure the inhibitory effect of isolated compounds on HCV NS5B polymerization, compounds dilutions were made in DMSO and different concentration of inhibitors were added to NS5B enzyme reaction mixture. Negative control consisted of 10% DMSO (Sigma–Aldrich) along with enzyme and activity was measured as mentioned above. Results represent the average of three independent experiments.

4.3 MTT assay to measure the inhibitory effect of isolated compounds

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was used for the screening of extract's toxicity using standard protocol [103]. Briefly, Huh7 cells were plated for 24 h at 37 ◦C on 96-well plates at a density of 5×103 cells per well to 50%

confluency, with 5% CO2 in a humidified atmosphere with Dulbecco's modified Eagle's medium.

After 24 hrs, isolated compounds were added to final concentrations of 5, 10, 15 and 20 µg/µl. The plate was incubated for further 78 h under the same conditions mentioned above. 20µl MTT (Sigma–Aldrich) solution (5 mg/ml in phosphate buffer) was added to each well and incubated at 37 ◦C for 4 h. The MTT solution was carefully decanted off, and formazan was extracted from the cells with 100µl of DMSO in each well. Color was measured with a 96-well ELISA plate reader at 550 nm, with the reference filter set to 620 nm. All MTT assays were repeated three times.

5. Insilco Studies on HCV NS3 and NS5B protease sequence

5.1. Ligand Preparation

Isolated compounds Ursolic acid (UA) from Clematis gouriana Roxb. and Berberine (BB), Taraxerol (TA), -sitosterol (BS) from Naravelia zeylanica and its in silico studies for the binding interact with inhibitors were built using Marvin sketch.The geometries of the investigated compounds are defined by performing an optimized geometry calculation using CHARMm, implemented in DS 3.5. Drug likeliness was evaluated using Lipinski rule of five. The ADME (Absorption, Distribution, Metabolism, and Excretion) studies were carried out using ADME pipeline in DS3.5 [104].

5.2. Homology Modeling

Homology modeling is usually the method of choice when there is a clear relationship of homology between the sequence of a target protein and at least one known structure. This computational technique is based on the assumption that tertiary structures of two proteins will be similar if their sequences are related, and is the most likely approach that gives accurate results [105].

Model building of NS3 protease sequences isolated from Indian patient (Q913D4) was carried out using the program SWISS—MODELL. SWISS—MODELL is an implementation of an automated approach to comparative modeling by satisfaction of spatial restraints [106]. The modeling procedure begins with an alignment of the sequence to be modeled (Q913D4) with related known three-dimensional structure (templates).

This alignment is usually the input to the program. The output is a three-dimensional model for the target sequence containing all main-chain and side chain non-hydrogen atoms. The optimization was carried out using Discovery studio 3.5 (Acclerys, USA) employing methods of conjugate gradients and molecular dynamics with simulated annealing. Several slightly different models can be calculated by varying with initial structure. A total of 1000 models were generated for each enzyme and the final models were selected based on steriochemical quality. The optimization process was performed on Intel cluster with 16 nodes (HP workstation Z200).

The overall steriochemical quality of the final models for each NS3 protease from hepatitis C virus was assessed by the program PROCHECK [107].

Model building of Nonstructural 5 B (NS5B) RNA dependent RNA polymerase as potent target for Hepatitis C Virus. The crystal structure of RNA Polymerase Genotype 2a In Complex with Non- Nucleoside Analogue Inhibitor PDB ID: 1YVZ was downloaded from Protein Data Bank. Water molecules and other heteroatoms including ligand were removed and the protein was subjected to energy minimization using CHARMM force field. The active site was found by reference ligand within a 4^{o} angstromregion.

Tested compounds were screened through Lipinski rules of five and ADMET properties and then 3D conformation of screened library had been done. The best conformations were selected based on the least energy values for molecular docking.

The above all process was done using Discovery Studio 3.5 (DS).For molecular docking studies, a flexible docking approach was done using the Lead IT software in which crystal structure of RNA polymerase was considered as receptor protein [108].

5.3. Mutants Analysis

The mutants have been prepared by replacing the residues with other ones using DS 3.5 and the protein mutant (A156T, A156V and R155K) [109] structures have also been optimized classically using CHARMm force field implemented in DS 3.5 with conjugate gradient energy minimize and convergence energy (0.001 kcal/mole) and become ready for docking simulations [92]. Active Site Prediction and Docking Active site residues (Q41, F43, H57, G58, D81, R109, K136, G137, S138, S139, G140, G141, F154, R155, A156, A157, D168, M485, V524, Q526 and H528) [110] have been selected for both the wild-type enzyme and mutant structures in order to study their interaction with Berberine, Ursolic acid, -sitosterol and Taraxerol.

All the docking calculations of compounds were performed using the LeadIT—FlexX docking [111]. The docking results for receptor-ligand complex comprised of intermolecular interaction energies namely hydrogen bonding, hydrophobic and electrostatic bonding was evaluated. The least binding energy of receptor-ligand complex was selected as best compound.

6. Identification of Viral Proteome by Two-Dimensional Gel Electrophoresis

The Huh7 cells were propagated as monolayer. The Replicon 2a RNA was Transfection to Huh7 cells and kept it for stabilization and the cells were scraped using a cell scraper at 48 h postinfection (p.i.), and centrifuged at 10,000×g for 5 min. After washing three times with ice-cold phosphate-buffered saline (PBS), the collected cells were lysed with lysis buffer contains 7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 65 mM DTT, 0.2% pharmalyte 4/7 and 1 mM PMSF containing 1% nuclease mix in the final concentration and were vertically vibrated until the cells were completely lysed. The supernatant was collected after centrifuging at 12,000×g at 4°C for 60 min. Samples were treated with a 2D clean-up kit (Biorad) according to the instructions of the manufacturers. Paralleled mock-infected Huh7 cells were used as control.

6.1.2-D Gel Electrophoresis

The 50μg of protein samples were taken to access biological variation, three experimental duplicates were carried out using the samples. The internal standards (equal amounts of both samples). Protein extracts from mock-infected cells, used as a reference state, IEF was performed using an IEF cell system (Bio-rad) and commercially available 7cm long IPG strips (Linear, pH 3–10, Biorad). The settings and conditions for active rehydration of the IPG strips were used as previously described [112]. The isoelectric-focused proteins in strips were incubated for 15 min in the equilibration buffer contains 6 M urea, 30% glycerol, 2% SDS, and 0.375 M Tris, pH 8.8 containing 1% DTT, followed by additional equilibration for 15 min in the equilibration buffer containing 2.5% iodoacetamide. The second dimension separation was performed using electrophoresis system (Bio-rad). Gels were poured between low fluorescent glasses plates, of which one plate was bind-silane treated. Two parallel gels were performed in running buffer (25 mM Tris, 192 mM glycine and 1% SDS). The equilibrated IPG strips were further resolved with 12% SDS-PAGE gels until the dye front reached the bottom of the gels.

6.2. Image acquisition and analysis of 2D gels.

Protein images were scanned directly between the glass plates using Imager reader. All gels were scanned. Determination of protein abundance and statistical analysis was performed using PDquest software package. Matching between gels was performed using the in-gel standard from each image pair. Pair of gels was tested for the protein spots showing significance and at least a 2-fold difference in abundance in the ratio of the mean of the normalized spot volume of the replicon infected samples versus mock-infected samples were considered as up-regulated or down-regulated.

6.3. Protein identification by MALDI-TOF mass spectrometry (MS) and a database search.

The protein spots of interest were manually excised from the gels and plated into eppendorfs tubes. Excised spots were firstly destained twice with 60 μl of 50 mM NH_4HCO_3 and 50% acetonitrile (ACN) and then dried twice with 60 μl of ACN. Afterwards, the dried pieces of gels were incubated in ice-cold digestion solution containing trypsin 12.5 ng/μl and 20 mM NH_4HCO_3 for 20 min and then transferred into a 37°C incubator for digestion overnight. Finally, peptides in the supernatant were collected after extraction twice with 60 μl extract solution contains 0.1% TFA in 50% ACN. The peptide solution was dried, and 0.8 μl matrix solution contains 5 mg/ml α-cyano-4-hydroxy-cinnamic acid diluted in 0.1% TFA, 50% ACN was pipetted to dissolve it. Then the mixture was spotted on a MALDI target plate (Applied Biosystems). MS analysis of peptides was performed on an AB SCIEX 5800 MALDI-TOF. The UV laser was operated at a 400 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV and mass resolution was maximized at 1,600 Da. Myoglobin digested with trypsin was used to calibrate the mass instrument with an internal calibration mode. Corresponding to a statistically significant ($p<0.05$) confident identification. Besides protein score, at least one ion score with $p<0.05$ was recommended to increase the reliability of identifications.

Statistical analysis. The statistical significance was determined at the 5% level using statistical software SPSS.

IV. RESULTS

1. Phytochemical investigation form plant extracts

Qualitative phytochemical analysis

The petroleum ether, chloroform and Methanol extracts of medicinal plants extract such as were subjected to preliminary phytochemical studies. The various groups of phytochemical constituents found to be present in these extracts are shown in Table.2.

The result of preliminary phytochemical analysis revealed that proteins were totally absent in the extract of *Clematis gouriana, Euphorbia geniculata, Gynandropis pentaphylla, Aeglemarmelos, Leucasaspera, Naravelia zeylanica and Phyllanthusamarus.*

In *Clematis gouriana* both sterols and tannins are present in pet ether, beside the only saponins are present in chloroform and methanol extracts shows carbohydrates, alkaloids, glycosides, saponins, Triterpenoids.

Euphorbia geniculata shows presence of steroids, carbohydrates, alkaloid, glycosides, saponins, triperpenoids are present in both petroleum ether and methanolic extract. Tannins, quinones and flavonoids are present in petroleum ether,chloroform and methanolic extract of *Euphorbia geniculata.*

Gynandropis pentaphylla shows carbohydrates only present in chloroform, flavonoids, sterols are present in petroleum, chloroform and Methanolic extracts. Tannins and terpenoids present both petroleum and chloroform while alkaloids, glycosides and saponins are present in both chloroform and methanolic extract of *Gynandropis pentaphylla.*

Aeglemarmelos shows carbohydrates present both chloroform and methanolic extracts. Only sterols are present in petroleum, chloroform and Methanolic extracts. Tannins and quinones are present both petroleum and methanolic extracts alkaloids present in chloroform and methanolic extracts, Saponins and triperpenoids are present in methanolic extract of *Aeglemarmelos.*

Leucasaspera shows carbohydrates present both chloroform and methanolic extracts. Only sterols and flavonoids are present in petroleum and Methanolic extracts. The saponins and alkaloids are present inchloroform and methanolic extracts. Tannins and glycosides are present in methanolic extracts.

Sterols were found to be present in petroleum ether extract of leaves of *N. zeylanica.* The Triterpenoids were present in methanol extracts of *N. zeylanica*whereas, it is totally absent in petroleum ether and chloroform extract. Except petroleum ether extract the remaining extracts of showed negative test for tannins. Quinones were all together absent in all the extract of present in *N. zeylanica* except chloroform extract. Saponins, Carbohydrates and Proteins were found to be present in the chloroform and methanol extracts of both the plants. In case of *N. zeylanica* the yield of chloroform extract was very less and more pigmented as compared with the petroleum ether and methanol extracts.

Phyllanthusamarus shows carbohydrates present both petroleum and methanolic extracts. Only alkaloids and sterols and flavonoids are present in petroleum, while the sterols and Tannins are present in petroleum and methanolic extracts. The quinones, saponins and triterpenoids Tannins and glycosides are present in chloroform and methanolic extracts.

Table.2.The phytochemical investigation of different solvent extraction of medicinal plants.

SL. NO	Plants name	Extracts	Tests									
			Carbohydrates	**Proteins**	**Alkaloids**	**Flavonoids**	**Sterols**	**Tannins**	**Quinones**	**Glycosides**	**Saponins**	**Triterpenoids**
1	*Clematis gouriana*	Petroleum ether	**-**	-	-	-	+	+	-	-	-	-
		Chloroform	**-**	-	-	-	-	-	-	-	+	-
		Methanol	**+**	-	+	-	-	-	-	+	+	+
2	*Euphorbia geniculata*	Petroleum ether	**+**	-	+	+	+	+	+	-	-	+
		Chloroform	**-**	-	-	+	-	+	+	+	+	-
		Methanol	**+**	-	+	+	+	+	+	+	+	+
3	*Gynandropis pentaphylla*	Petroleum ether	**-**	-	-	+	+	+	-	-	-	+
		Chloroform	**+**	-	+	+	+	+	-	+	+	+
		Methanol	**-**	-	+	+	+	-	-	+	+	-
4	*Aegle marmelos*	Petroleum ether	**+**	-	-	-	+	+	+	-	-	-
		Chloroform	**-**	-	+	-	+	-	-	-	+	-
		Methanol	**+**	-	+	+	+	+	+	+	+	+
5	*Lecuas aspera*	Petroleum ether	**+**	-	-	+	+	+	-	-	-	-
		Chloroform	**-**	-	+	-	-	-	-	-	+	-
		Methanol	**+**	-	+	+	+	-	+	+	+	-
6	*Naravelia zeylanica*	Petroleum ether	-	-	-	-	+	+	+	+	-	-
		Chloroform	+	-	+	-	-	-	+	+	+	+
		Methanol	+	-	+	-	-	-	-	+	+	-
7	*Phyllanthus amarus*	Petroleum ether	**-**	-	-	-	+	+	-	-	-	-
		Chloroform	**+**	-	-	-	-	-	+	+	+	+
		Methanol	**+**	-	+	+	+	+	.	+	+	+

2. Isolation and characterization of active bio-constituentsignificant effect on medicinal plants against HCV such as *clematis gouriana* and *Naravelia zeylanica*.

Isolation of active constituents from the methanol extract of the leaves of *clematis gouriana*

The yield of the methanol extracts of *in vivo* leaves and *in vitro* derived leaf of *C. gouriana* were 64 g per 800 g powder and 400 mg per 1.37 g of calli respectively. Both the extracts were subjected to column chromatography using the solvents methanol: chloroform in the ratio of 8.5:1.5, which yielded a brown colored crystalline compound which were labeled as CGM and CGM^1. The yield of these compounds was 450 mg per 20 g of crude extract (*in vivo* leaves) and 250 mg per 1.37 g of crude extract respectively. Both the compounds showed the positive results for Salkowaski and Liebermann-Burchard tests and it was found to be a triterpenoid. On TLC the compounds CGM and CGM^1 exhibited the same spot with same R_f value and the melting point of this compound was found to be 289^0 c. The characterization of the compound was done by the following spectral studies.

Spectral Characteristics of Code – CGM

IR(KBr)	3434.8 cm^{-1} (br, OH),
(Fig 2)	2926.0 cm^{-1}, 2368.5 cm^{-1} (C-H str. in CH_3 and CH_2)
	1749.7 cm^{-1} (C=O str. of COOH group),
	1595.2 cm^{-1} (C=C str.),
	1350.1 cm^{-1} (C-H deformation in gem dimethyl),
	1059.3 cm^{-1} (C-O str. of secondary alcohol)
^{1}H NMR ($CDCl_3$)	δ 0.88 to δ 0.9 (m, methyl protons),
(Fig 3)	δ 1.29 to δ 1.93 (m, 25H for CH_2 and CH protons),
	δ-1.3, 2H (cyclohexane),
	δ-2.0, 1H (O-H of alcohol),
	δ-4.52 (olefinic protons)
	δ-11.0 (1H (O-H of COOH).

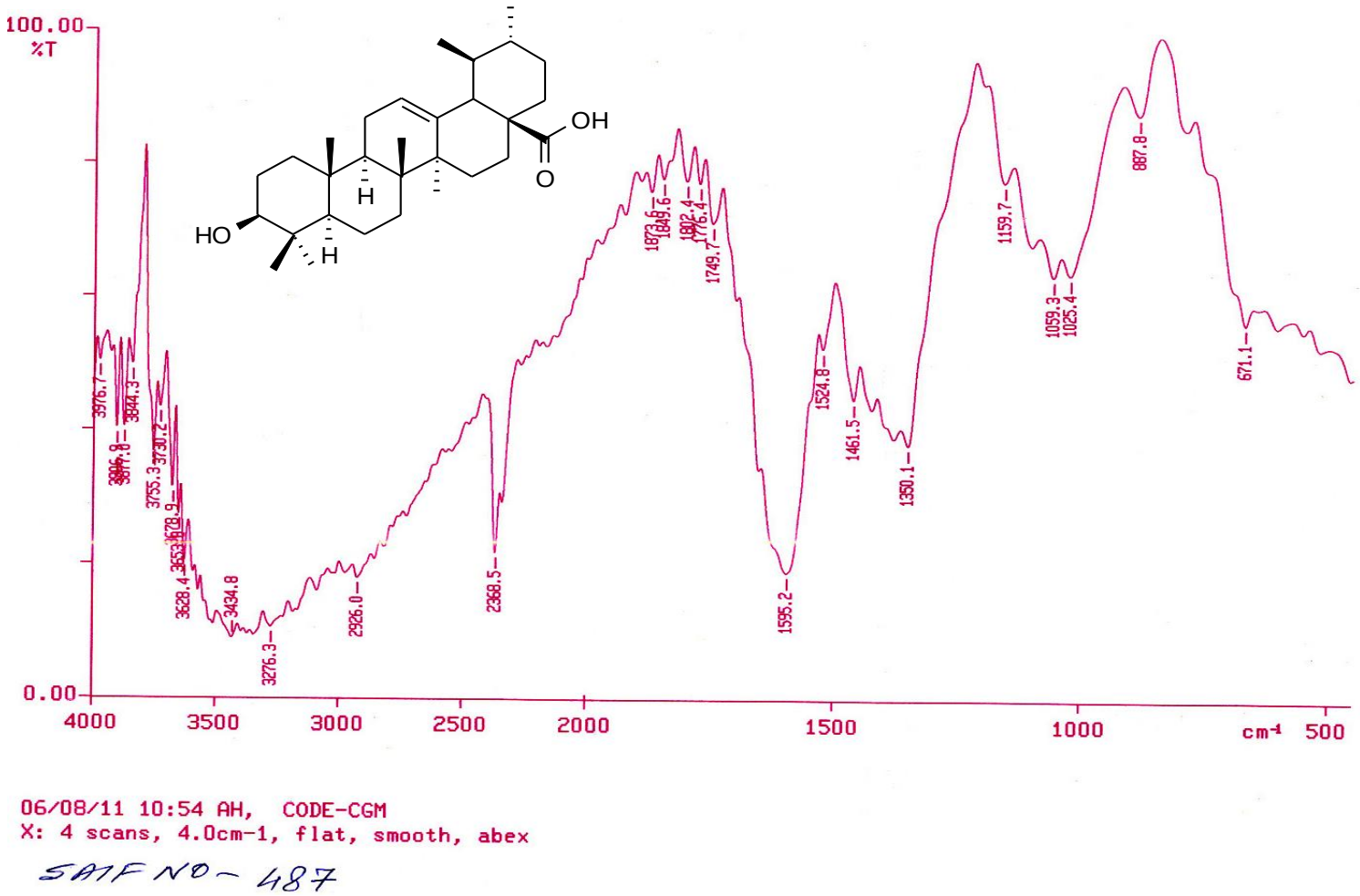


Fig 2: IR Spectra of Code – CGM (Ursolic acid)

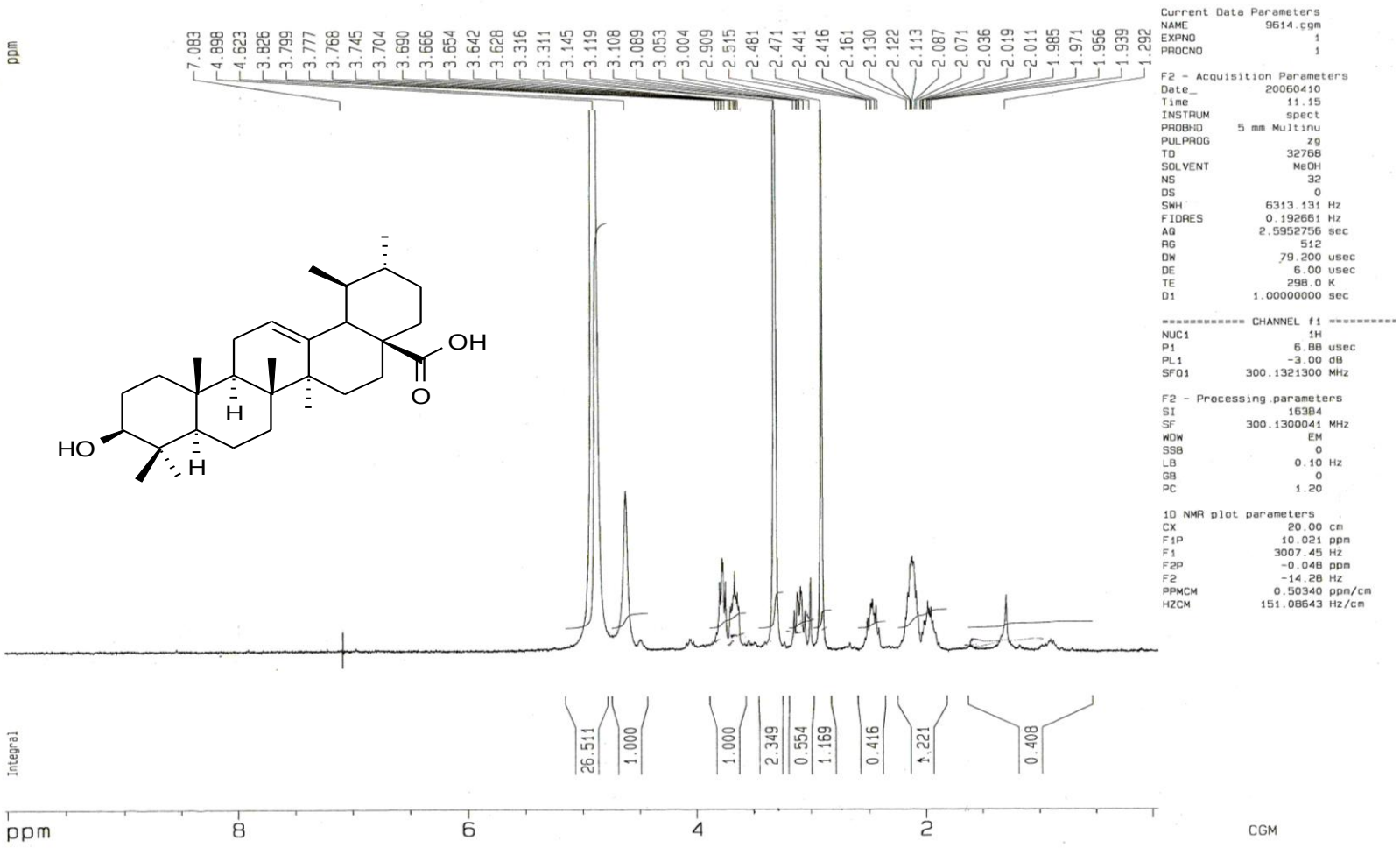


Fig 3: ^{1}H NMR Spectra of Code – CGM (Ursolic acid)

Mass spectra (ES^+)

Molecular formula : $C_{30}H_{48}O_3$

Molecular weight : 457

Mass spectrum shows molecular ion peak at m/z 475(4%). The other peaks appeared at 424 (14.58%), 423 (56.25%), 407 (46.87%), 385 (7.29%), 331 (6.25%), 317 (9.15%), 219 (10.13%), 182 (8.74%), 181 (43.75%), 168 (18.75%), 149 (20.62%), 130 (54.16%), 100 (22.91%), 91 (55.20%), 84 (100%) and 41 (41.6%) (Fig.5).

The IR spectrum of the compound Code – CGM showed absorptions bands at 3434.8 cm^{-1} and 2926.0, 2368.5 cm^{-1} for hydroxyl group and terminal methylene groups. The ^1H NMR spectrum revealed the presence of terminal methyl groups at δ 0.88 to δ 0.9, a multiplet at δ 4.52 due to olefinic protons. The ES^+ mass spectrum showed the molecular ion peak at m/z 457 corresponding to the molecular formula $C_{30}H_{48}O_3$. From the above spectral data, compound Code -CGM was identified as Ursolic acid (Fig.4)

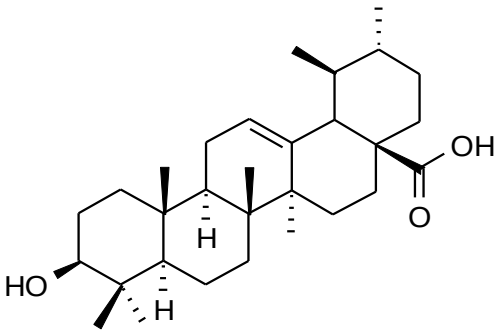


Fig 4: Ursolic acid

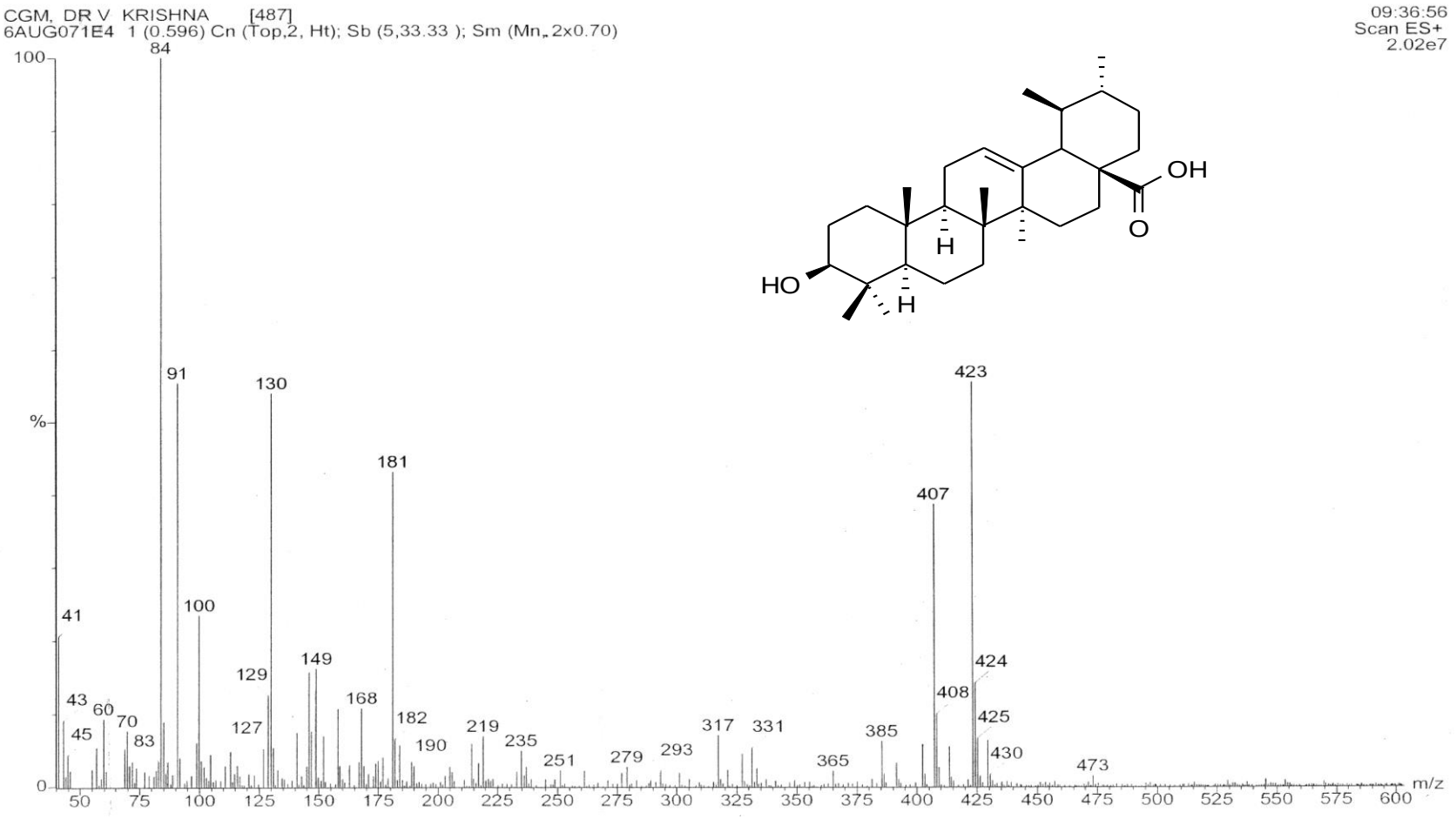


Fig 5: Mass spectrum of code - CGM (Ursolic acid)

Isolation of the constituents from the leaves of*Naraveliazeylanica*

In *N. zeylanica* a triterpenoid, sterol and an alkaloid compound were isolated. The triterpenoid (NZP-1) and sterol (NZP- 2) compounds were isolated from the petroleum ether extract and an alkaloid compound (NZM) was isolated from the methanol extract of the leaves of *Naravelia zeylanica* from the leaf only the alkaloid compound NZM [1] was isolated with more yield 650 mg. The yield of the other two compounds were very less, therefore they will subjected for isolation and characterization.

The compounds NZP-1 and NZP- 2 eluted from petroleum ether extract gave positive color tests for vanillin-sulfuric acid, Salkowaski test and Lieberman- Burchard reactions indicated the presence of triterpenoids and sterols. IR, ^{1}HNMR and MASS spectroscopic studies confirmed the structures of the compounds. The structure and molecular formula of compound NZP- 1, NZP- 2 and NZM were established by the following spectroscopic data.

Spectral Characteristics of Code - NZP- 1

IR (KBr)	3429.7 cm^{-1} (br, OH),
(**Fig 6)**	2923.5 cm^{-1}, 2853.7 cm^{-1},
	2364.7 cm^{-1} (C-H str. In CH_3 and CH_2),
	1629.6 cm^{-1} (C=C str.),
	1446.6 cm^{-1}, 1359.5 cm^{-1} (C-H deformation in CH_2/ CH_3)
^{1}H NMR ($CDCl_3$)	δ 0.806 to δ 1.1 (m, 24 H, 8 X CH_3)
(**Fig 7)**	δ 1.303 to δ 1.997 (m, 22H for 11 X CH_2 protons)
	δ 2.320 to δ 2.808 (m, 5H for CH proton)
	δ 1.316 (m, 1H) and 1.430 (m, 1H), CH_2 protons.
	δ 3.6 (m, 1H, OH).

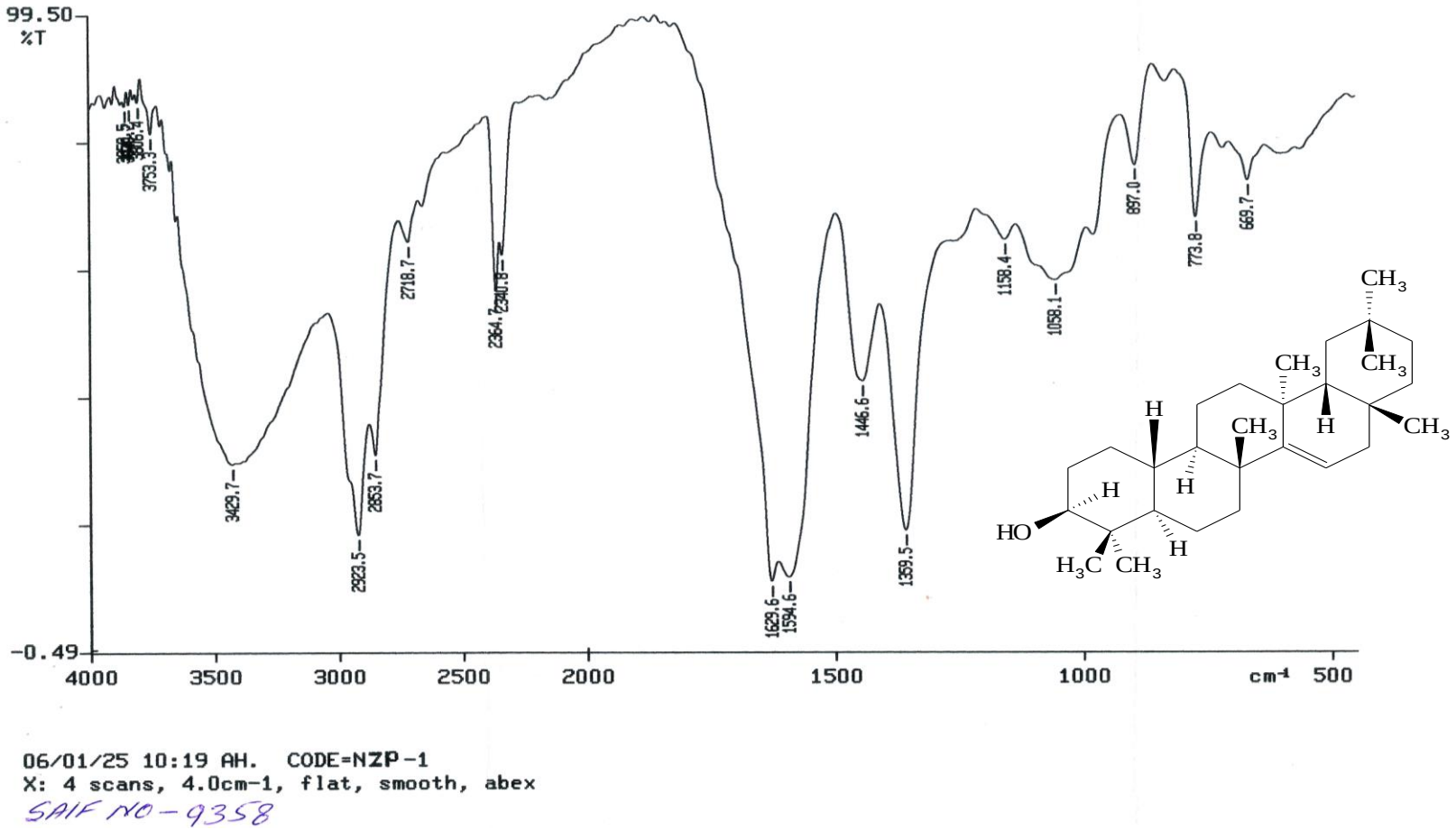


Fig 6: IR Spectra of Code – NZP-1 (Taraxerol)

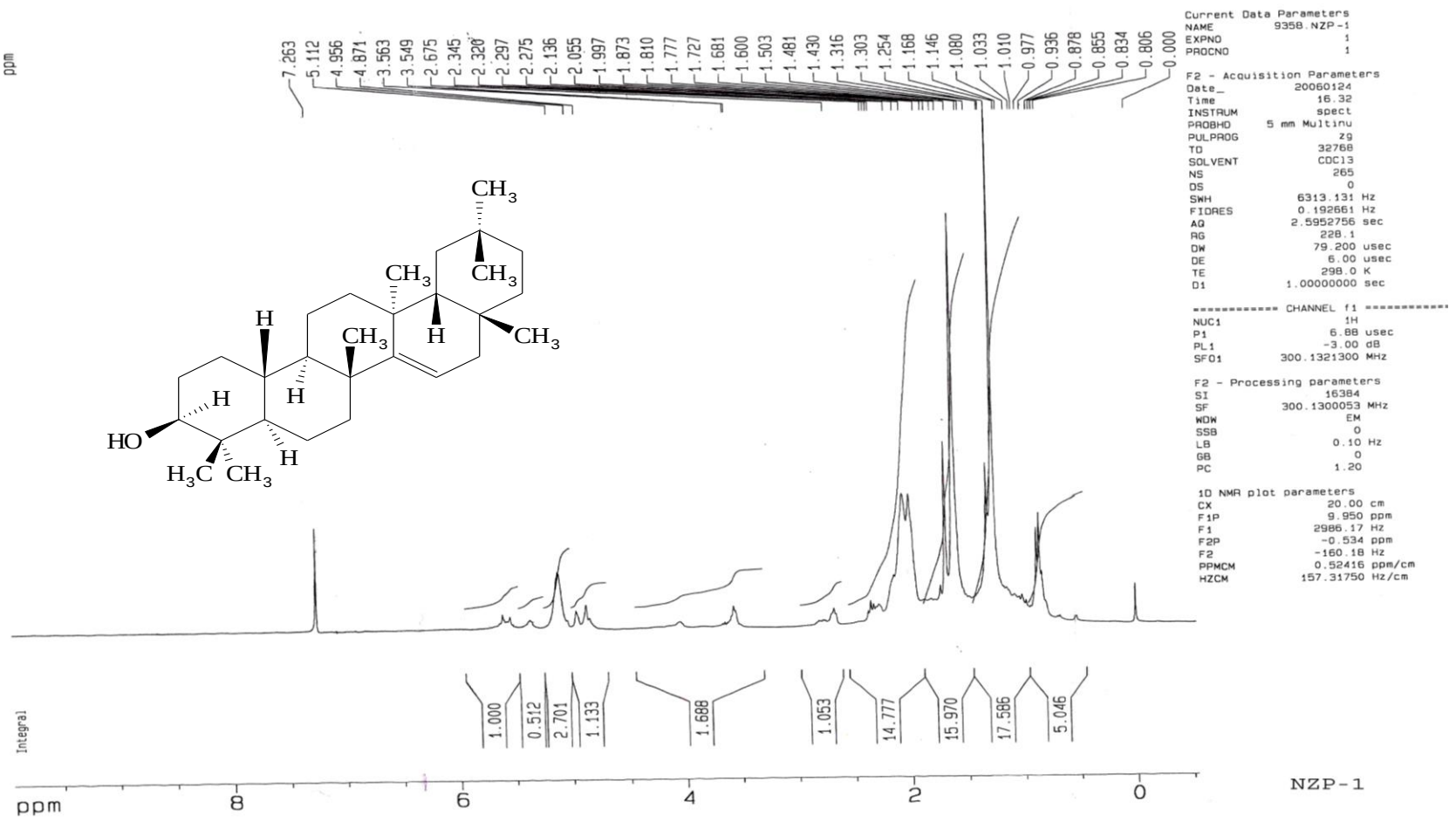


Fig 7: ^{1}H NMR Spectra of Code – NZP-1 (Taraxerol)

Mass spectra (FAB^+-MS)

Molecular formula : $C_{30}H_{50}O$

Molecular weight : 426

: 427 (11%) $[M]^+$, 411 (13%), 405 (15%), 395 (11%), 378 (13%), 327 (15%), 301 (16%), 287 (7%), 284(8%), 268 (11%), 257 (13%), 241 (13%), 213 (21%), 203 (29%), 185 (25%), 135 (96%), 121 (92%), 95 (100%) (Fig 9).

The IR spectrum showed a broad band due to OH at 3429.7 cm^{-1} and 1629.6 cm^{-1} due to C=C stretching. The ^1H NMR spectrum of Code-NZP- 1 showed absorption at δ 0.806 to δ 1.10 due to the presence of methyl groups. The presence of hydroxyl group was observed as a doublet at δ 3.6. The FAB^+-MS indicated a molecular ion peak at *m/z* 427 $[M^+]$ corresponding to the molecular formula $C_{30}H_{50}O$.

Based on the evidential data from IR, ^{1}HNMR and MASS spectra of compound Code-NZP- 1 was identified as taraxerol(Fig 8).

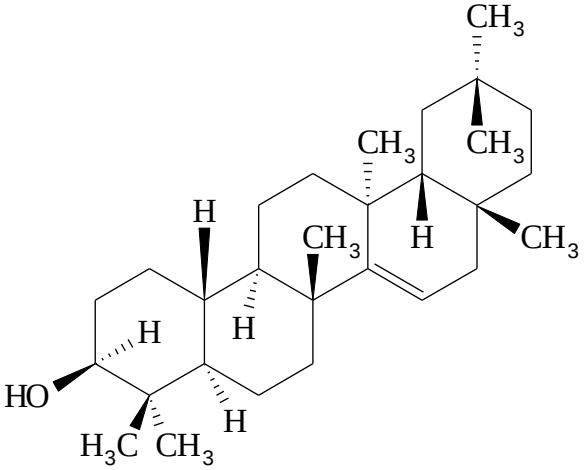


Fig 8: Taraxerol

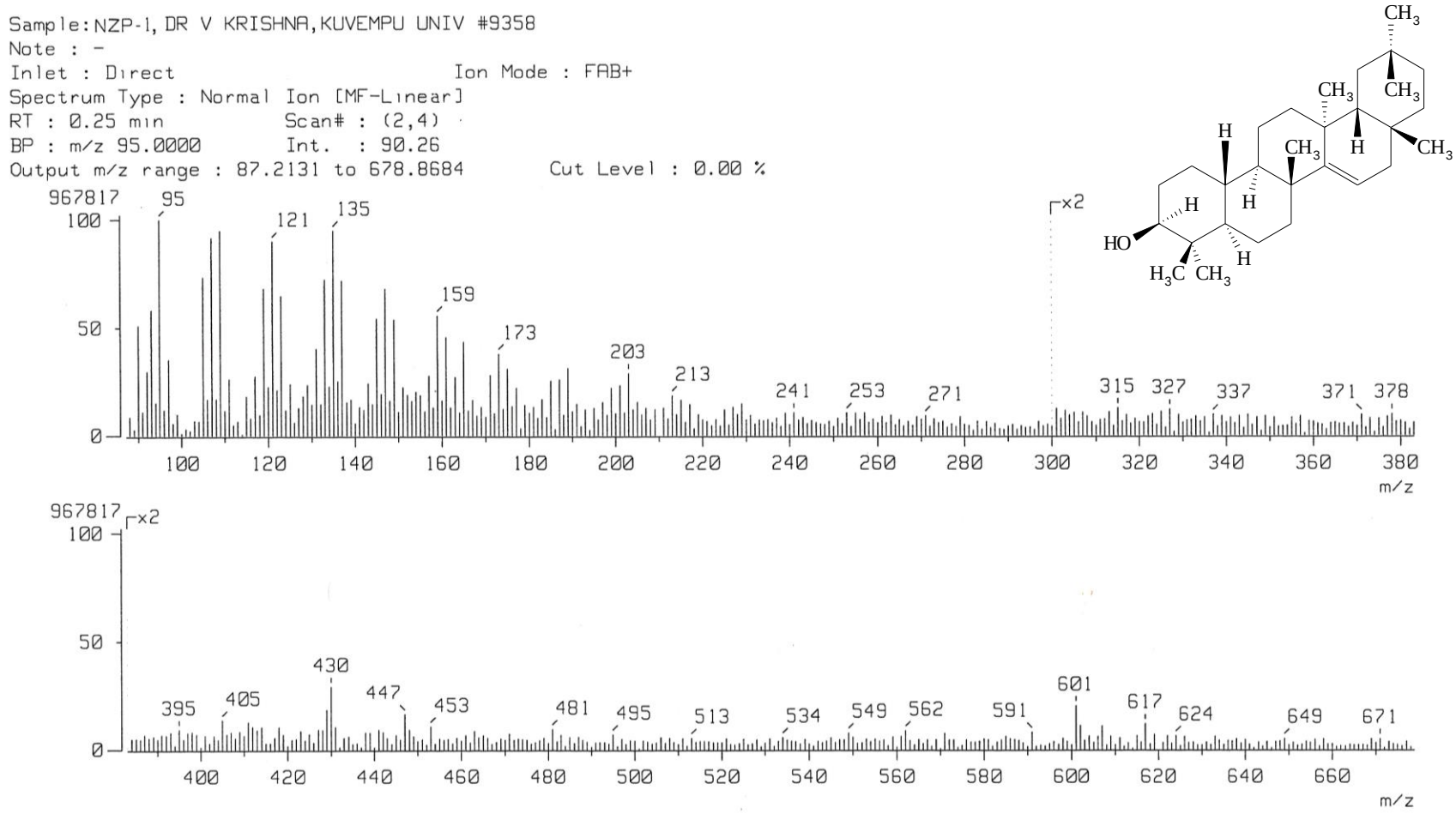


Fig 9: Mass Spectra of Code – NZP-1 (Taraxerol)

Spectral characteristics of Code- NZP- 2

IR (KBr)	3409.9 cm^{-1} (br, OH),
(**Fig 10)**	2924.2 cm^{-1} (C-H str. in CH_3),
	1629.2 cm^{-1} (C=C str.),
	1461.3 cm^{-1} (C-H deformation in CH_3),
	1360.0 cm^{-1} (C-H deformation in gem dimethyl),
	1064.4 cm^{-1} (C-O str. of secondary alcohol)
^{1}H NMR ($CDCl_3$)	δ 0.834 to 0.979 (18 H,6 X CH_3)
(**Fig 11)**	δ1.022 to 1.255 (22 H,11XCH_2)
	δ 1.448 to 2.286 (m, 8H, methine protons)
	δ 3.541 (m, 1H, OH), δ5.345 (m, 1H, Vinylic proton).

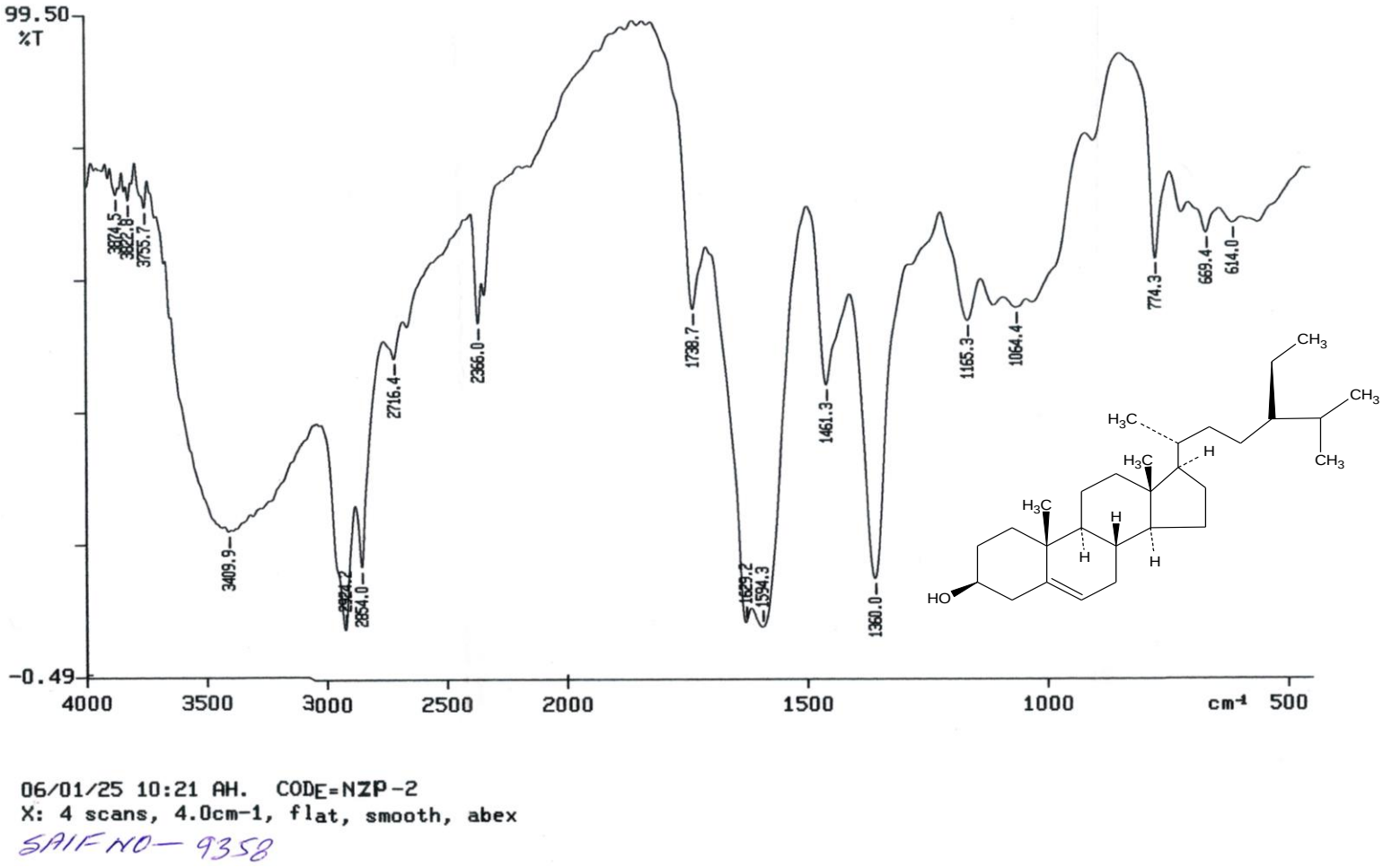


Fig 10: IR Spectra of Code – NZP-2 (β - sitosterol)

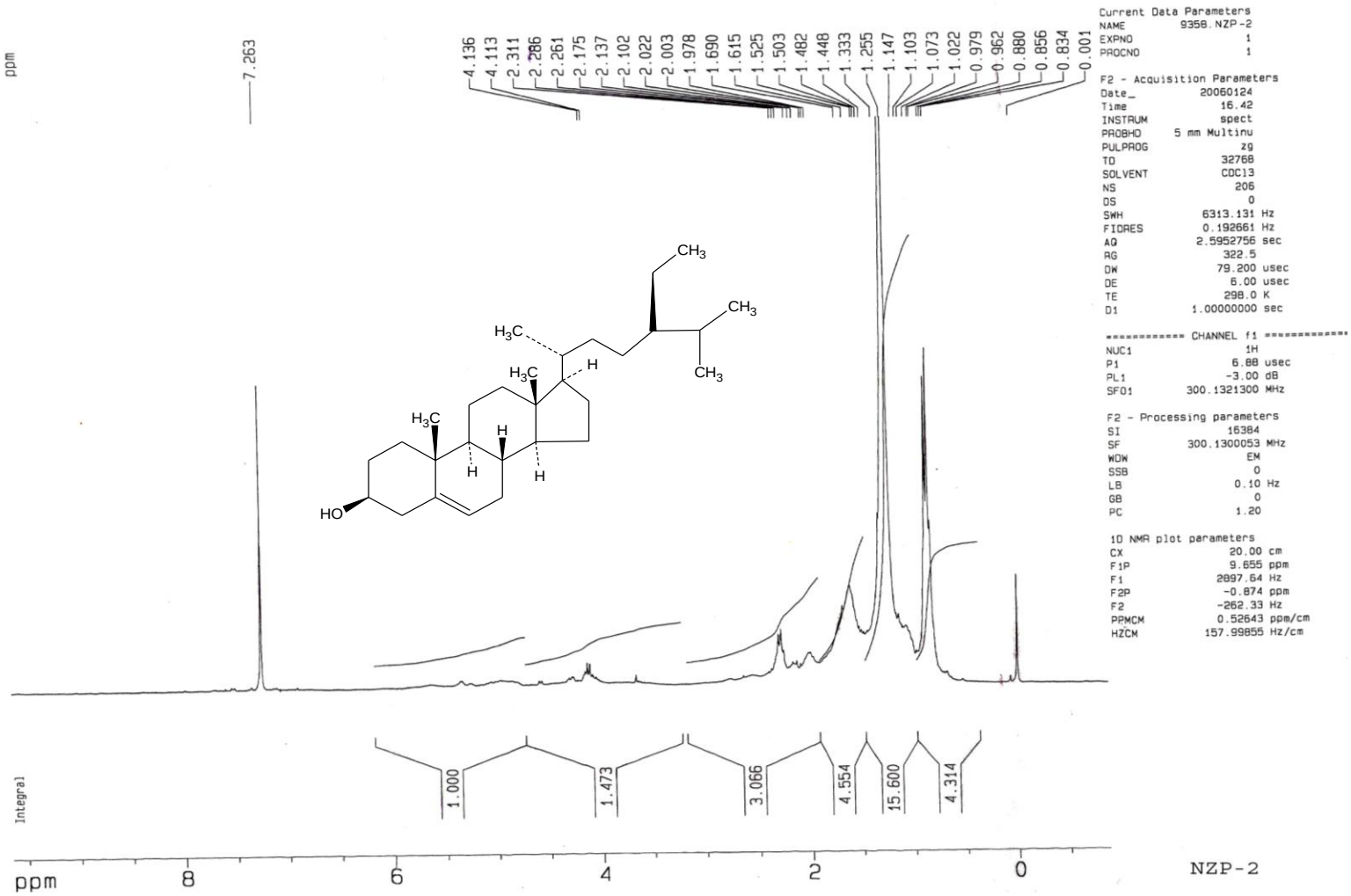


Fig 11: ^{1}H NMR Spectra of Code – NZP-2 (β - sitosterol)

Mass spectra (FAB$^+$-MS)

Molecular formula : $C_{29}H_{50}O$

Molecular weight : 414

: 414 (M^+, $C_{29}H_{50}O$, 9%), 397 (23%), 341 (17%), 327(11%), 313(21%), 295 (11%), 285(19%), 257 (M^+ - Side chain + H_2O, 21%), 239 (17%), 203 (15%), 189(21%), 165 (27%), 147 (46%), 133 (42%), 121 (52%), 105 (57%), 95 (100%) (Fig 13)

From the results of IR, ^{1}H NMR, and mass spectral data, the compound Code-NZP- 2 was identified asβ-sitosterol. The structure of the compound is shown in the Fig 12.

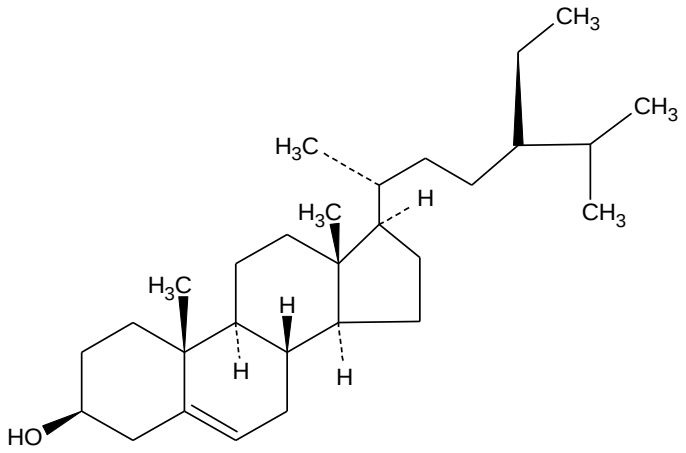


Fig 12: β - sitosterol

The compound code NZM eluted from methanol extract gave positive tests alkaloids. IR, ^{1}H NMR, and MASS spectroscopic studies confirmed the structure of the compound. The structural elucidation and molecular formula of compound Code- NZM was established by the following spectroscopic data.

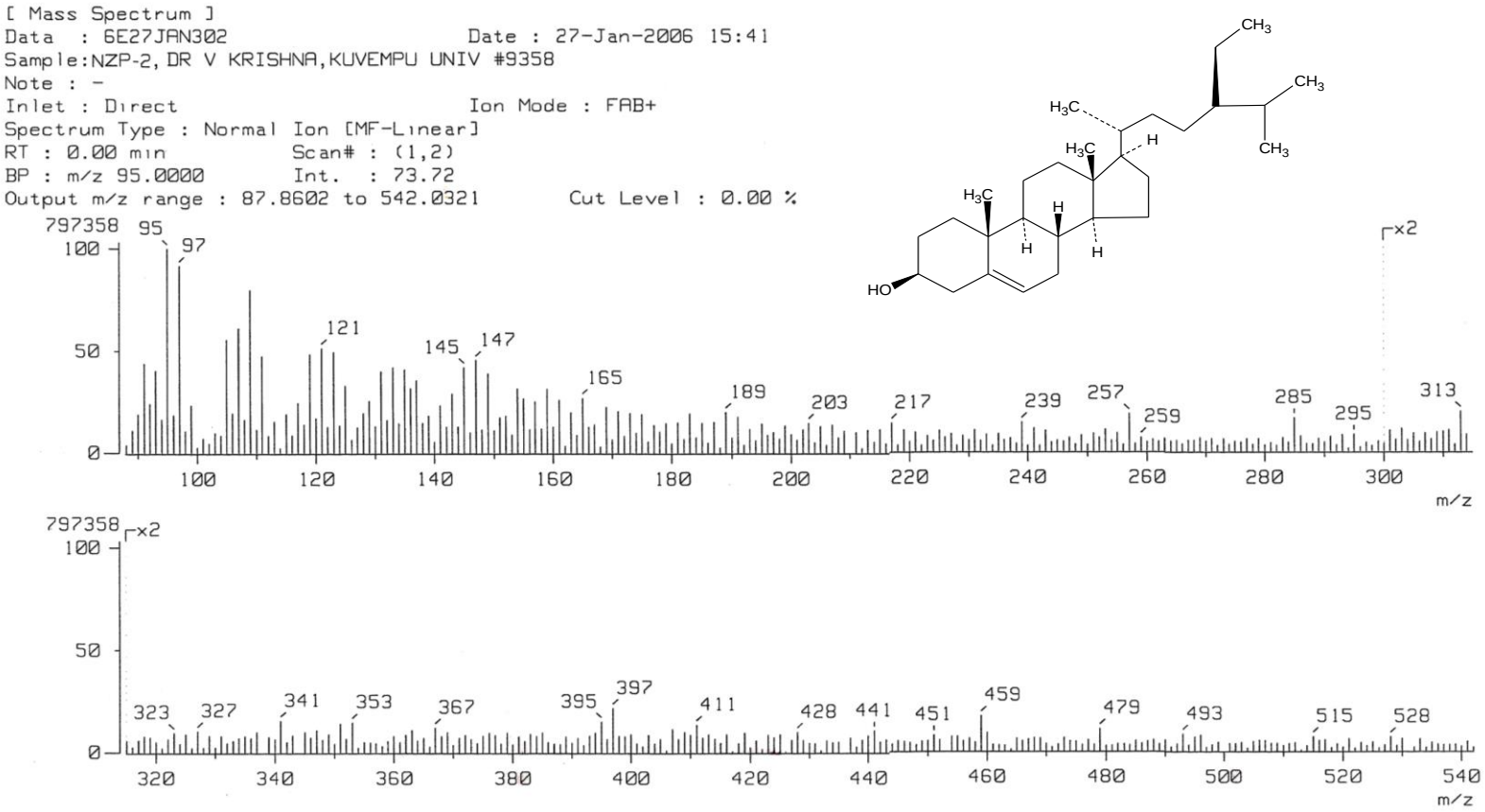


Fig 13 – Mass Spectra of Code – NZP-2 (β - sitosterol)

Spectral Characteristics of Code- NZM

IR (KBr)	3418.5 cm^{-1} (br, NH),
(**Fig 14)**	2820.8 cm^{-1}, 2372.1 cm^{-1} (C-H str. In CH_3 and CH_2),
	1597.6 cm^{-1} (C=C str.),
	1060.4 cm^{-1} (C-O str.).
^{1}H NMR ($CDCl_3$)	δ 0.837 to δ 0.992 (m, 6 H, 2 X CH_3)
(**Fig 15)**	δ 1.164 to δ 1.981 (m, 8H for 4 X CH_2 protons)
	δ 2.138 to δ 2.499 (m, 5H for CH proton)
	δ 6.9 to δ 8.7 (m, Ar-H).

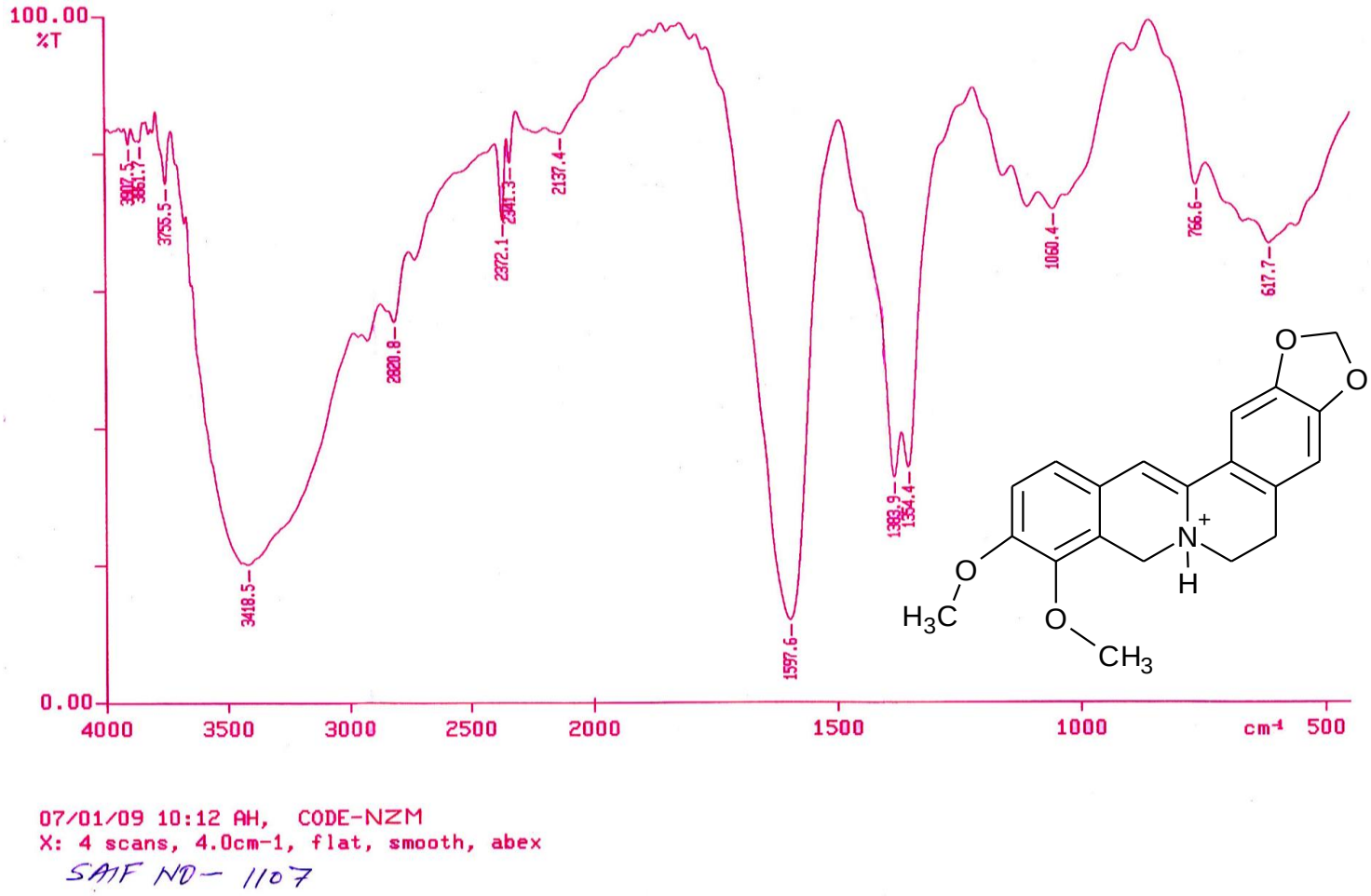


Fig 14 – IR Spectra of Code-NZM (Berberine)

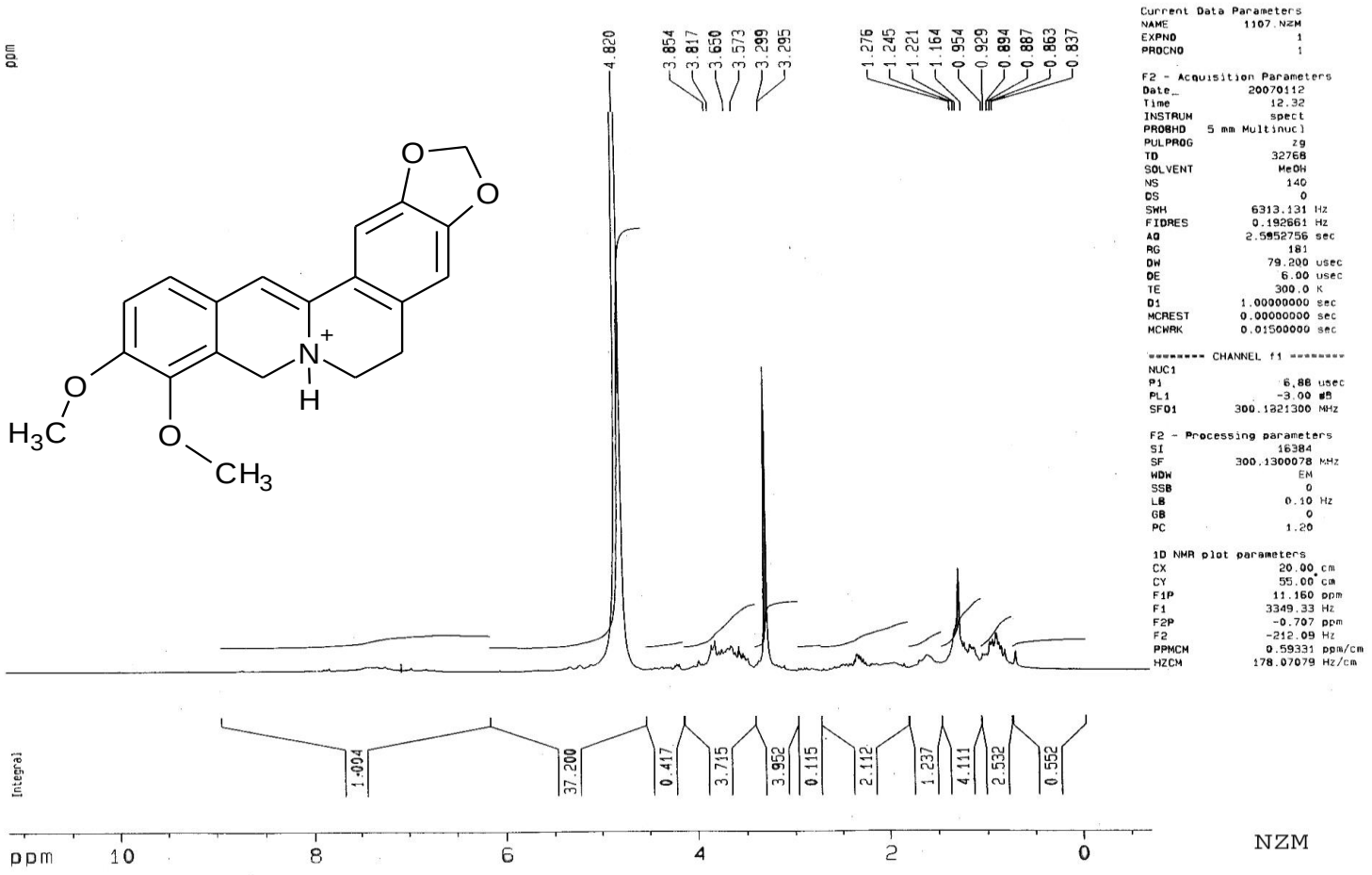


Fig 15 – ^{1}H NMR Spectra of Code-NZM (Berberine)

Mass spectra (FAB^+-MS)

Molecular formula : $C_{20}H_{20}NO_4$

Molecular weight : 338.37

: 339 (9%) $[M]^+$, 338 (23%), 326 (44%), 281 (57%), 267 (36%), 221 (48%), 207 (81%), 193 (42%), 147(100%), 133 (42%), 90 (67%) (Fig 17)

The IR spectrum showed a band due to NH at the vibration frequency 3429.7 cm^{-1} and 1597.6 cm^{-1} due to C=C stretching. The ^1H NMR spectrum of Code-NZM showed absorption at δ 0.837 to δ 0.992 due to the presence of methyl groups. The FAB^+-MS indicated a molecular ion peak at *m/z* 339 $[M^+]$ corresponding to the molecular formula $C_{20}H_{20}NO_4$.

Based on the evidential data from IR, ^{1}HNMR and mass spectra of compound Code-NZM was identified as Berberine.

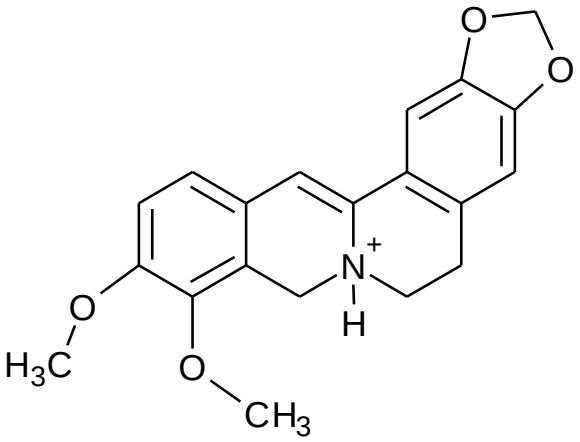


Fig 16: Berberine

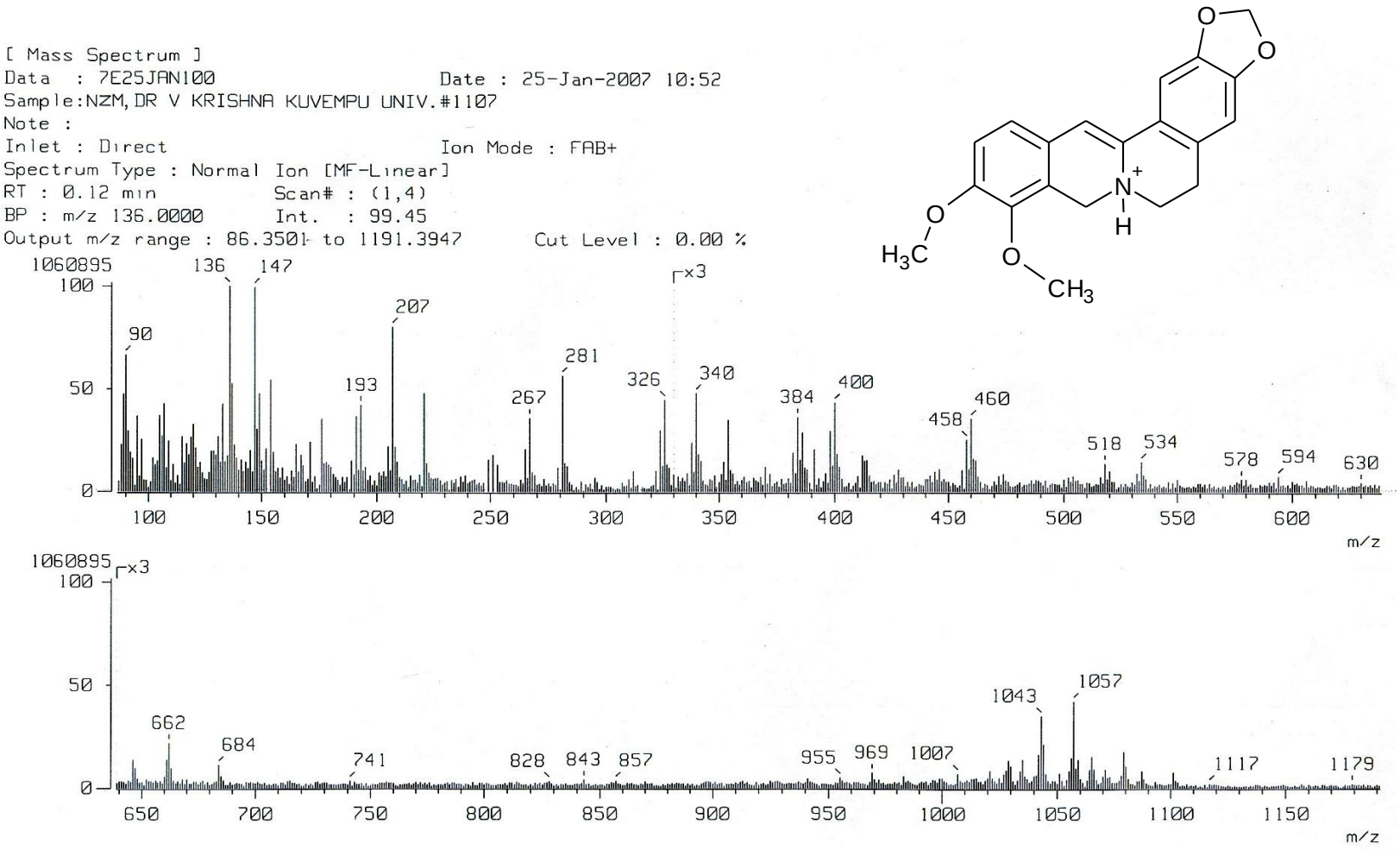


Fig 17 –Mass Spectra of Code-NZM (Berberine)

The yield of Methanolic extracts of leaves of *C. gouriana* was found to be 21%. From the extract we have isolated and characterized by spectral studies and identified as Ursolic acid. The yield of the Methanolic extracts of leaves of *N. zeylanica* was found to be 30% and Petroleum ether extract of leaves of *N. zeylanica* was found to be 25 %. In *N. zeylanica* triterpenoid, sterol and alkaloid compound were isolated. From the petroleum ether extract the triterpenoid Taraxerol and sterol β - sitosterol compounds were isolated from the alkaloid compound Berberine was isolated from the Methanolic extract of the leaves of *Naravelia zeylanica*.

Above bio constituents is isolate from leaves of *C. gouriana* and *Naravelia zeylanica* already reported for further pharmolocological activities [113-115]. Apart from these bio constituents to analysis the effective HCV on NS3 and NS5B Protease*invitro* and*insillico* studies.

3. Evaluation of plant extract and bio constituents of *Clematis gouriana* and *Naravelia zeylanica* against NS3 –NS5B protease.

3.1. Effect of plant extracts on NS3Protease

The Plants listed inTable-1 is based on the information collected from literature, Existing knowledge of plants is being used conventionally to treat viral infection, liver disorders, compositions of liver tonics and the patents information on treatment of hepatitis using herbal plants material used as a medicine.

Firstly, these plants extracts were screened for their inhibitory effect against HCV NS3 protease. The effect of screening inhibitory activity, recombinant HCV-NS3 protease weretaken assubstrates and Plant extracts. Purified recombinant protease efficiently cleaved the substrate at NS3 cleavage site and separated EGFP from the CBD.

The effect of Methanolic extracts from different Plants against HCVNS3 protease activity was examined and the result is depicted in Fig.18. The significant inhibition of HCV NS3 protease activity was observed inthe Methanolic extracts of*C.gouriana which* exhibited moderate inhibitory effect in*E.geniculataP.amarus,G. pentaphylla, A.marmelos, L. aspera, N. zeylanica*methanolic extracts against HCV NS3 protease.

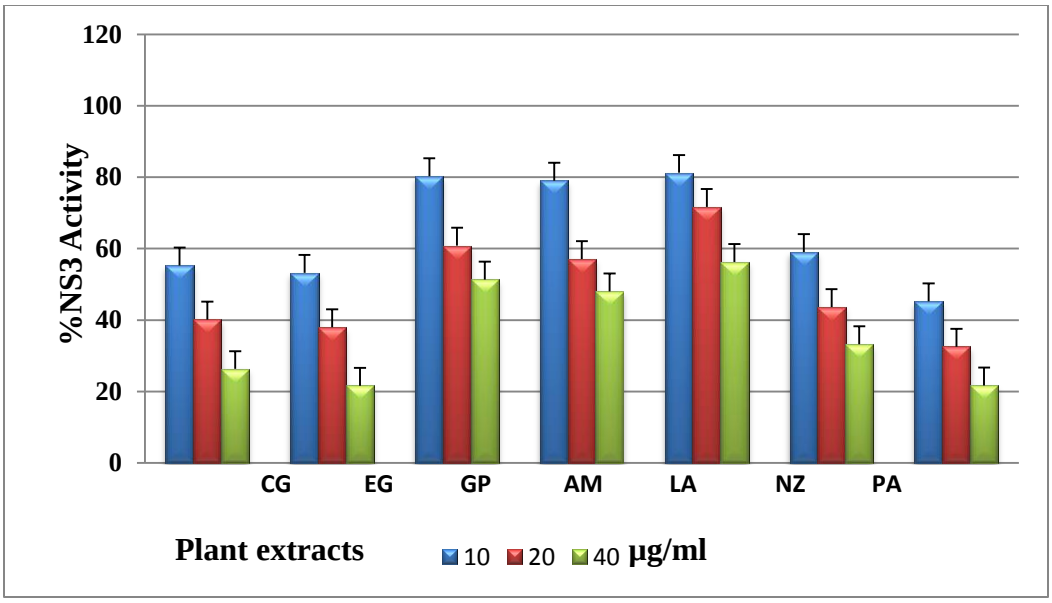


Fig.18 Inhibitory effect of methanolic plant extracts on HCV NS3 protease

The effect of petroleum ether extracts from different Plants against HCV NS3 protease activity was examined and the result is depicted in Fig.19.

The significant inhibition of HCV NS3 protease activity was observed in the petroleum ether extracts of *N. zeylanica which* exhibited moderate inhibitory effect in *E.geniculata P.amarus,G. pentaphylla, A.marmelos, C.gouriana L. aspera,* methanolic extracts against HCV NS3 protease

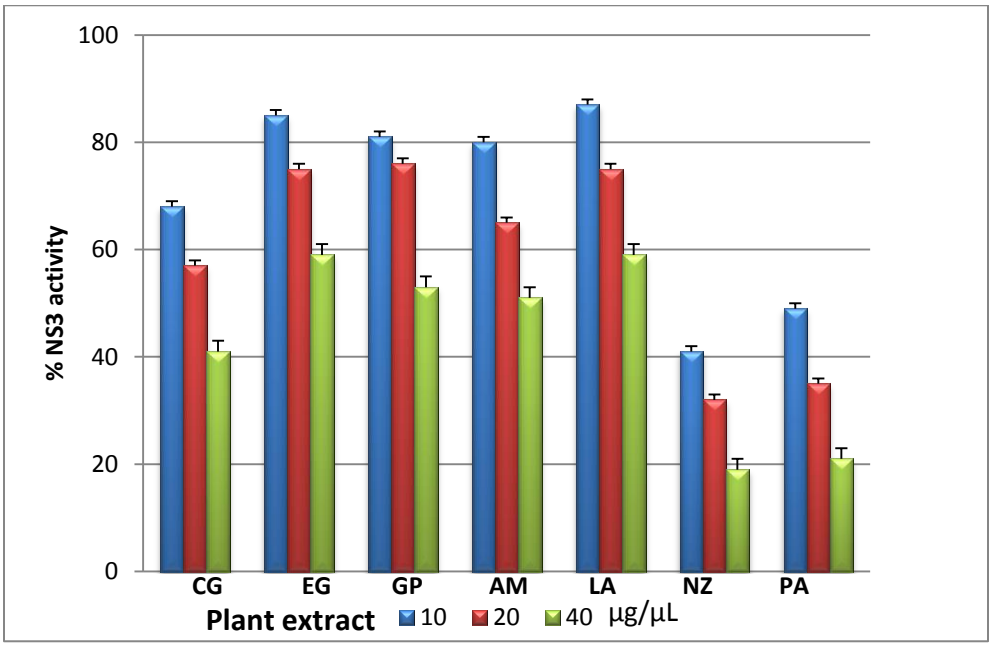


Fig.19 Inhibitory effect of petroleum ether plant extracts on HCV NS3 protease

The effect of chloroform extracts from different Plants against HCV NS3 protease activity was examined and the result is depicted in Fig.20.

The significant inhibition of HCV NS3 protease activity was observed in the chloroform extracts of *N. zeylanica*and*P.amarus which* exhibited moderate inhibitory effect in *E.geniculata,G. pentaphylla, A.marmelos, C.gouriana L. aspera,* methanolic extracts against HCV NS3 protease.

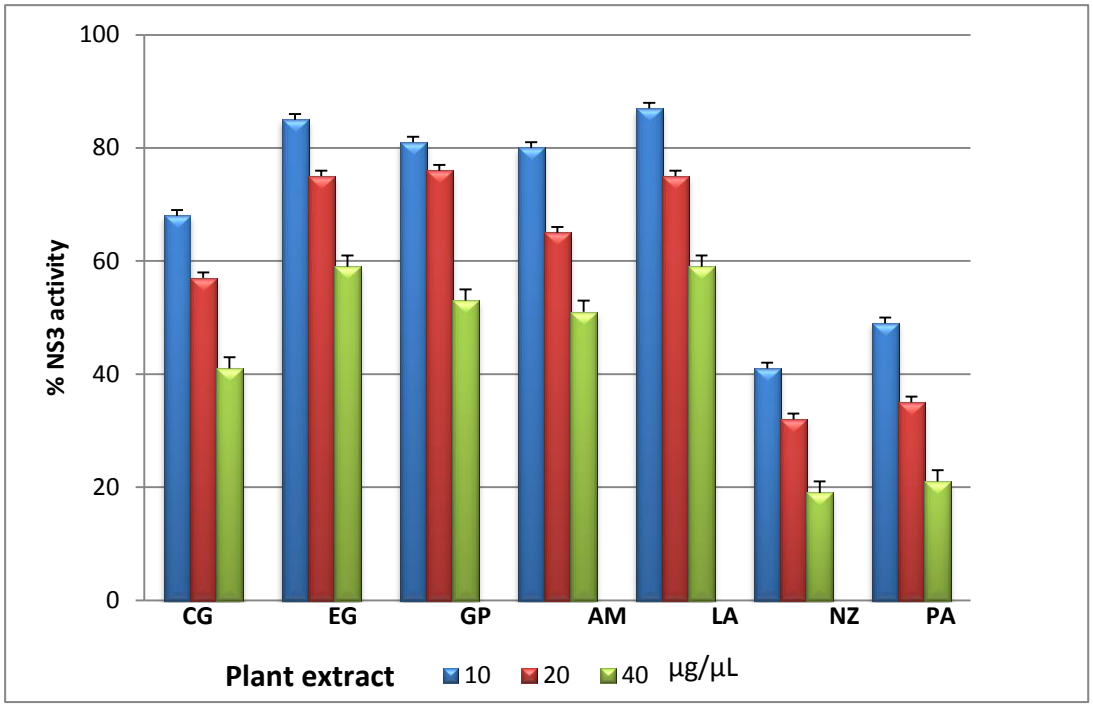


Fig.20 Inhibitory effect of chloroform plant extracts on HCV NS3 protease

3.2. Effect of plant extracts on NS5BProtease

The different plant extraction are listed in Table 1 is based on the information collected and potential effects on the conventionally to treat viral infection, liver disorders, compositions of liver tonics and the patents information on treatment of hepatitis using herbal plants material used as a medicine. Firstly, these plants extracts were screened for their inhibitory effect against HCV NS5B protease. The effect of screening inhibitory activity, recombinant HCV-NS5B protease was taken as substrates and Plant extracts. Purified recombinant protease efficiently cleaved the substrate at NS5B cleavage site and separated EGFP from the CBD. Effect of different extracts of medicinal plants.

In methanolic extracts from different Plants against HCVNS5B protease activity was examined and the result is depicted in Fig.21. The significant inhibition of HCVNS5B

protease activity was observed inthe Methanolic extracts of *N. zeylanica* is higher while compare to *P.amarus, E.geniculata,C.gouriana, G. pentaphylla, A.marmelos, L. aspera,* did not exhibit significant inhibitory activity against HCV NS5B protease.

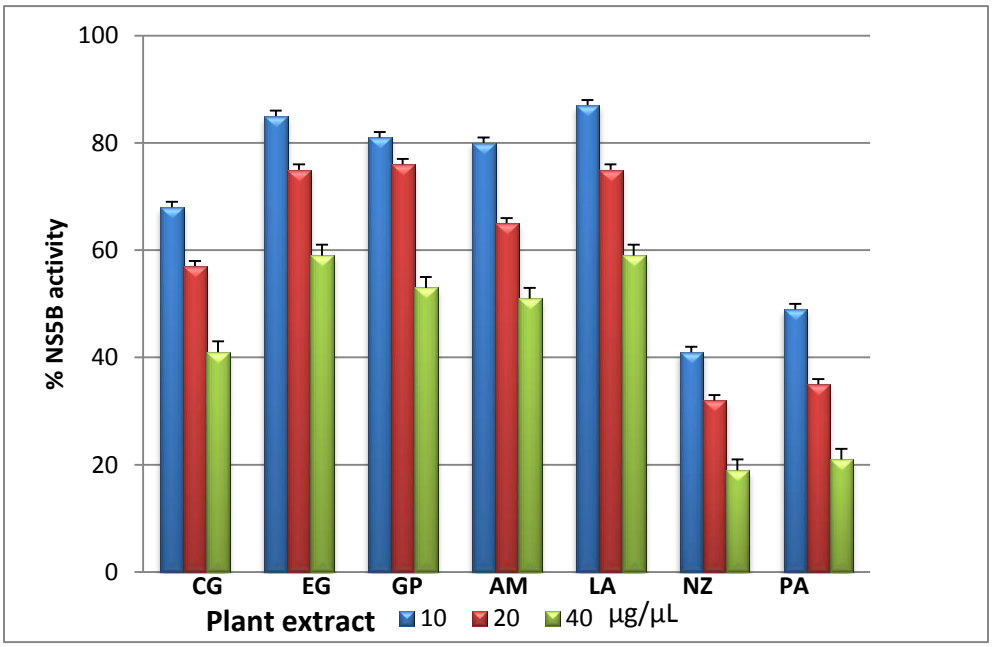


Fig.21 Inhibitory effect of methanolic plant extracts on HCV NS5B protease

In Petroleum ether extracts from different Plants against HCVNS5B protease activity was examined and the result is depicted in Fig.22.

The significant inhibition of HCVNS5B protease activity was observed in the Methanolic extracts of *N. zeylanica* is higher while compare to *P.amarus, E.geniculata, C.gouriana, G. pentaphylla, A.marmelos, L. aspera,* did not exhibit significant inhibitory activity against HCV NS5B protease.

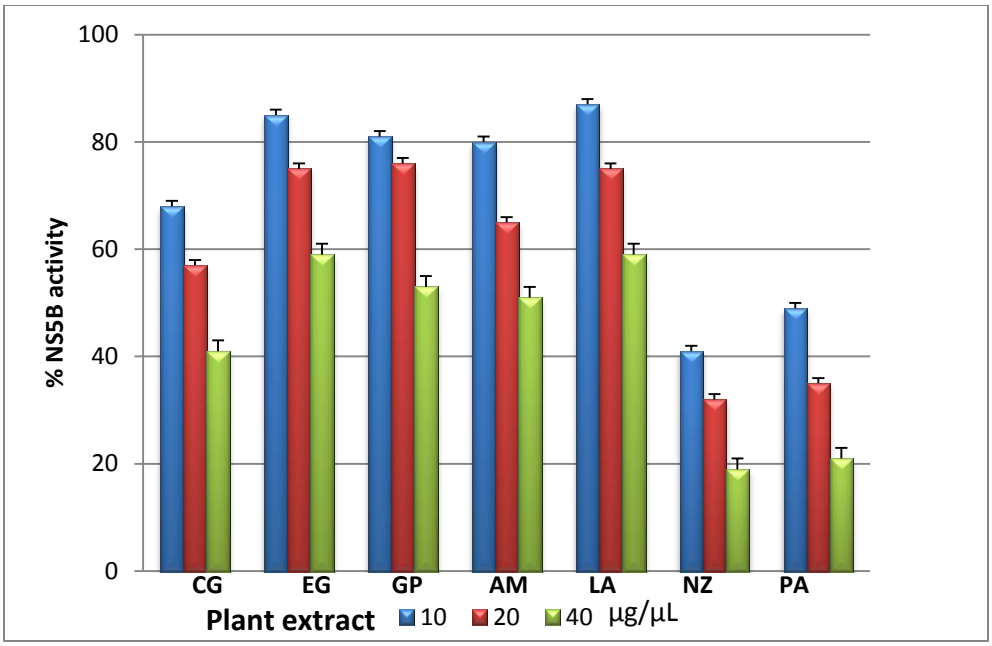


Fig.22 Inhibitory effect of petroleum ether plant extracts on HCV NS5B protease

In Chloroform extracts from different Plants against HCVNS5B protease activity was examined and the result is depicted in Fig.23.

The significant inhibition of HCVNS5B protease activity was observed in the Methanolic extracts of *N. zeylanica* and *P.amarus,* is higher while compare to *E.geniculata, C.gouriana, G. pentaphylla, A.marmelos, L. aspera,* did not exhibit significant inhibitory activity against HCV NS5B protease.

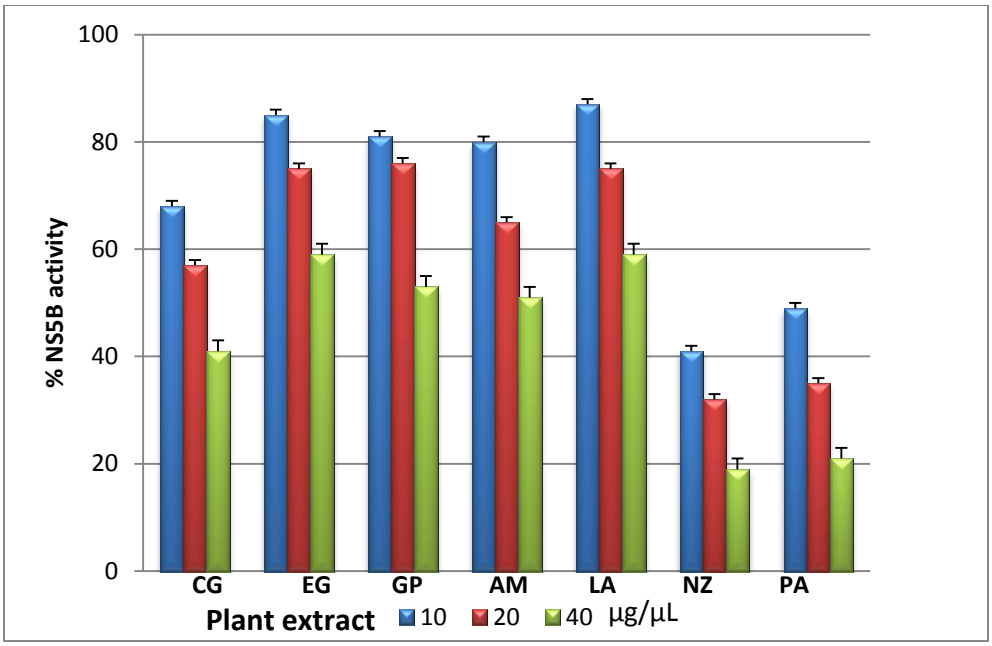


Fig.23 Inhibitory effect of Chloroform plant extracts on HCV NS5B protease

3.3. Effect of phyto constituents on HCV enzymes

Naturally, isolating the compounds from *Clematisgouriana* (**UA**) and the leaves of *Naravelia zeylanica* (as UA, BB, BS and TA.Isolated compounds such as UA, BB, BS and TA were first screened for their inhibitory effect against HCV NS3 protease enzyme. To screen inhibitory activity, recombinant HCV-NS3 protease and substrate were used following the methods described. Effect of isolated compounds from different plants on HCV NS3 protease activity was examined (Fig.24).The inhibitory activity of isolated compounds on HCV NS3 protease activity was studied. Inhibition of HCV NS3 protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of UA and BB showed moderate inhibitory effect, while the remaining compounds such as BS and TA did not exhibit significant inhibitory effect on HCV NS3protease activity

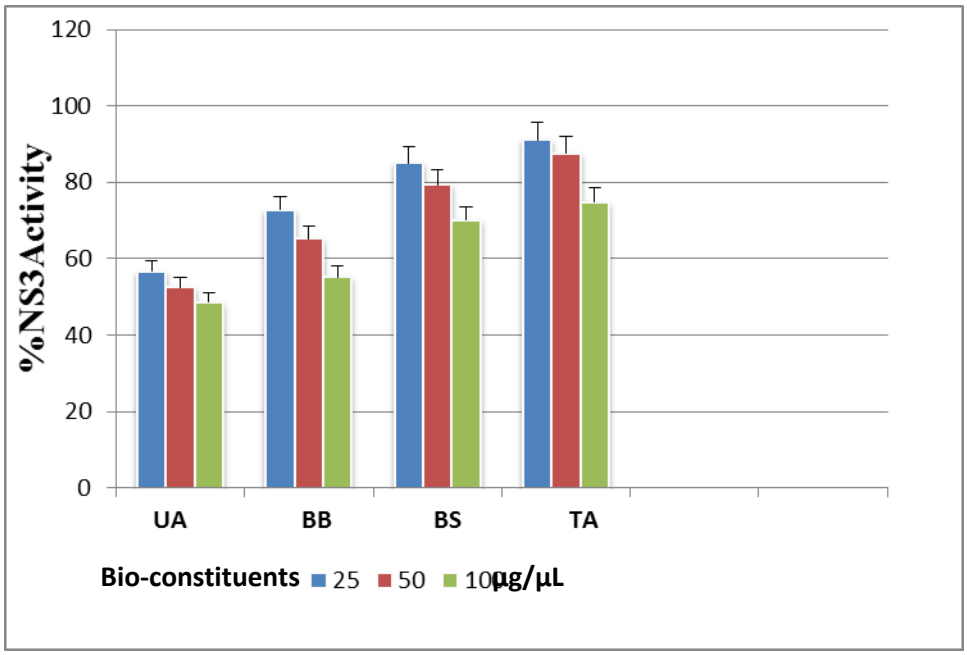


Fig.24.NS3 protease enzyme was pre-incubated with increasing concentrations of bio-constituents followed by addition of pYB-44 substrate containing EGFP. The resulting fluorescence was determined using Fluorometer and the relative enzyme activity normalized with the control (absence of compounds) was plotted against the Bio-constituents.

The inhibitory effect of isolated compounds from plants against HCV NS3 protease enzyme was pre-incubated with the compounds was the enzyme at different concentration followed by addition of pYB-44 substrate containing EGFP. As a resulting fluorescence was determined using Flurometer, its relative enzyme activity normalized with the control and plotted against the Compounds. To investigate the NS3 protease inhibition assay ex vivo, Huh7 cells were co-transfected with plasmid constructs encoding HCV NS3 protease (pYB-43) and the substrate (pYB-44). Since these plasmid constructs are in prokaryotic expression vector (with T7 promoter), we have taken help of the recombinant vaccinia virus expression system (VTF-7.3 construct) to express T7 RNA polymerase [116]. Huh7 cells were first infected with vaccinia virus followed by co-transient transfection with the above mentioned plasmid constructs.

Further, the above isolated compounds were tested for inhibitory effect on HCV NS5B activity. UA and BB showed significant inhibition when compare to TA and BS. The Ursolic acid and Berberine significant inhibitory effect with lower to higher concentration and then less significant inhibitory effect in Taraxerol and β- sitosterol with various concentration of these compounds in µg/µL.

Isolated compounds such as UA, BB, BS and TA were first screened for their inhibitory effect against HCV NS5b protease enzyme. To screen inhibitory activity, recombinant HCV-NS5B protease and substrate were used following the methods described. Effect of isolated compounds from different plants on HCV NS5B protease activity was examined (Fig. 25).The inhibitory activity of isolated compounds on HCV NS5B protease activity was studied. Inhibition of HCV NS5B protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of BB, BS and TA showed moderate inhibitory effect, while the remaining compounds such as UA and did not exhibit significant inhibitory effect on HCV NS5B protease activity

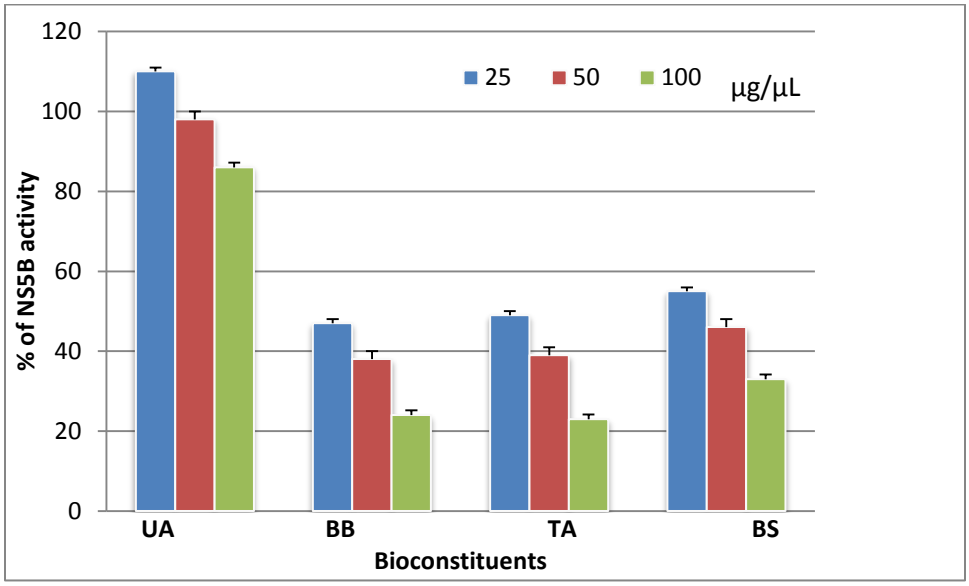


Fig.25. NS5B RdRp enzyme was pre-incubated with increasing concentrations of bio constituents followed by addition of EGFP substrate. The resulting fluorescence was determined using Fluorimeter and the relative enzyme activity normalized with the control (absence of compounds) was plotted against the bio-constituents.

3.4. MTT assay to measure the toxicity of phytoconstituents

HCV replication in cell culture is limited to Huh-7 cells and its derivatives. Hence, we first verified the toxicity caused by isolated compounds against host cell. For this purpose, isolated compounds were screened for their possible cytotoxicity against Huh7 cells by MTT assay. Isolated compounds such as UA,BB, shows significant cytotoxicity when compare to BS and TA toxicity (Fig. 26).

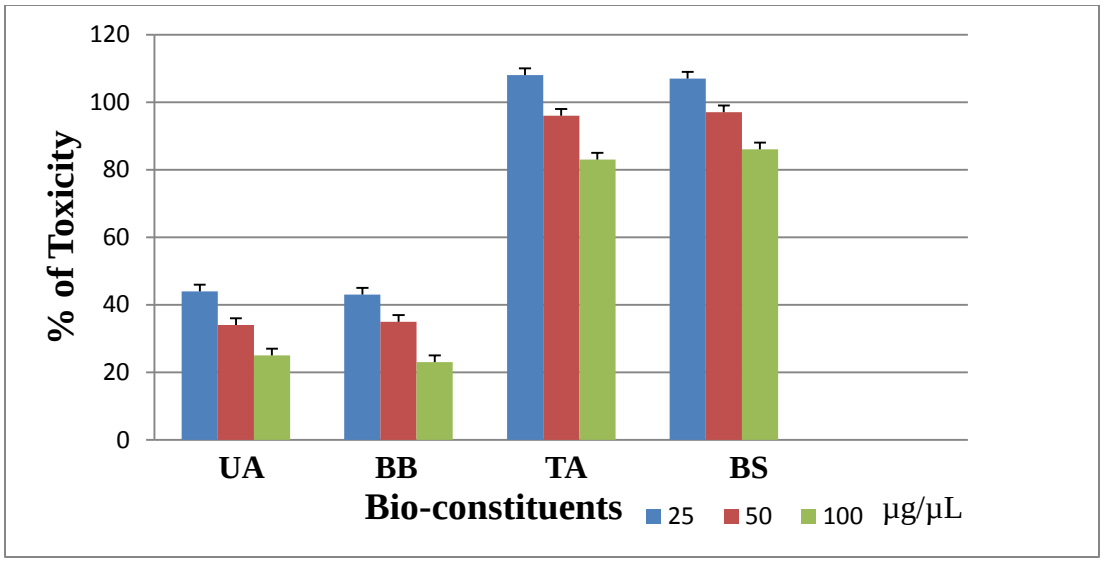


Fig.26.MTT assay representing the relative percentage of toxicity was plotted against increasing concentrations of bioconstituents in Huh7 cells.

4. *Insilico* studies on Phyto-constituents against NS3 and NS5B protease sequence analysis.

a) Molecular docking studies of active constituents against NS3 Protease

Homology modeling methods use structural templates that have been highest sequence homology with the target protein. Homologous proteins were identified by scanning in the target protein sequence of Q913D4 against 3D structures deposited in the protein data bank using PDB BLAST. The 3O8BA was found be the best template structure for NS3 protease since it had 89% identity and least value. The target and template structure (3O8B_A) was aligned using sequence alignment protocol. The percentage of identity was found to be 89.35. The final alignment was carefully evaluated and was found to match the conserved residues fairly well as shown in Fig.27.

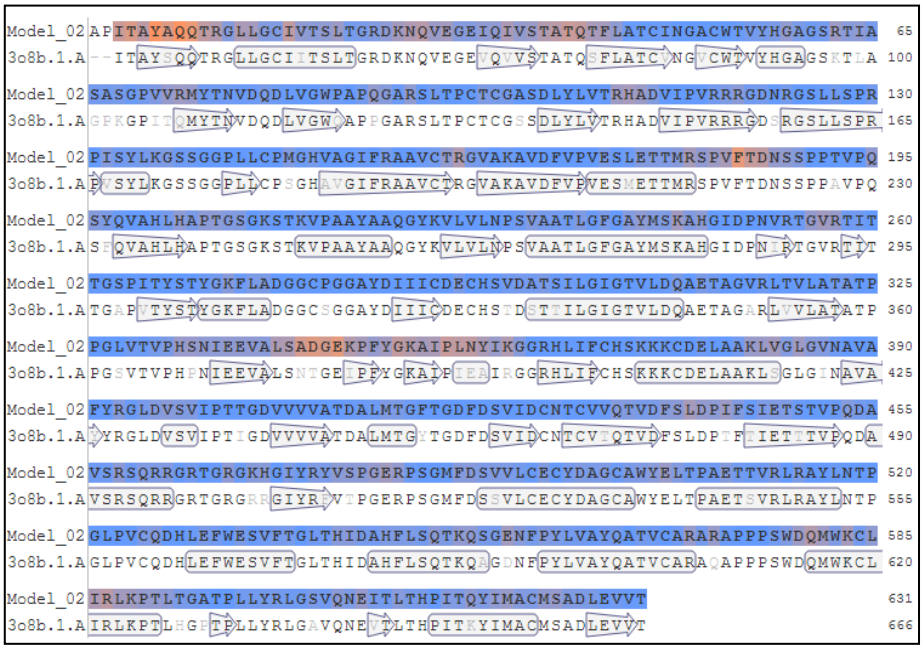


Fig.27: Sequence alignment of NS3 protein with template 3O8B_A

The 3D model of target protein was generated using Swiss – Model automated method. The model structure and template structures are depicted in Fig.28.

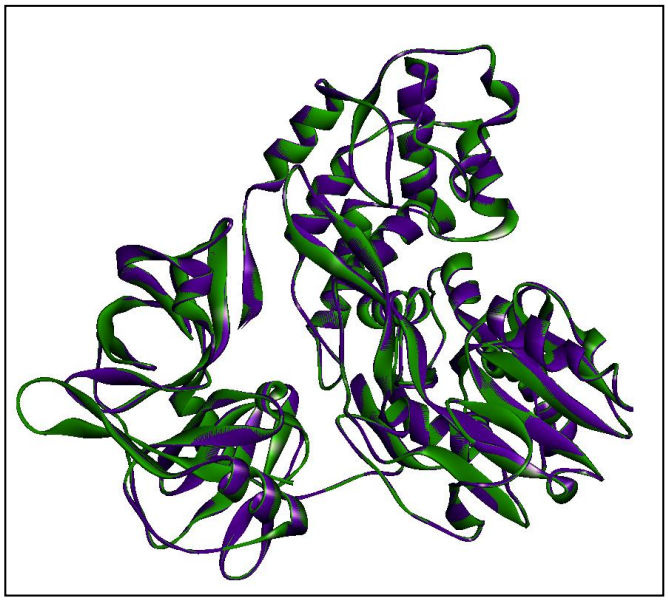


Fig.28. Homology Modeled Structure (Violet color – Model, Green color – Template (3O8B_A)

All compounds satisfied Lipinski rule and ADME properties and the results are tabulated in Table -3.

Table-3: Results of ADME properties

Compound Name	ADMET Solubility Level	ADMET BBB Level	ADMET EXT CYP2D6	ADMET EXT Hepatotoxic	ADMET Absorption Level	ADMET EXT PPB	ADMET AlogP98	ADMET PSA 2D
Berberine	1	4	-4.36461	-10.0226	1	1.6932	6.492	58.931
Ursolic acid	2	1	0.750826	4.32503	0	6.79225	3.446	41.068
β-sitosterol	1	1	-.3.56773	-9.8775	1	2.8769	5.987	49.908
Taraxerol	1	3	-4.90672	-8.9087	0	2.7658	4.098	45.098

The docking scores of the screened compounds against wild-type protein and three different mutations (R155K, A156V, and A156T of NS3 protease with ligand are tabulated in Table-4.

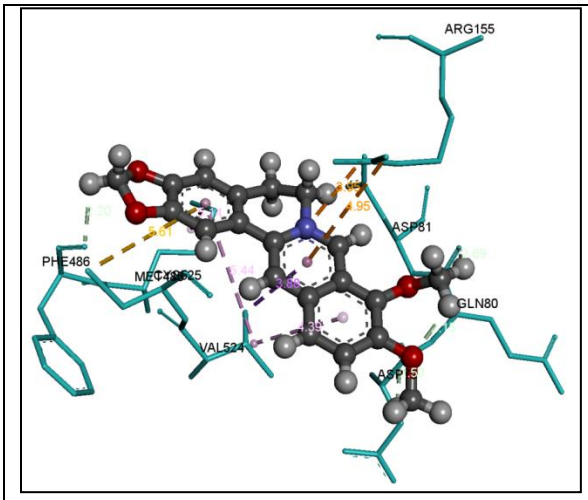
Table -4: Results of Docking score

Compound	Wild(kcal/mol)	R155K(kcal/mol)	A156T(kcal/mol)	A156V(kcal/mol)
Berberine*	**-10.678**	**-11.321**	**-11.532**	**-10.782**
Ursolic acid *	**-10.513**	**-10.342**	**-11.231**	**-10.675**
β-sitosterol	-3.871	-5.875	-4.765	-5.768
Taraxerol	-3.321	-2.876	-3.987	-5.876

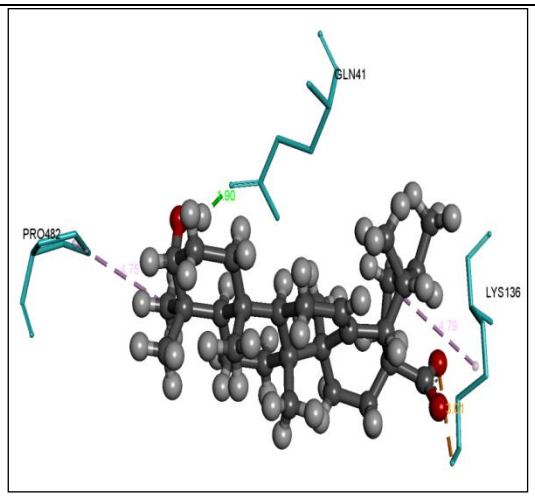
The compound Berberine and its charged counterpart displayed best scores against the wild type protein and mutant proteins -10.678,-11.321, -11.532 and -10.782kcal/mol, respectively. They also have moderately good scores against different mutations compared to Ursolic acid, β-sitosterol, and Taraxerol compounds

The Fig.29(a,b,c,d,e,f,g and h) represents the docked conformation of top two compounds (Berberine and Ursolic acid) with wild- type and mutation protein active site using discovery studio visualizer 4.0.

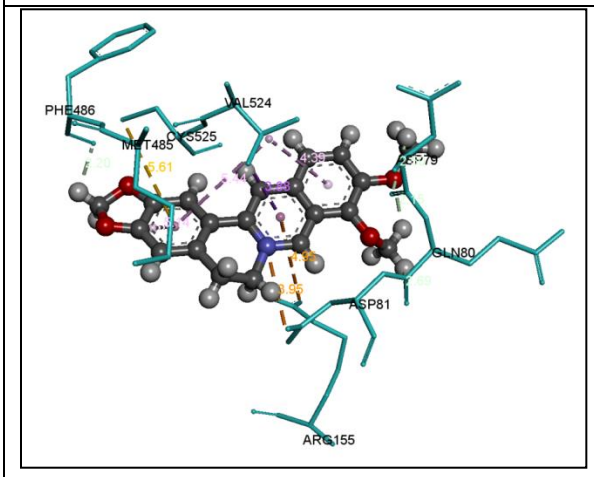
For all docked conformations of inhibitors, active site amino acid residues are represented as sticks. Inhibitor is colored with the atoms as carbon: grey, hydrogen: white, nitrogen: blue and oxygen: red (ball and stick model).


ARG155
ASP81
PHE486
GLN80
VAL524
ASP79

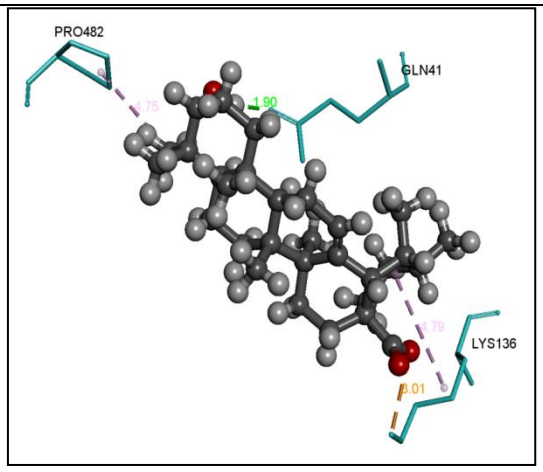
a


GLN41
PRO482
LYS136

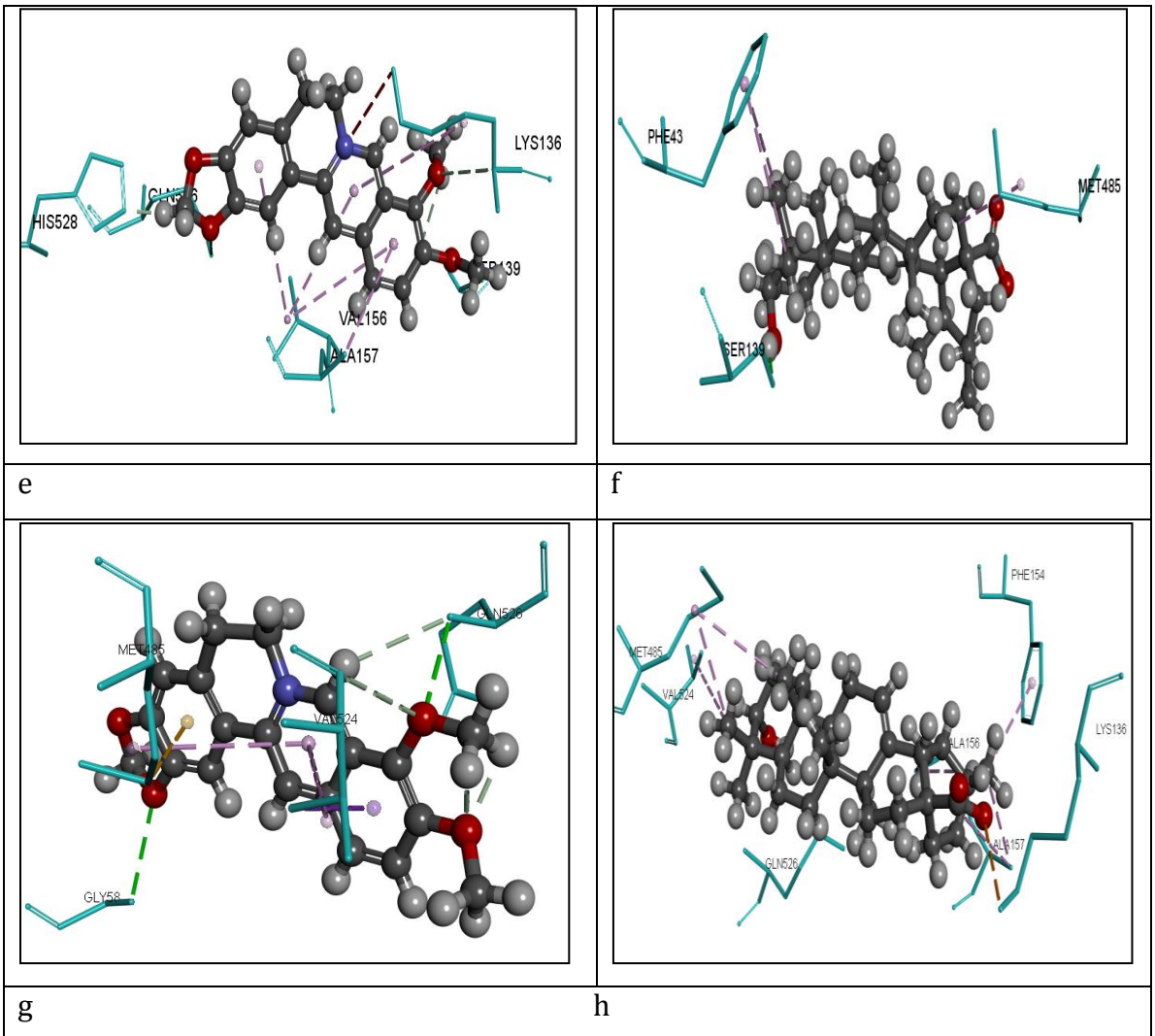
b


PHE486
VAL524
MET485
ASP79
GLN80
ASP81
ARG155

c


PRO482
GLN41
LYS136

d



e f

g h

Fig 29: Top 2 docking results for wild and mutant protein

a) Docked conformation of Berberine compound with the wild-type protein active site
b) Docked conformation of Ursolic acid compound with the wild-type protein active site
c) Docked conformation of Berberine with the NS3/4A protease A156T mutant active site
d) Docked conformation of Ursolic acid with the NS3/4A protease A156T mutant active site
e) Docked conformation of Berberine with the NS3/4A protease A156V mutant active site
f) Docked conformation of Ursolic acid with the NS3/4A protease A156V mutant active site
g) Docked conformation of Berberine with the NS3/4A protease R155K mutant active site
h) Docked conformation of Ursolic acid with the NS3/4A protease R155K mutant active site

*****_note: Inhibitor is colored with the atoms as carbon: gray, hydrogen: white, nitrogen: blue and oxygen: red (ball and stick model). Active site amino acid residues are represented as (stick model)_

Wild-type Protein

The Berberine compound had hydrogen bonding interaction with PHE486, GLN80 and ASP79, electrostatic interaction with ASP81 and ARG155, pi interaction with VAL524, CYS525, MET485 and VAL524 respectively. The Ursolic acid compound indicated hydrogen bonding interaction with GLN41, electrostatic interaction with LYS136, hydrophobic interaction with PRO482 and LYS136 respectively.

A156T Mutant

The Berberine compound displayed hydrogen bonding interaction with PHE486, GLN80 and ASP79, electrostatic interaction with ASP81 and ARG155, pi interaction with VAL524, CYS525, MET485 and VAL524 respectively. The Ursolic acid compound illustrated hydrogen bonding interaction with GLN41, electrostatic interaction with LYS136, hydrophobic interaction with PRO482 and LYS136 respectively.

A156V Mutant

The Berberine compound had hydrogen bonding interaction with HIS528, GLN56, LYS136, pi interaction with ALA157, VAL156. The Ursolic acid compound indicated hydrogen bonding interaction with SER139, MET485, pi interaction with PHE43.

R155K Mutant

The Berberine compound had hydrogen bonding interaction with MET485, GLY58, VAL624, and GLN528. The Ursolic acid compound shows hydrogen bonding interaction with MET485, VAL524, GLN526, ALA157, LYS136, ALA156, pi interaction with PHE154.

b) Molecular docking studies of active constituents against NS5B Protease

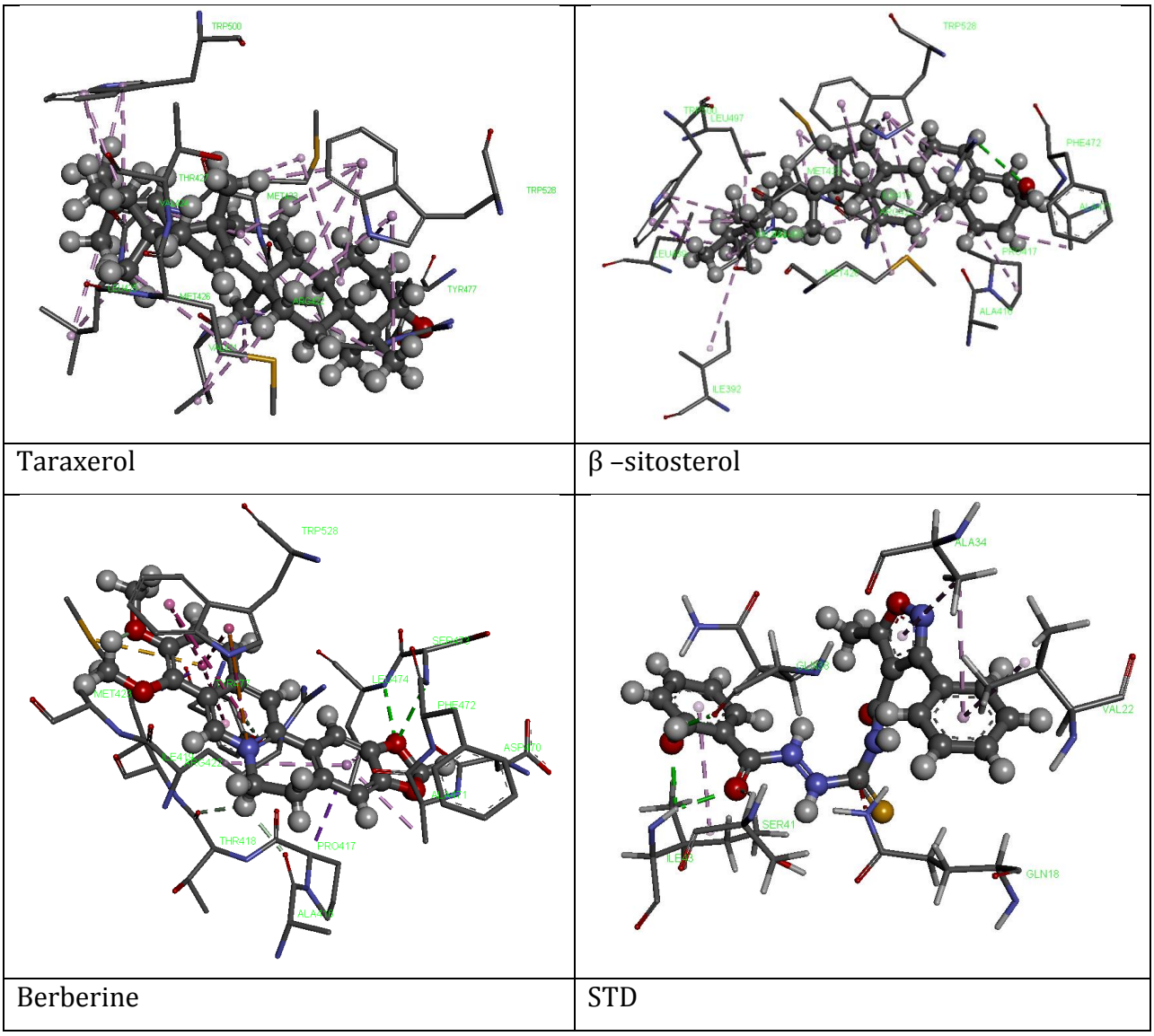
Insilico studies of phyto constituents of against NS5B inhibitors such as Ursolic acid, Taraxerol, β - sitosterol and Berberine, which are naturally occurring compounds present in various plants, based on the selected molecule structure.All tested molecules and slandered compound, have not more than 5 hydrogen bond donors, not more than 10 hydrogen bond acceptors, a molecular weight under 500 dalton, and a partition co-efficient log P less than 5 and ADMET results are shown in (Table-5),

Table-5 Results of ADME properties

S. No	Name	ADMET Solubility Level	ADMET BBB Level	ADMET EXT CYP2D6	ADMET EXT Hepatotoxic	ADMET EXT PPB
1	Taraxerol	0	4	-3.57933	-8.25473	8.59399
2	β -sitosterol	0	4	-1.66327	-7.13406	2.19303
3	Berberine	0	4	-2.78645	-4.19872	7.45673
4	Ursolic acid	1	4	-4.36461	-10.0226	.6932

Note: Solubility: 0-2 highly soluble, BBB: 1-high penetration, 2- medium penetration and 3- Low penetration, CYP2D6: -ve - non-inhibitors & +ve – inhibition. HEPATOX: <1: Non-toxic, PPB: Greater the value greater the binding capacity

These result indicating that these compounds satisfy Lipinski's rule of five and ADMET properties. Docking and binding energies of the compounds have been calculated after docked into the active site of Nonstructural 5 B (NS5B) RNA dependent RNA polymerase (PDB code 1YVZ). Based on these energy values, it can be concluded that the compounds are potent inhibitors of the target Nonstructural 5 B (NS5B) RNA dependent RNA polymerase and the compounds bind more selectively towards 1YVZ protein active site (Fig. 30).



Taraxerol	β –sitosterol
Berberine	STD

Fig.30. Top 2 docking results for wild and mutant protein in NS5B

Table 6: Results of Docking score

Compound name	Hydrogen bonds	Score
Berberine	7	-21.756
Taraxerol	6	-9.01
β -sitosterol	6	-6.474
STD	5	-5.876

The rank of each compound was determined on the basis of the binding free energy of the lowest energy cluster. The tested compounds showed a binding energy ranging from -21.756 kcal/mol to -6.474 kcal/mol (Table 6). The top three compounds with low binding energies Berberine, Taraxerol and β -sitosterol, whereas the compound with the highest binding energy (--21.756 kcal/mol) Berberine.

5. Identification of Viral Proteome by Two-Dimensional Gel Electrophoresis

The analysis of protein sample for proteomics to study the components of cellular protein interactions as well as host cellular pathophysiological processes that occur during virus infection. The sample prepared from Huh7 cells as control and in replicon cell pellate were sonication by using Lysis buffer. The supernated treated with RNAse, DNAse, DTT, and IAA. The protein samples were run for isoelectric focusing of pH 3-10 and followed by 1-D Gel Electrophoresis. The results revealed that some of the proteins were up regulated, down regulated, differentially expressed, and unaltered in the Fig.31.

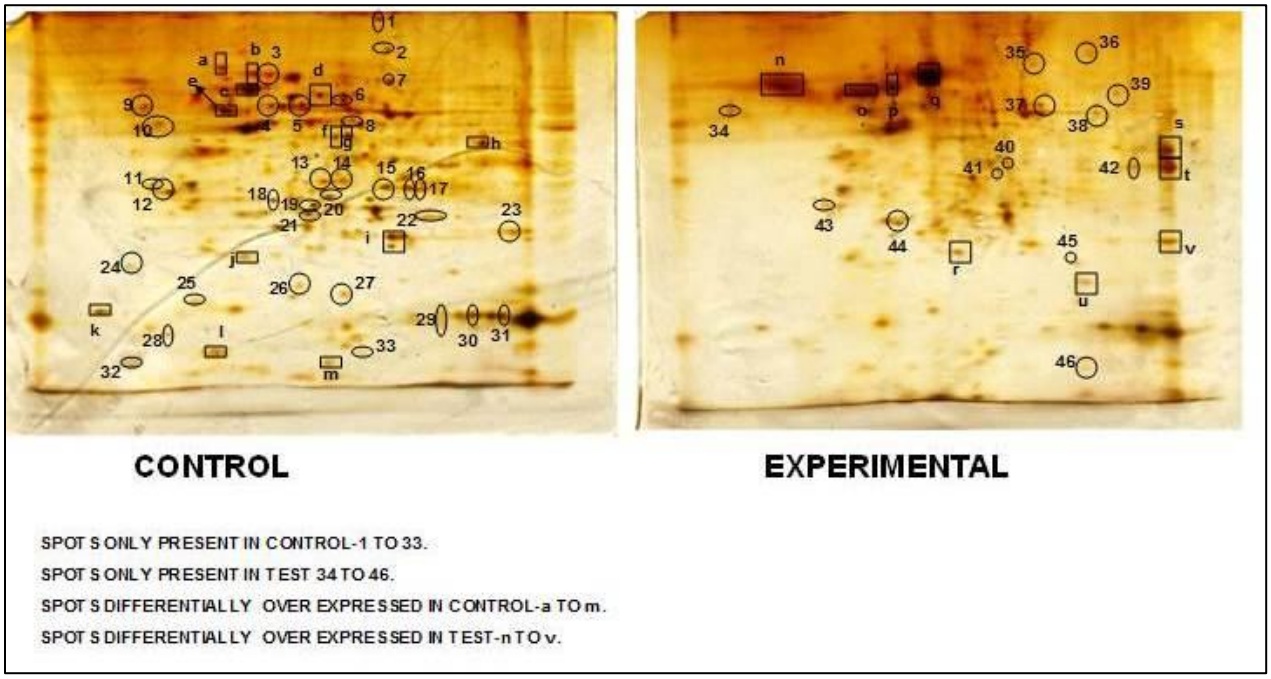


Fig. 31. Differential expressed protein spots

The 33 differential expressed protein spots were successfully identified of which function in diverse biological processes. The differentially expressed protein spots, of which 23 were up-regulated and 10 were down-regulated were identified. All the protein spots were

successfully identified. The identified proteins were involved in the regulation of essential processes such as cellular structure and integrity, RNA processing, protein biosynthesis and modification, vesicle transport, signal transduction, and the mitochondrial pathway.

Mass spectral identification of differentially expressed proteins, to identify the differentially expressed proteins in replicon infected Huh7 cells some of protein spots with a threshold greater than 2-fold were excised manually from gels and subjected to in-gel trypsin digestion and subsequent MALDI-TOF-MS identification. As shown in Fig.32, the differentially expressed protein spots, comprising 23 up-regulated and 10 down-regulated protein spots, were successfully identified. According to the protein function and subcellular annotations from the Swiss-Prot and EMBL protein database and Gene Ontology Database, the identified cellular proteins were comprised of cellular structure and integrity, RNA processing, protein biosynthesis and modification, vesicle transport, signal transduction, and mitochondrial pathway.

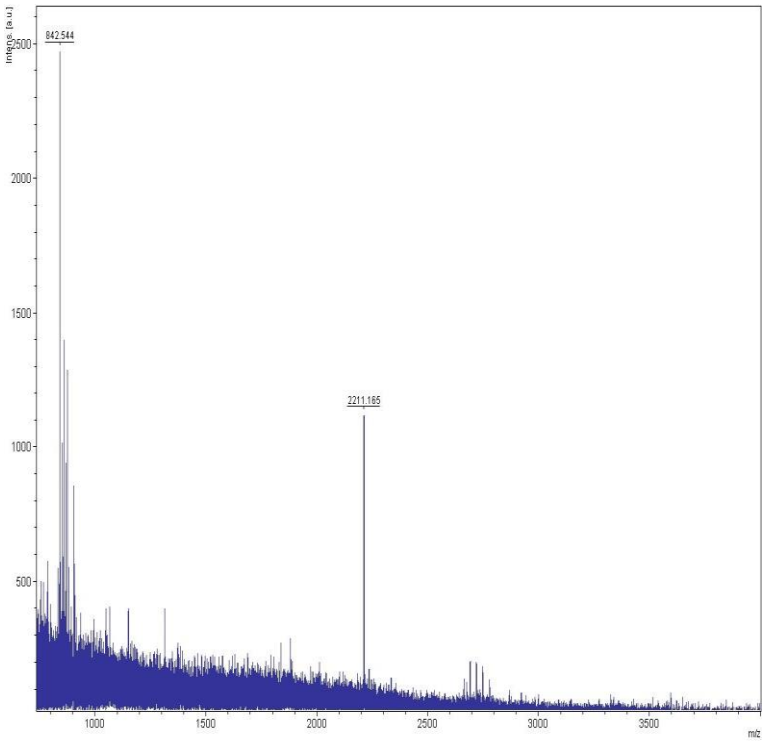
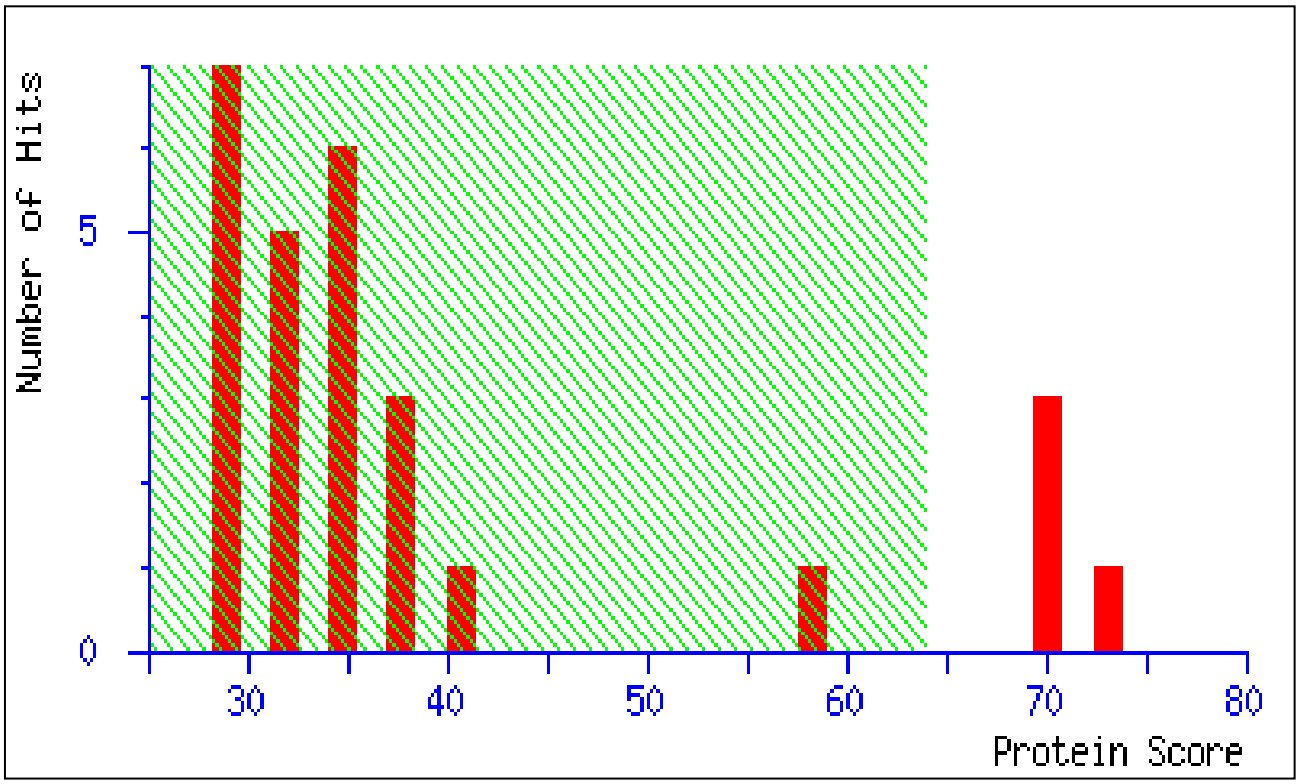


Fig.32 Mass Spectrometry of Spot 1 of rep2a Protein gel Profile

Fig.33 the acquired spectral data of samples were processed using TOF/TOF Explore™ Software in a default mode. The data were searched by GPS Explorer (V3.6) with the search engine MASCOT (2.1). The following parameters were used in the search: National Center for Biotechnology information non-redundant (NCBInr) database. All identified proteins had a protein score greater than 70.



Search title : Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Taxonomy : Homo sapiens (human) (148148 sequences)
Timestamp : 27 Apr 2010 at 14:07:09 GMT
Top Score :73 for AAH12854, BC012854 NID: - Homo sapiens
Mascot Score Histogram
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).

Fig.33 the acquired spectral data of samples were processed using TOF/TOF Explore™ Software in a default mode.

Fig.34 shows analysis of identified proteins at the transcriptional level, the alterations in expression of a protein may be due to a change in its mRNA level. In order to confirm the results of the proteomics analysis at the mRNA level, the transcriptional alterations in six selected proteins were measured by PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control housekeeping gene. The mRNA levels of cells were increased in replicon 2a cells. These data provide transcriptional information complementary to those differentially-expressed proteins detected by 2D gel analysis.

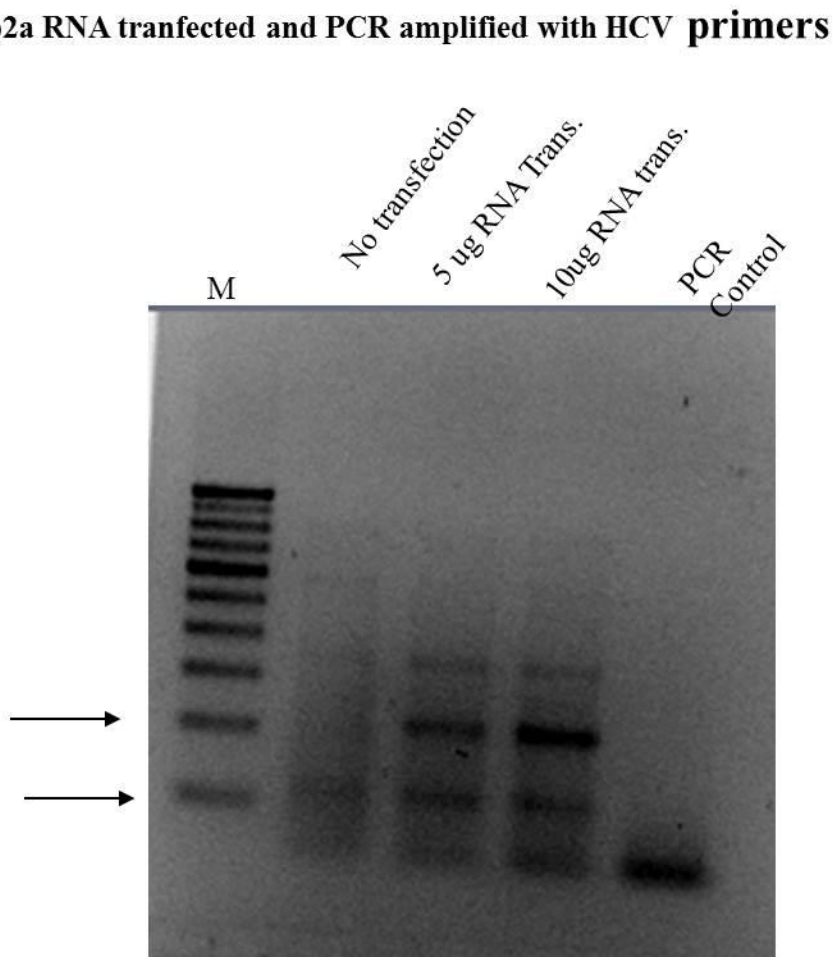


Fig.34Rep2a RNA transfected and PCR amplified with HCV primers

V. DISCUSSIONS

Naturally, Bio-constituents are derived from medicinal plants have been extensively studied in terms of their antiviral activity. Several hundreds of naturally active compounds have been identified all over the world. Many of them have more than complementary and overlapping mechanisms of action, either by inhibiting viral replication or by blocking synthesis of the viral enzymes. These naturally active compounds offer the major opportunities for finding novel structures, active against a wide range of assay targets. In addition, natural occurring compounds from plants, biologically active in assays are generally small molecules with drug-like properties [117]. Moreover, they are capable of being absorbed and metabolized by the body. Hence, the developmental costs of producing orally active medicines are likely to be much lower than that of biotechnological products or most compounds produced to date from combinatorial chemistry [118]. Therefore, natural products including in the traditional medicinal plants offer great promise as potentially effective new antiviral drugs. HCV NS3 and NS5B protease has been used as target enzyme for screening anti-HCV compounds and its vital role in replication of the hepatitis C virus (HCV) an important human pathogen causing in its chronic hepatitis, cirrhosis, and hepatocellular carcinoma [119]. A number of inhibitors have been designed based on the cleavage of native substrates in recent years for the inhibition of HCV NS3 and NS5B protease, many of which are large peptidomimetic compounds with less pharmacokinetic properties [120].

In vitro and *insilico* studies revealed that the 181-residue N-terminal protease domain of protein formed a hetero dimer with the small 54- residue peptide cofactor, leading to the subsequent downstream cleavage of the HCV polyprotein at the junctions between viral proteins. Therefore, drug design targeting the NS3/NS5B to block viral replication and restore hepatocyte innate immune control of HCV replication has been significantly investigated. Varying in their all over the world prevalence and response to treatments in majority of phytoconstituents against HCV Protease enzyme.

In this study, we have selected two plants such as *Clematis gouriana* Roxb. And *Naravelia zeylanica* (L.) DC. The crude extracts of *C. gouriana* and *N. zeylanica* viz., petroleum ether, chloroform, methanol extracts from leaves were subjected to the qualitative tests to detect the Bio-active chemical constituents such asUrsolic acid from

methanolic extract of *C. gouriana* and Berberine from methanolic extract *N. zeylanica,* petroleum ether extract of *N.* zeylanica is isolated compounds such as terpenoid Taraxerol and sterol β- sitosterol. The above isolated compound screening *invitro* and *in silico* activity against NS3/NS5B Protease Domain of Hepatitis C Virus.

Inhibition of HCV NS3 protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of UA and BB showed moderate inhibitory effect, while the remaining compounds such as BS and TA did not exhibit significant inhibitory effect on HCV NS3 protease activity. *Insilico* studies of inhibitor of NS3protein, screened compounds against wild-type protein and three different mutations (R155K, A156V, and A156T of NS3 protease with ligand. The compound Berberine and its charged counterpart displayed best scores against the wild type protein and mutant proteins -10.678 and-11.321, -11.532 and -10.782kcal/mol, respectively. They also have moderately good scores against different mutations compared to Ursolic acid, β-sitosterol, and Taraxerol compounds.

Further, the inhibition of HCV NS5b protease activity was observed with isolated compounds such as UA, BB, BS and TA. The isolated compounds of BB,BS and TA showed moderate inhibitory effect, while the remaining compound UA did not exhibit significant inhibitory effect on HCV NS5B protease activity. *Insilico* studies of inhibitor of NS5B protein, screened compounds against wild-type protein and three different mutations. The rank of each compound was determined on the basis of the binding free energy of the lowest energy cluster. The tested compounds showed a binding energy ranging from -21.756 kcal/mol to-6.474 kcal/mol. The top three compounds with low binding energies Berberine, Taraxerol and β -sitosterol, whereas the compound with the highest binding energy (--21.756 kcal/mol) Berberine.

Viral infections are the major ailment inflicting the human population. Conventional treatment with interferon-alpha is very expensive and has many serious side effects. Alternative medicine using different routes has been reported to be effective against viral infections. Viral infection is a major health problem worldwide developing effective antiviral therapy is the need of the hour. The viral enzymes are essential for polyprotein processing in viral multiplications thus can be potential targets for screening and

identification of viral proteins. The comparative protein expression of host cells in response to viral infection. In this study, cellular protein response to viral infection in Huh cells was analyzed, using the proteomic method of two-dimensional gel electrophoresis (2D) coupled with MALDI-TOF-MS identification. The total of 17 altered cellular proteins that differentially expressed in replicon infection was identified in this study. Most of these proteins were involved in transcription and translation processes, vesicle transport, signal transduction, and alteration of the cytoskeleton networks. The study provides large scale protein-related information that should be useful for understanding the pathogenesis of viral infection. The purpose of this study is to determine the proteins which are responsible for viral multiplications.

VI. REFERENCES

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Annexure – XI

Final report assessment /Evaluation certificate

(Two Members Expert Committee Not belonging to the Institute of Principal Investigator)

(To be Submitted with the Final Report)

It is certified that the final report of Major Research Project entitled **"Phytochemical investigation and antiviral properties of some potential medicinal plants by proteomic approaches"** Dr. /Prof. **Raja Naika H.**, Dept. of **Biotechnology**, has been assessed by the committee consisting the following members for final submission of the report to the UGC, New Delhi under the scheme of Major Research Project.

Comments/Suggestions of the Expert Committee:-

(1) The project objectives have been successfully accomplished through well designed experimental strategy.

(2) The findings of the research have been published in reputed journals and contribute to significant findings in the research area.

Name & Signatures of Experts with Date:-

Name of Expert	**University/College name**	**Signature with Date**
1. Prof. V. Shyamkumar	Karnataka University, Darward	9/1/2018
2. Dr. M.K. Satheesh	Bangalore University, Bangalore	9/1/18

It is certified that the final report has been uploaded on UGC-MRP portal on 17-03-2018.
It is also certified that final report, Executive summary of the report, Research documents, monograph academic papers provided under Major Research Project have been posted on the website of the University/College.

17/3/18

(Registrar/Principal)

Seal

Registrar
Tumkur University
Tumkur