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N-butanol fraction of Decalepis hamiltonii root shows potential to possess antiproliferative activity and combat oxidative damage

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Abstract:

In Ayurvedic system of medicine *Decalepis hamiltonii* has been used as traditional medicine to treat various disorders like cytotoxicity, oxidative stress, bacterial infections, inflammation. The aim of the current study was to determine the cytotoxicity, antioxidant potential of different fractions of *D. hamiltonii* including n-butanol, aqueous, chloroform. Among the fractions studied n-butanol, exhibited good antioxidant DPPH assay, superoxide Phenols and flavonoids were quantified and analysed using HPTLC. *Declapeis hamiltonii* cytotoxicity on Hep G2 cells. HPTLC detected bioactive substances. Comparing rf values of tested chemicals with standard values predicted flavonoids, glycosides. Flavonoid glycosides and those with RF values of 0.23 and 0.35 may exist.The peaks at 0.17, 0.67, 0.57, 0.74 and 0.82 steroid or triterpene or glycoside. Bioassay-guided extraction and fractionation was utilized to identify Hydromethanolic extract of *D. hamiltonii* root which demonstrated Hepatoprotective efficacy on CCl4-induced toxicity on Hep G2 cells. n- butanol fraction has the most protective effect. Percent cell viability was found to be 94.2 %.In addition, LC-MS/MS was used to analyze chemical characterization of bioactive molecules through bioassay guided approach. The result showed n-butanol fraction of *D. hamiltonii* extract was most promising regarding antioxidant, hepatoprotective activities because of presence of flavonoids specially isoflavones. Glucocaffeic acid compound showed an excellent docking score of 95.78, 125.78, 162.14 and 120.62 Kcal/mol with all four targeted enzymes and receptors TGF-beta, NF-kB, iNOS and COX-2 respectively. The results indicated n-butanol fraction exihibit protective effect activity against CCl4 induced toxicity in Hep G2 cell lines.

Keywords: Root extract, quantitative and qualitative analysis, HPTLC *D. hamiltonii,* Hep G2 cell lines

1. Introduction

The liver is an important organ that regulates several bodily activities such as detoxification, storage, secretion, and metabolism.Some of these functions are commonly disrupted as a result of liver injury caused by numerous agents and environmental variables. It is reported as hepato-toxic agents act by generating oxidative stress, reactive oxygen species and oxidative damage in proteins and DNA and reducing ATP [1]. Notably, protecting the liver from hepato-toxic agents and their harmful effects i.e. altering the anti-radical defensive mechanism, is known as hepatoprotection [1]. Despite the presence of several advancements in the modern era, the incidence of the hepatic disease has not reduced and on the contrary, an exponential increase is observed.The utilization of medicinal herbs of potential therapeutic significance has been increased during last few years. The usage of natural products in cancer treatment has been attracting the cancer survivors to pick out the material which they consider to be safe as compared to synthetic therapy[2]. The phytochemical and pharmacological estimation of all medicinal plants which lead to drug discovery is called as screening of natural products [2]. Pharmacological activity is based on the secondary metabolites from the plants [3,4].Geographically, *D. Hamiltonii* Wight &Arn. species, which is well-known as barresugandhi, maredukommulu, maraud gaddalu, or makaliberu, is widespread and endangered in southern India. It is a member of the Asclepediaceae family and is usually called these names[5,6]. It has been found in dry and moist evergreen forests of Andhra Pradesh, Karnataka and Tamil Nadu. The roots part of the plant has been used since long for several disorders like skin diseases, hemorrhoids, diaphoretic, rheumatism, asthma, diaphoretic, general tonic, stimulate appetite in Indian Traditional medicines [7]. *D. Hamiltonii* is used as a preservative, blood purifier, respiratory disorders, diarrhea, pyrexia, asthma, bronchitis, eye diseases, appetite loss, urinary disorders, rheumatism and burning sensation as a source of bio insecticide for stored food grains [8]. Previous studies have shown that roots containsinositols, aldehyde, lupeols and amyrins [9] also volatile compounds like 2- hydroxy-4-methoxy benzaldehyde, 2-phenyl ethanol, vanillin, benzaldehyde and others [10]. The roots are bitter then sweet. The study of the NMR, MS analysis and phytochemical investigation, antioxidant studies of the *D. hamiltonii* of root extracts and qualitative phytochemical estimation of flavonoids, alkaloids, tannins and saponins. Phenols, steroids, terpenoids was carried out. For a better interpretation of the molecular basis of the physiological activity of natural products, in silico studies have been effectively used to the theoretical prediction of ligand-target interactions [11]. Additionally, it offers more information on the potential method of action and manner of binding of

substances that are active against enzymes.A relative evaluation of the cytoprotective and antioxidant potential of various stem extracts of D. Hamiltonii will form effective, applicable information about the future drug designing to reverse the hepatotoxicity in patients. The present study was designed to evaluate the in vitro antioxidant and hepatoprotective potentials of Swallow root extract fraction with the objective to elucidate earlier results on antioxidant and anticancer activities of Swallow root extract fraction. Standard analytical procedures were also used to assess total phenols and total flavonoid content. The antioxidant properties of a wide variety of chemicals contained in the swallow root extract fraction were determined using a combination of DPPH, H_2O_2, and the reducing power assay. The in vitro hepatoprotective potentials of a fraction of swallow root extract was investigated using the MTT test using Hep G cell lines.

2. Materials and methods:

2.1 Extract preparation:

From NaattuMarundhuKadai evergreen Eco Farms Private Limited in Coimbatore, Tamil Nadu, India, plant roots were purchased. The roots were cleaned with distilled water, allowed to air dry for 7 to 10 days at room temperature, and then dried in an oven at 400°C to remove any remaining moisture. The dried plant materials were ground into a powder and kept at 40°C for later use. Methanol was used to do a soxhlation extraction on 50 g of powdered gum samples between 60 and 80^0C independently concentrated in a water bath at 40^0C and evaporated were the three filtrates.

2.2 Drugs and Chemicals:

HepG2 Cell lines were purchased from NCCS, Pune India. The drugs and chemicals were purchased from various companies and the details are as mentioned: Hi Media Laboratories Pvt. Ltd., silymarin, Folin- Ciocaltaeu reagent - Sigma Aldrich, Spruce Street, St. Louis, China; Biochemical kits - Merck Specialties Private Limited, Mumbai, India; Formic acid-Merck Specialities Pvt. Ltd., DMSO- Research Lab Fin Chem Industries, Mumbai; Ethanol – ChangshuYangyuan Chemicals, China. Ecoline diagnostic kit was obtained from EeMerck Ltd., Mumbai, India. All other chemicals and solvents used were of analytical grade. All chemicals were analytical grade and were purchased from Research Lab Fine Chem Industries, Mumbai.

2.3 Extraction and fractionation:

Preparation of the Extract:

D.hamiltonii was shade-dried to retain its chemicals. A sensitive digital balance (Denver (BT-224S)) weighed 100 g of *D. hamiltonii* root powder. Powder was macerated in methanol and water for 24 hours in a 1 L beaker. Following 24hrs of stirring, the extract was recovered

from the marc using cloth and suction-filtered by Whatman No. 1 filter paper [12,13]. Three times remaceration removed all plant components. In a rotary evaporator at 40°C and reduced pressure, maceration filtrates were dried. The methanolic extract was dehydrated for 24 hours. Brown crude methanolic extracts were stored at 4°C [13]. 100 mL of chloroform was added to a separating funnel containing 5.3 g of hydro methanolic crude extract diluted in 100 mL of methanol-water to separate chloroform sub extract. Then, two transparent layers developed. Draining the flask gathered the dark methanolic extract layer. Add 70 mL of n-butanol to 100 ml of methanolic extract in a separating funnel (brown). The mixture was mixed and left to stand until two clear layers developed. The separating funnel's n-butanolsubextract was emptied. Repeating this method three times allowed chloroform to remove all moderately polar chemicals. After separating n-butanolsubextract, methanolicsubextract remained (M1). Aqueous subextract was emptied from the separating funnel into methanolicsubextract three times. The three sub extracts were evaporated at 40^{o} C and kept at 4° C until use.The flow chart for bioguided fractionation (**Fig 1).**

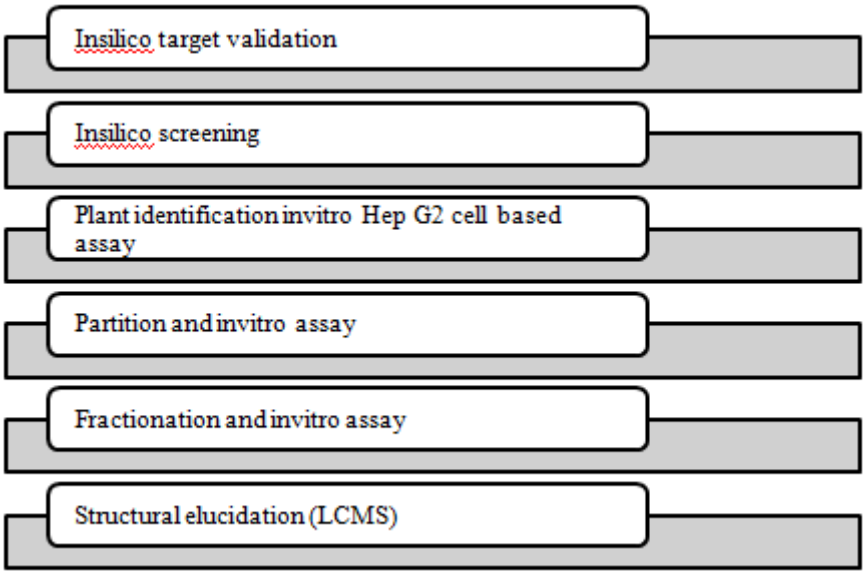

Fig 1: The flow chart of bioguided fractionation procedure for study of *D. hamiltonii* root extract hepatoprotective activity

2.4 Phytochemical Investigation

The extracts were processed to phytochemical screening utilizing conventional technique to determine the phytoconstituents. Presence or absence of several phytochemical like Alkaloid, cardiac glycosides, carbohydrate, protein, saponins, phenol, terpenoids. Presence

or absence of several phytochemicals like Alkaloid, carbohydrate, glycosides, glycosides, saponins, protein, phenol, terpenoids.

2.5 Quantitative analysis

Estimation of total flavonoids and total phenols.

2.5.1 Determination of total flavonoids: The reaction between flavonoids and aluminum trichloride ($AlCl_3$) produces a red color compound. The total flavonoid content (TFC) is estimated by their intensity [14, 15,16].

To the diluted extracts of DH, the methanolic solution of AlCl¬3 (1 ml) was mixed and left undisturbed at 25±2 ℃ for about 1.5 h and further absorbance measured by UV-spectrophotometer at λ max 420 nm. The prepared samples concentrations were 1 mg/mL and were formed in triplicate to determine the mean value and standard deviations. Absorbance of standard quercetin solution was determined using the same procedure and calibration curve was plotted. The flavonoids concentration was determined from the calibration plot based on their absorbance. The flavonoids content was denoted in terms of quercetin equivalent (QE), which is amount of quercetin (mg) per gram of extract.

2.5.2 Estimation of total phenols: Folin- Ciocalteu reagent (10 ml) got diluted with distilled water in 1:10 ratio and mixed with different extract of DH [10,15,16]. The solution was first vortexed and then kept aside for a time period of 5 min. The prepared 7% w/v sodium carbonate solution (10 ml) was then mixed followed by volume adjustment with distilled water up to 25 ml. Further, the solution was left to stand at 25±2 ℃ for about 1.5 h and absorbance measured through UV-spectrophotometer at a λmax of 765 nm. Similar procedure was carried out with standard gallic acid solution and calibration curve was plotted. The polyphenol concentration was determined from the calibration plot based on their absorbance. The polyphenols content was denoted in gallic acid equivalent (GAE) terms, which is amount of gallic acid (mg) per gram of extract.

2.5.3 Antioxidant studies:

2.5.3.1 DPPH Radical Scavenging Activity:

The scavenging reaction between DPPH and any antioxidant compound results in reduction of DPPH to DPPH turning solution yellow and as a result the absorbance of the solution decreases. The degree of discoloration indicates the scavenging potential i.e., hydrogen donating ability of antioxidant compounds or extracts. DPPH quenching ability of Sample was measured [17,18]. Serial dilutions of the chemical (200–1000 g/ml) were combined with the ethanol DPPH solution (0.15%), and after 10 minutes, the absorbance was measured at

515 nm. The amount of antiradical substance needed to provide a 50% inhibition, or IC50 (g/ml), was used to express the antiradical activity. The standard was vitamin C. All samples were analyzed in triplicate. The DPPH radical scavenging ability was calculated using the following equation:

DPPH scavenging effect (%) = (Ao - A1)/ Ao* 100

Where Ao is the absorbance of the control at 30 min

A1 is the absorbance of the sample at 30 min.

2.5.3.2 Hydroxyl radical scavenging activity

The extracts ability for OH° radical scavenging activity was determined via measurement of the hydroxylated salicylate complex intensity, which is formed due to reaction of sodium salicylate with OH° radical [14,19]. The reaction mixture of Vit C 1 mL (10-200 µg/mL) or different concentrations of extracts of DH, 0.3 mL sodium salicylate solution, 0.7 mL H_2O_2 solution and 1 mL $FeSO_4.7H_2O$ solution incubated at a temperature of 37±2 °C for time interval of 1 h. The reaction mixture's absorbance was taken post-incubation at 562 nm. The OH° radical scavenging activity was determined by following equation:

Hrdroxyl radical scavenging activity (%) = (Ao - A1)/ Ao* 100

Where, A_0= Control absorbance, A_t= Sample absorbance

The graph of inhibition (%) against concentration and IC 50 was predicted. The experiment was repeated three times at each concentration.

2.5.3.3 Reducing power assay

To assess the extracts' reducing capabilities, chromophore absorbance at a wavelength of 700 nm was taken into account. [19,20]. The DH extracts were taken in different concentrations. The extract and 1 ml Vitamin C (20-200 µg/ml) was separately added to 2.5 ml phosphate buffer and potassium ferricyanide solution. The solutions were then incubated for 20 min maintaining 50±2 °C temperature. After incubation, 2.5 ml of TCA added and mixture is centrifuged for 10 min at 3000 rpm. The solution separated into two layers. The upper layer (2.5 ml) was taken and 2.5 ml distilled water and 0.5 ml $FeCl_3$ solution was added. The absorbance was taken at 700 nm. Increased reductive ability is demonstrated by an increase in absorbance (9).

2.5.4 HPTLC:

The sample was dissolved in a mixture of Methanol and spotted (10 µL) with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) in the shape of a band that was 6 mm wide on a precoated silica gel aluminium plate 60 F254 (5 cm 10 cm) with 250 m thickness (E.

MERCK, Darmstadt, Germany) (Switzerland). Slit dimensions of 5 mm 0.45 mm and a scanning speed of 20 mm/sec were used. The linear development was conducted using toluene, ethyl acetate (EA), methanol, and formic acid (5: 4: 2: 0.5) as the mobile phase in a 10 cm x 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland). 10 minutes was the ideal chamber saturation period for the mobile phase. Chromatogram development took 8 cm of run length.

2.5.5 In-vitro Hepatoprotective activity on Hep G2 cell lines:

2.5.5.1 Evaluation of cell viability by MTT assay

Tetrazolium measurements of mitochondrial synthesis were used to determine CTC 50 (10). In 96-well plates, Hep G2 cells (5.0 103 cells/well) were cultured for 72 hours in 100 l of three fractions of HADHE at 50, 100, and 200 g/ml each. After the drug solutions had been incubated, each well received 50 l of 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which had been made in modified Eagle's medium (MEM) with no phenol red. At 37°C and 5% CO2, the plates were incubated with a gentle shake. The supernatant was removed after three hours. After adding 50 l of propanol to dissolve the formed formazan, it was shaken for 30 minutes at room temperature. Microplate reader reading absorbance at 540 nm (OD) (Bio-Tek Instruments, Inc., Winooski,VT).All concentrations of plants extracts were in triplicates on the same cell batch.The percentage growth inhibition was calculated using the following formula:

$$\%\ Growthinhibition = \frac{MeanODofnormalcontrol - MeanODoftestgroup}{MeanODofNormalControl} X\ 100$$

The extract concentration (g/ml) and percent growth inhibition (%) were used to create a dose-response curve. The dose-response curve is used to get the CTC 50 value.

Assessment of hepatoprotective activity

Using well-maintained HepG2 cells, the hepatoprotective activity of all three fractions of HADHE was assessed. As a hepatotoxicant, carbon tetrachloride was utilized, while silymarin served as a typical positive control. The amount of ethanol that was ingested was 100 mM, which is hazardous. Based on the findings of the MTT experiment, the concentrations of HADHE and standard were chosen.

Statistical analysis:

Results were expressed as mean ± SD One-way ANOVA and the Dunnett's multiple comparisons test were used to examine the data (Graph Pad Software, La Jolla California USA). The significance level is based on $p<0.05$.

2.5.5.2 Serum Alanine transaminase, Aspartate Transaminase and Lactate dehydrogenase leakage:

The cells and culture medium were collected individually The (0.2 ml) medium was utilized to determine concentration of ALT, AST and LDH as an evidence of cell cessation by Ecoline diagnostic kit.

2.5.6 LC-MS profiling:

HPLC determination was done using 1 mg/ml of DHF2, on Agilent 1260 infinity II with Agilent 6540 UHP Accurate –Mass Q-TOF LCMS using Agilent eclipse XDB-C18, 3.0X150 mm, 3.5 micro column. The gradient elution was carried out with 0.1 % fromic acid in acetonitrile (B). A-0.1 % formic acid in H_2O. The flow rate was 0.3 ml/min. The fraction was dissolved in H_20/MeOH followed by direct injection into the M-QTOF LCMS. Capillary temp was 350 ^{0}C, voltage -3500V, Nozzle voltage 1000 V, fragmentor voltage -175V. However, full scan mode with mass range of m/z 100-1700 with the positive polarity was applied.

2.5.7 In-Silico Molecular Docking Analysis

19 phytoconstituents present in *D. hamiltonii* were selected. Pubchem database was used to download the structures of all the phytoconstituents. Silymarin (PubChem CID 5213) was the standard used for the database**.** The three-dimensional crystallographic structures of targeted receptors and enzymes including transforming growth factor beta (TGF-β), Nuclear factor kappa B (NF-κβ), Inducible nitric oxide synthase (iNOS), and Cyclooxygenase-2 (COX-2)were retrieved from RCSB PDB database (https://www.rcsb.org/pdb)1vjy, 1vkx, 2orq and 5jvy respectively and were used for further processing. The preparation of proteins was performed with the aid of CHARMM force fields. In protein structure modification initially requires various tasks such as insertion of atoms missing in incomplete residues, deletion of alternate confirmations, missing loop region modelling, protonation of titrable residues, prediction of pKs (a measure of negative logarithm of dissociation constant of an acid), providing standardized atom names and removal of water molecules or heteroatoms and addition of hydrogen atoms. According to the present investigation, the binding interactions of the identified compounds from LC-MS analysis with the binding active pockets of TGF-β,

NF-κβ, iNOS, and COX-2 enzymes are consistent with the previously published literature [21,13,22,23,30].

3. Results

In this research work, we performed LCMS profiling of *Decalepis hamiltonii* root to identify its metabolites. Also, depth biological study was performed out invitro to find substantial evidence of the hepatoprotectivee activity of the plant extract fraction F2. In vitro study was carried out by determination of antioxidant characteristics by DPPH scavenging, SOD activity, H_2O_2 assay; RO assay as well as invitro Hep G2 cell model to invitro assesses activity of plant fraction F2 as initial screening to get an overview about the activity.

3.1 LC- MS Profiling:

LCMS profiling revealed 14 major peaks which were predicted by comparing their MS data (in negative ionization mode) and their UV/Vis spectra earlier recorded in the PSM literature **(Table 1)**. The compounds recognized belongs to classes mainly glycosides, flavonoids, Phenols, tannins **(Table 1) (Fig 1)** and comprises of bis-2,3,4,6-galloyl-α/β D-glucopyranoside (Decalepin) (1), Rutin(2), Apigenin 7-[galactosyl-(1->4)-mannoside](3), Quercetin 3-(4"-Acetyl rhamnoside) 7-rhamnoside (4), Ellagic acid (5), Catechin 3',7-diglucoside(6), 6-Methoxyluteolin 7-rhamnoside (7), 6-C-Glucopyranosyl-8-Cgalactopyranosylapigenin (8), Diosmetin (9), Epicatechin-(4beta->8)-gallocatechin (10), 2-hydroxy- 4 methoxy benzaldehyde (11), Kaempferol 3-(6-acetylgalactoside) (12), Diterpenes ent-kaur-16- en-19 oic acid (Kaurenoic acid) (13), p-anisaldehyde (14). Structures of identified compounds **(Fig 2).** Compounds 2, 6 and 10 are glycosides however compounds 1, 3, 4, 7, 8, 9 and 12 belong to flavonoids while compound 5, 11 and 14 is a polyphenols and compound 13 belongs to diterpenes.

Table 1: The characterization of phenolic, flavonoids, glycosidic compounds in the n-butanol fraction of D. hamiltonii extract by LC-MS/MS analysis

Sr. No.	Rt (min)	$(M-H)^-$ *m/z*	Molecular formula	Compounds
1.	4.730	657.9	C20 H20 O14	bis-2,3,4,6-galloyl-α/β D glucopyranoside (Decalepin)
2.	9.791	610.1527	C27 H30 O16	Rutin
3.	14.167	594.1585	C27 H30 O15	Apigenin 7-[galactosyl-(1->4)-mannoside]
4.	16.308	464.0951	C29 H32 O16	Quercetin 3-(4"-Acetyl rhamnoside) 7-rhamnoside

5.	16.657	302.0055	C14 H6 O8	Ellagic acid
6.	17.135	614.1874	C27 H34 O16	Catechin 3',7-diglucoside
7.	17.260	462.1163	C22 H22 O11	6-Methoxyluteolin 7-rhamnoside
8.	17.670	594.1590	C27 H30 O15	6-C-Glucopyranosyl-8-Cgalactopyranosylapigenin
9.	17.316	462.1152	C22 H22 O11	Diosmetin
10.	22.856	594.1371	C30 H26 O13	Epicatechin-(4beta->8)-gallocatechin
11.	23.859	152.0475	C8 H8 O3	2-hydroxy- 4 methoxy benzaldehyde
12.	23.086	490.1114	C23 H22 O12	Kaempferol 3-(6-acetylgalactoside)
13.	26.872	302.2247	C20 H30 O2	Diterpenes ent-kaur-16-en-19 oic acid (Kaurenoic acid)
14.	37.660	136.0525	C8 H8 O2	p-anisaldehyde
15.	6.399	342.0945	C15 H18 O9	Glucocaffeic acid

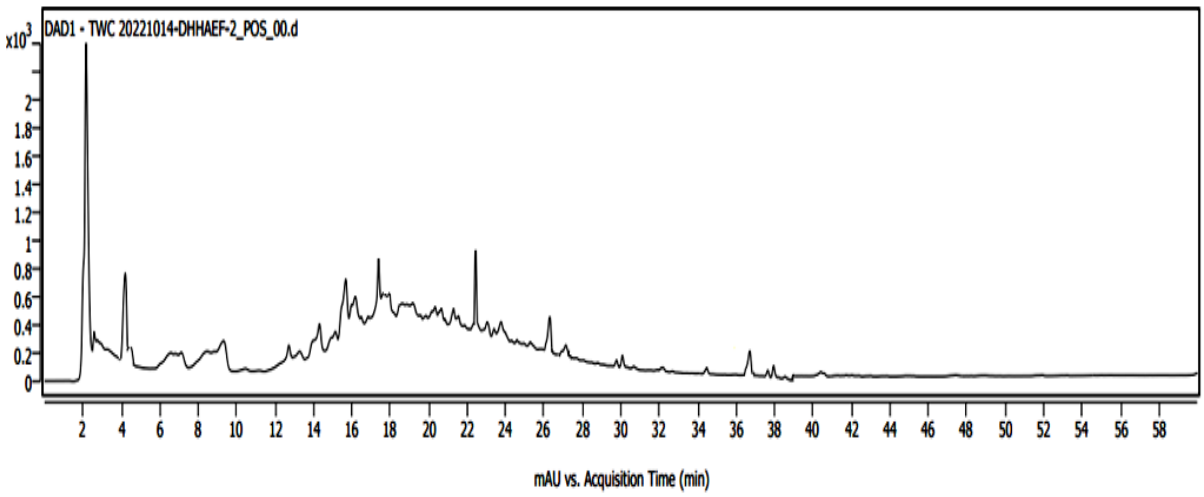

Fig2: LC-ESI-MS (Liquid Chromatography coupled with Electrospray Ionization Mass Spectrometry) profiling of the total Hydroalcoholic extract fraction (F2- n-butanol fraction) of *Decalepis hamiltonii*) root.

3.2 Phytochemical Screening:

D. hamiltonii roots subjected to successive extraction resulted in 3 extracts, DHHAE (*D. Hamiltonii* Hydroalcohol extract), DHEE (*D. hamiltonii* ethanol extract), DHAE (*D. hamiltonii* aqueous extract). Preliminary phytoconstituents present in different extracts of root of *D. hamiltonii* were revealed in **Table 2**. Carbohydrates, flavonoids, phenols, tannins, terpenes and phytosterols were found as most of constituents in Hydroalcoholic extract. Quantitative analysis for the plant extracts were done for Flavonoids and Phenol **(Table-3).**

Table 2: Phytochemical studies of *Decalepis hamiltonii* using various extracts

Test	Hydroalcoholic	Ethanol	Chloroform	Aqueous	Hexane
Alkaloids	+	+	-	-	+
Carbohydrates	-	-	-	-	-
Steroids & terpenoids	+	+	-	+	-
Tannins	+	+	-	+	-
Amino acids and proteins	-	-	-	-	-
Flavonoids	+	+	-	+	-
Phenols	+	+	-	+	-
Saponin	-	-	-	-	-
Glycosides	+	+	-	+	-

Table 3: Quantitative analysis of the hydroalcoholic root extracts of *D. hamiltoni* for determination of Phenols and Flavonoids

Sr. No	Plant Extract	Phytochemicals	Estimation
1.	Root	Phenols	15.22± 0.86
2.	Root	Flavonoids	8.15±0.85

3.3 High Performance Thin Layer Chromatography:

D. hamiltonii hydroalcoholic extract photo documentation profile using high performance thin layer chromatography at UV 366 nm and UV 254 nm was performed **(Fig4)**. The spots' Rf values and colour **(Table 4)**. The colour of the spots under UV 254 nm reveals that the TLC profile contains various constituent types. The spots at Rf values 0.17, 0.23, 0.35, and

0.67 were major spots, while spots at Rf values 0.13, 0.35, and 0.67 were minor spots. The spots at Rf values 0.13, 0.17, 0.75, 0.78, and 0.84 may be steroid or triterpene or their glycoside, and the spots at Rf values 0.23 and 0.35. Nine chemical components were found in the HPTLC finger print profile of F2 hydroalcoholic extract of D. Hamiltonii root **(Table 4).** Since the intensity of the peak area was at 1042.1, 2195.4, 2561.1, 2778.7, 3486.4, and 3492.7 AU, respectively, the components with Rf values of 0.17, 0.23, 0.35, 0.67, 0.13, 0.35, and 0.67 were determined to be more numerous. The Rf values of AU for all other peaks were less than 323.4, 486.3, showing the presence of at least 9 different components separated in the solvent system. The other components were found to be very insignificant in number **(Table 4and Fig4).** Flavonoid glycosides and those with RF values of 0.23 and 0.35 may exist. The peaks at 0.17, 0.67, 0.57, 0.74 and 0.82 steroid or triterpene or glycoside.

Table 4. HPTLC finger profile and Rf Values of hydroalcoholic root extract of 10 µL at 575 nm

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.03 Rf	1.7 AU	0.00 Rf	562.5 AU	47.40 %	0.12 Rf	24.6 AU	11684.6 AU	41.66 %
2	0.13 Rf	24.3 AU	0.16 Rf	42.2 AU	3.56 %	0.17 Rf	39.4 AU	1042.1 AU	3.72 %
3	0.17 Rf	39.6 AU	0.21 Rf	59.9 AU	5.04 %	0.23 Rf	39.4 AU	2195.4 AU	7.83 %
4	0.23 Rf	39.6 AU	0.26 Rf	50.9 AU	4.29 %	0.34 Rf	7.2 AU	2561.1 AU	9.13 %
5	0.35 Rf	5.8 AU	0.38 Rf	10.9 AU	0.92 %	0.41 Rf	2.0 AU	323.4 AU	1.15 %
6	0.67 Rf	12.8 AU	0.74 Rf	98.2 AU	8.28 %	0.75 Rf	31.8 AU	2778.7 AU	9.91 %
7	0.75 Rf	92.3 AU	0.76 Rf	194.5 AU	16.39 %	0.78 Rf	75.9 AU	3486.4 AU	12.43 %
8	0.78 Rf	77.7 AU	0.80 Rf	145.7 AU	12.28 %	0.84 Rf	11.3 AU	3492.7 AU	12.45 %
9	0.84 Rf	11.7 AU	0.86 Rf	21.8 AU	1.84 %	0.89 Rf	0.7 AU	486.3 AU	1.73 %

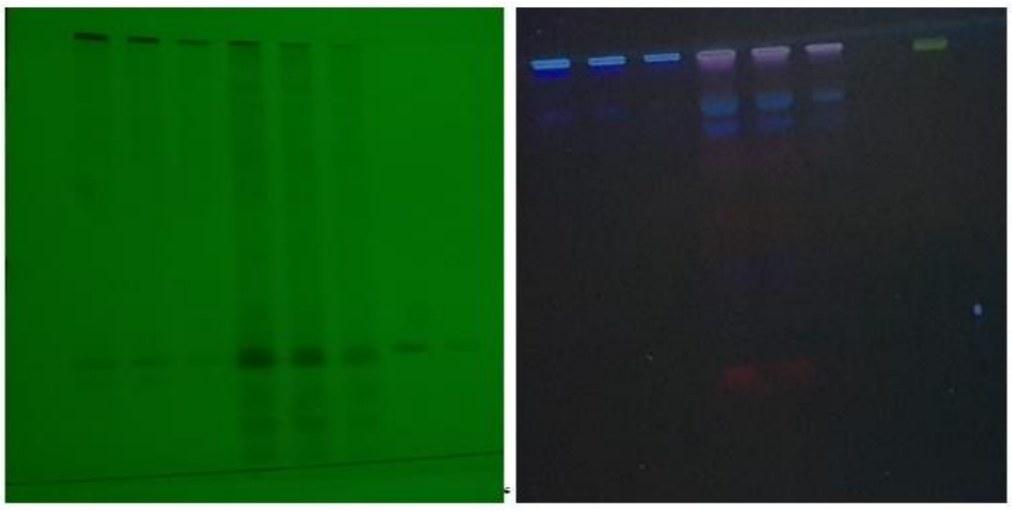

Fig3: TLC profile of DHHMEF2 fraction root of *D. Hamiltonii*

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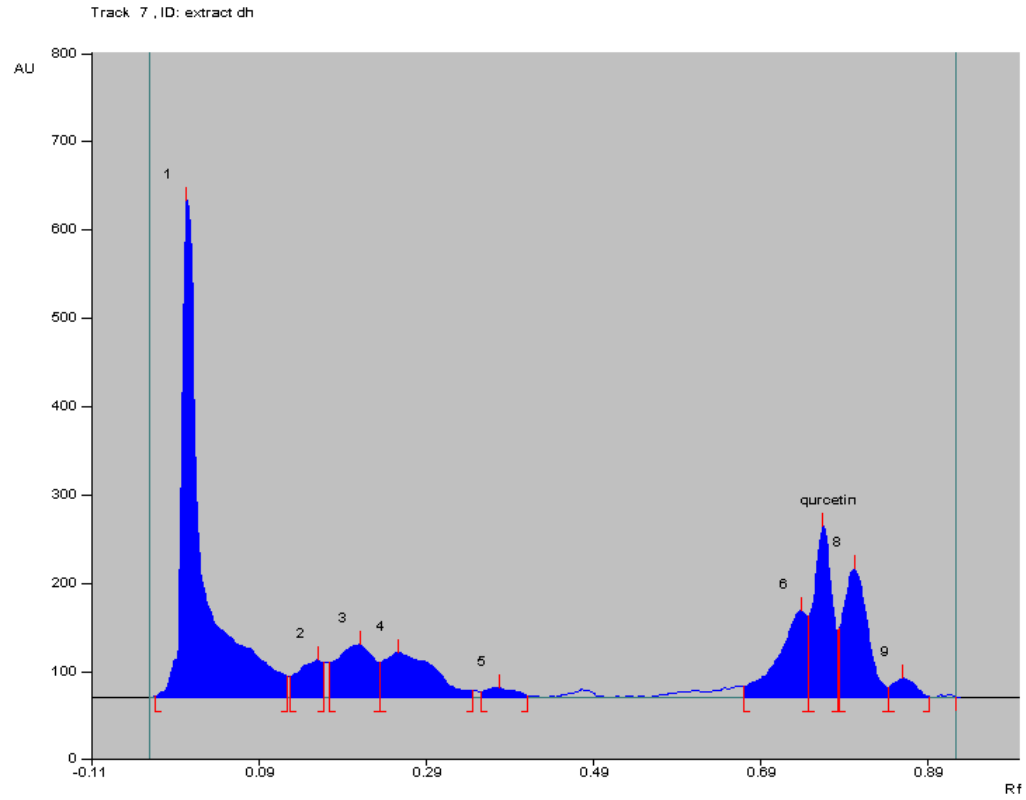

Fig4: HPTLC finger print of DHHMEF2 fraction root of *D. Hamiltonii*

3.4 Antioxidant Assay:

Antioxidant activity of different extracts of *D. Hamiltonii* was measured by DPPH scavenging assay, reducing power and HSRA assay. In the DPPH assay**,** the percentage inhibition of DH extracts is shown in **Table 5 and Fig 5**. It is observed that the IC50 of Vitamin C was 11.29. Therefore the order of scavenging activities was found to be Vit C (11.29 µg/ml)> DHHAE (169.29µg/ml)> DHEE (190.6µg/ml)>DHAE (237.54 µg/ml). IC 50 OF DHHAE was found to be 14.99 times lesser compared to Vit C. At 200 µg/ml DHHAE showed 60.4 % DPPH radical scavenging activity. In HRSA OH- is the most reactive-oxygen-centered species. It causes severe damage to an adjacent biomolecules. Radical is formed when H_2O_2 reacts with ferrous ions using the Fenton reaction and is predicted to in vivo cell injury. The scavenging property of different DH extracts on OH° radical has been represented in **Table 6** and **Fig 6**. High scavenging activity was detected with DHHAE. IC 50 of Vit C was found to be 29.41µg/ml. The sequence of scavenging action of Vit c and other extracts was as follows: Vit C (29.41µg/ml) > DHHAE (165.693 µg/ml) >DHEE (188.07µg/ml) > DHAE (319.95 µg/ml). The IC 50 of DHHAE was found to be 5.64 times lesser than vit C. At 200 µg/ml concentration displayed 49.3% OH° radical scavenging action. In RPA assay, the increase in absorbance is a major indicator of the antioxidant action

potential as represented in **Table 7 and Fig 7.** The outcome showed the obvious reducing action of DH extracts might appear because of the content of flavonoid or polyphenol presence, which may act like reductants by giving hydrogen or electrons and also reacting through free radicals to translate them into further stable compounds and dismiss reaction of chain radical. Absorbance at 700 nm was taken and an increase in absorbance demonstrated increased reductive ability.

Table 5: Percent inhibition by DH extract in DPPH radical scavenging assay

Sample	**% Radical Scavenging activity**								
	10 µg/ml	**20 µg/ml**	**40 µg/ml**	**60 µg/ml**	**80 µg/ml**	**100 µg/ml**	**150 µg/ml**	**200 µg/ml**	**IC50**
Ascorbic acid	34	43.1	58.3	77.1	82.6	84.6	86.6	89.1	11.29
DHAE	7.3	10.3	12.2	15.2	17.1	28.5	38.5	39.5	237.54
DHHAE	4.8	11.3	20.5	28.4	32.4	43.5	50.3	60.4	168.9
DHEE	4.5	7.2	11.3	19.1	28.2	35.5	39.4	48.5	190.6

Table 6: Percent inhibition by DH extract in hydroxyl radical scavenging assay

Concentration (µg/mL)	**10**	**20**	**40**	**60**	**80**	**100**	**150**	**200**	**IC50**
Vit C	32.7	45.3	50.1	65.2	70.3	80.9	81.5	83.8	29.41
DHHAE	7.2	17.1	25.6	32.1	38.1	43.5	48.1	49.3	165.693
DHAE	5.4	7.2	11.5	19.1	28.2	35.5	41.3	48.5	188.07
DHEE	3.1	7.3	12.1	18.2	22.3	24.5	26.1	30.2	319.95

Table 7: DH extracts absorbance in reducing power assay

Concentration (µg/mL)	**20**	**40**	**60**	**80**	**100**	**150**	**200**
Vit C	0.572	1.275	1.549	1.842	1.87	1.927	1.899
DHHAE	0.117	0.184	0.444	0.596	0.821	0.854	0.867
DHAE	0.199	0.686	1.102	1.198	1.249	1.741	1.792
DHEE	0.098	0.179	0.191	0.201	0.216	0.271	0.22

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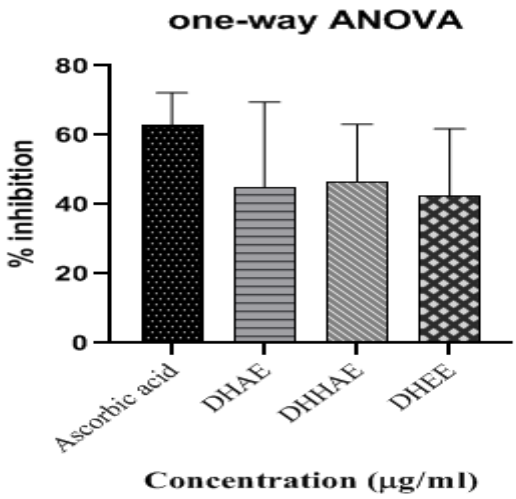

Fig5: Percent inhibition by DH extracts in DPPH radical scavenging assay

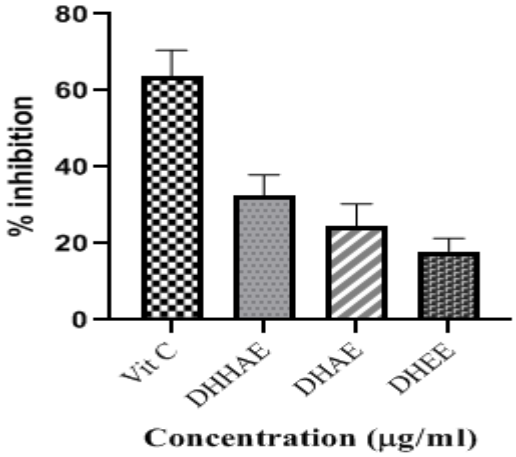

Fig6: Percent inhibition by DH extracts in hydroxyl radical scavenging assay

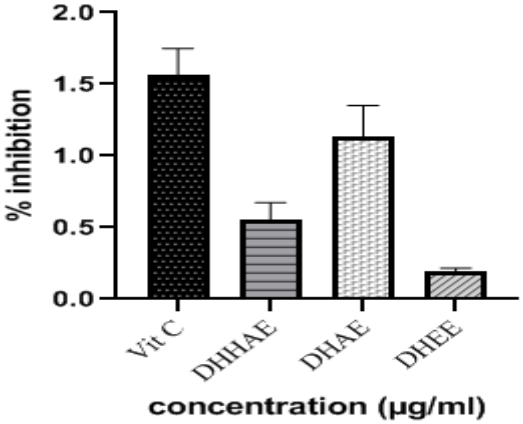

Fig7: Reducing power of different DH extracts

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3.5 Hepatoprotective activity:

Based on the bioguided fractionation of extracts for Hepatoprotective activity study reported for 3 fractions, butanol fraction was found to be more potent as compared to other 2 fraction using MTT assayis shown in **Table 7, Fig 8 and Fig 9**. Non toxic dose of DHE tested for its Hepatoprotective activity on Hep G 2 cell line intoxicated with CCl4 showed 94.2 % protective effect (200µg/ml) whereas standard Silymarin at 200µg/ml exhibited. Fig 7 depicts the time-dependent toxicity of CCl4 in HepG2 cells. No significant difference in cell viability, ALT, AST, or LDH levels was observed when HepG2 cells toxicated by CCl4 were exposed for 3 hours. When compared to the vehicle control, there was a time-dependent significant (p 0.01) increase in AST, ALT, LDH leakage and a significant loss of cell viability. Table 7 depicts the results of cell viability, leakage parameters AST, ALT, LDH levels in all experimental groups. A significant ($p < 0.01$) decrease in viability of cells and a significant ($p < 0.01$) increase in the levels of AST, ALT, LDH was observed in the HepG2 cells exposed to CCl4 as compared with Group 1 (normal control). These cells, when treated with DHHME of different fraction (200 mg/ml) showed a significant restoration of the altered biochemical parameters towards the normal compared to CCl4 treated group and is dose dependent. F2 fraction showed significant restoration as compared to other fractions which was further used for docking.

Table 8: *In vitro* hepatoprotective activity of DHHM F2 using HepG2 cell lines

		% viability of cells	AST (U/L)	ALT (U/L)	LDH (U/L)
I Control					
Normal control		99.32	13.46±0.40	9.96±0.30	109.56±1.35
DMSO control (0.25%, v/v)		97.56	13.57±0.62	10.01±0.57	107.46±1.57
Silymarin control (200 µg/ml)		98.72	12.48±0.56	9.87±0.78	109.56±1.35
DHHAE control (200 µg/ml)		97.98	13.59 ±0.44	9.78 ± 0.21	109.78±0.68
II Silymarin treatment					
Standard (Silymarin+ CCl4)		58.2	34.01±0.28	19.38±0.25	175.56±2.38
		59.8	28.27±0.52	15.33± 0.65	147.41±3.11
		65.59	19.47± 0.54	13.61±0.38	128.01±1.14
IIIToxin treatment					
	CCl4	53.06	47.21± 0.78	21.29 ±0.57[a]	208.60 ±2.57[a]
IV DHHME treatment					
n-butanol + CCl4	50 µg/ml	94.42	43.25±0.48	21.14±0.31	195.56±3.34
	100 µg/ml	92	35.41±0.58	17.34±0.46	167.41±2.13
	200µg/ml	97.70	26.21±0.64	15.35±0.51	148.01±1.15
Aqueous + CCl4	50 µg/ml	81	45.21±0.29	20.01±0.71	192.53±2.14
	100 µg/ml	88.35	36.21±0.31	16.21±0.29	155.23±1.16

	200µg/ml	87.72	28.01±0.46	14.47±0.36	133.21±1.18
chloroform +CCl4	50 µg/ml	68.34	46.52±0.39	18.21±0.49	179.52±1.23
	100 µg/ml	32.41	36.25±1.12	15.21±0.85	143.23±3.15
	200µg/ml	43.82	29.81±1.10	12.08±.56	121.35±2.15

Values are mean ±S.E.M., n = 6 in each group. [a]p< 0.01, [b]p < 0.05, Group III compared to Groups I and Group II and IV compared to Group III, No significant difference was notedbetween groups 1, 2, 3 and 4, (One-way ANOVA followed by Dunnett's test). DHHAE, hydroalcoholic extract from D. Hamiltonii root; AST, aspartate aminotransferase; ALT, alanine aminotransaminase; LDH, lactate dehydrogenase; MDA, malondialdehyde;GSH, glutathione.

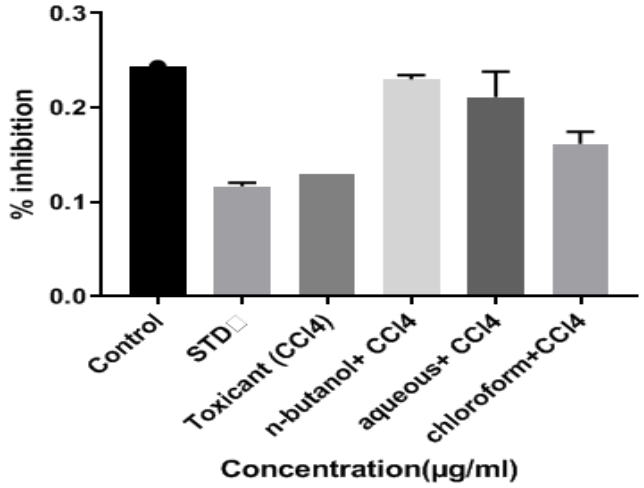

Fig 8: graph of % inhibition of hep G2 cells with different conc of *DHHAE f2* extract of D. Hamiltonii on intoxication with CCl_4 (1 mm)

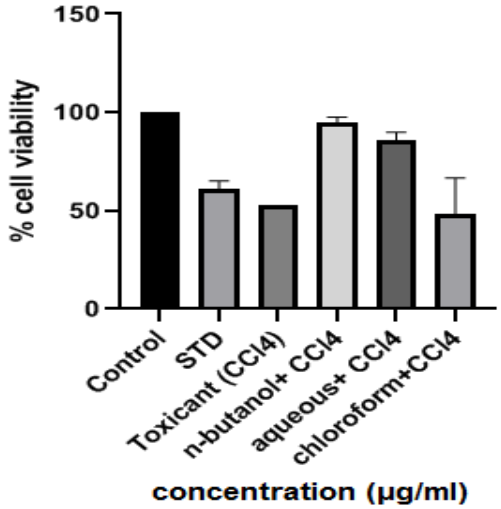

Fig9: graph of % viability of hep G2 cells with different conc. of *DHHAE* extract fractions (F2) of *D. Hamiltonii*

3.6 Molecular docking studies:

In LC-MS of *D. hamiltonii*, 51 compounds were identified of which 19 compounds were docked including active binding sites of TGF-β, NF-κβ, iNOS, and COX-2 enzymes and their binding energies were found between 40.10 – 105.15 Kcal/mol as shown in **Table 9**. These identified showed various binding interactions including Vander walls interaction, hydrogen bond interactions, pi-pi bond interactions and hydrophobic bond interactions with the active binding cavities of the targeted enzyme. Asp351, Asn338, Lys232, Ala350, Val219, Leu340, Asp400, Ala403, Tyr378, Asn338, Gly212, Lys335, Lys213, Lys337, Asp351, Arg215 and Asp333 amino acid reissued of TGF-beta receptors were involved in the formation of binding interactions with identified compounds **Table 10**. Similarly, Ser508, Asp336, Ala545, Thr399, Glu504, Asn40, Gly401, Asp636, Tyr538, Val509, Arg351, Asn547, Asn544, Arg633, Leu548, Asn400, Ser546, Glu638 amino acids residues of NF-kB were formed binding interactions with the identified compounds **Table 11**. Amino acid residues of iNOS enzyme Asn364, Tyr483, Trp188, Phe363, Leu203, Ala237, Ala191, Ile238, Thr184, Cys194, Tyr485, Arg193, Arg260, Ser236, Val346, Ala345, Pro344 were involved in the formation of binding interactions with identified compounds. Interestingly, compounds identified from plant extracts were involved in the formation of various binding interactions with active binding amino acid residues ofCyclooexgenase-2 enzymes *viz* Ala203, Thr207, Leu391, Leu392, Trp388, His208, Ala200, His389, Ser531, Leu535, Phe210, Ala528, Ser534, Leu353, Gly527, Val350, Tyr386, Gln204, Ala203 and Tyr386 **Table 12**. Glucocaffeic acid compound showed an excellent docking score of 95.78, 125.78, 162.14 and 120.62 Kcal/mol with all four targeted enzymes and receptors TGF-beta, NF-kB, iNOS and COX-2 respectively as shown in **Fig 9-12**.

Table 9: Binding interactions of the identified compounds with TGF-β receptor

Compounds	Docking score (Kcal/mol)	Binding residue	H-bond distance (Å)
Glucocaffeic acid	95.7852	O9-Lys335 H30-Asp33 O8-Arg215 O2-Gly212 O7-Gly212	1.47396 2.37539 1.8962 2.67096 2.85978
Apigenin	71.9034	O5-Lys335 O3-Gly212	1.56146 2.34226
Ivalin	70.7005	H19-Lys337 O3-Lys335	2.15739 2.12797
2-phenyl ethanol	68.2097	H13-Asp400	2.26468

Vanillin	53.3756	O3-Arg215 O3-Arg215	2.42714 2.36798
Grandiflorenic acid	52.8321	H23-Lys337 H23-Asn338 O2-Gly214	2.04863 2.85475 2.9358
Ethyl salicylate	48.7038	O2-Asn338 H1-Lys337	2.12047 1.97356
2-hydroxy-4 methoxybenzaldehyde	47.14	O2-Asn338 H12-Asn338 H16-Asp351 H12-Asp351 O3-Lys232 O3-Lys232	2.23152 2.98389 2.76817 2.10612 2.65662 2.65662
Methyl salicylate	45.9257	H12-Asp351 H12-Asp351 H17-Lys337	2.57 2.47802 1079596
Isocinnamic acid	44.6764	O2-Asn338 H12-Asn338 H12-Asp351	2.4775 2.359 2.34027
4-methoxy benzaldehyde	43.252	H17-Asn338 O2-Gly212	2.24184 2.25581
4-methyl benzaldehyde mol	41.793	O1-Lys335 O1-Lys335	2.29676 2.86832
Salicylaldehyde	38.2695	O1-Asn338 H10-Asp351	2.40727 2.05736

Table 10: Binding interactions of the identified compounds with NF-κβ receptor

Compounds	**Docking score (Kcal/mol)**	**Binding residue**	**H-bond distance (Å)**
Glucocaffeic acid	125.786	O3-Asn547 H25-Ser546 H25-Ser546 O5-Arg633 O1-Arg351 H30-Asn400	2.19765 2.3458 2.13775 2.75076 1.99958 2.20161
Ellagic acid	103.189	H24-Asn544 H26-Asn544 O7-Ala545 O1-Arg351 O3-Arg351	3.08648 2.24759 2.88935 2.43109 3.0443
Apigenin	101.185	H23-Asn547 O5-Asn547 H21-Asp636 C12-Als545 H22-Ser508	2.36893 1.92464 2.66753 2.82632 2.78332
Ivalin	93.1024	O2-Ala545 H26-Asn544	2.93634 2.1064
Alpha atlantone	90.7089	O1-Arg633 O1-Arg633	1.91959 2.02433

Ethyl salicylate	79.8072	O2-Arg633 O3-Arg633 O3-Arg633	2.51543 1.9168 1.99686
Grandiflorenic acid	79.1804	O2-Arg351 O2-Arg351	1.14738 2.89252
4-methoxy catechol	77.9592	H15-Ser508 O2-Ser508 H16-Asn544	2.65565 2.95946 3.00888
2-hydroxy-4 methoxybenzaldehyde	73.3057	O3-Asn547 H12-Arg351	2.53581 2.57688
Vanillin	73.1126	O3-Arg351 O3-Gly352 O2-Arg633	2.41001 2.90205 1.75766
Methyl salicylate	72.2203	O1-Asn547 O3-Asn547	2.32418 2.49842
Kaurenoic acid	68.3865	O1-Arg351	1.66944
4-methoxy benzaldehyde	65.8093	O2-Asn547 O2-Arg351	2.50691 2.51627
Salicylaldehyde	63.756	O1-Arg633 H10-Asp636	1.98958 2.34114
4-methyl benzaldehyde	57.1937	H17-Asp636	2.33533
Borneol	48.152	H12-Ser546 O1-Ala545 H14-Glu638	2.96106 2.97974 2.50083

Table 11: Binding interactions of the identified compoundswithiNOSenzyme

Compounds	**Docking score (Kcal/mol)**	**Binding residue**	**H-bond distance (Å)**
Glucocaffeic acid	162.144	O3-Ser236 O3-Gly365 H30-Tyr483 O9-Tyr483 O9-Phe482	2.33406 3.03221 2.95986 2.07039 2.97321
Flavine mononucleotide (FMN)	157.325	O6-Tyr483 O4-Val346	2.51836 2.90924
Silymarin	142.122	H39-Thr184 H39-Tyr483 O10-Asn348 H57-Gln257 H38-Asn364 O6-Gly365	2.46092 2.26895 2.77635 2.7691 2.03891 2.6346
Diosmentin	138.415	H40-Thr184	2.922
Decalepin	137.734	H43-Asn364 H40-Gln257 O11-Ser256	2.50577 3.04255 2.65213

		O10-Ser256 H39-Asn348	2.77107 1.75219
Piceatannol 4'-galloylglucoside	137.055	H46-Ala237 H46-Asn364 H42-Arg193	2.45177 2.14004 3.09626
Phenylethyl	135.334	H45-Pro344 H35-Pro344	2.92919 2.07028
Apigenin	120.813	H21-Asn364 H22-Thr184	2.47139 2.70521
Peumoside	115.548	H64-Tyr483 H51-Asn348 H44-Ser256	2.74156 2.51499 1.66608
Epicatechin 3-O-(4-o-methylgallate)	113.956	O7-Arg193 H35-Gln257 H38-Asn364	2.72215 2.90143 2.30864
Ellagic acid	106.672	H25-Asn364	2.23331
Epicatechin-(4beta-8)-gallocatechin	96.7944	H50-Asn304 O13-Ala191	2.73696 2.8295
Cyanidin 3-rhamnoside	93.8615	O13-Pro344 O13-Ala345 H58-Tyr485 O15-Trp188	2.39633 2.64228 3.00526 2.64312
4-methoxy catechol.mol	93.182	H15-Ala237 H15-Asn364	2.41797 2.55415
2-hydroxy-4 methoxybenzaldehyde	85.9169	O3-Tyr483 O2-Asn364	2.03812 2.79095
Vanillin	85.6395	H12-Ala237 H12-Asn364	2.86773 1.99883
Methyl salicylate	82.0063	O3-Gly365 O3-Gly365 O3-Ser236 O2-Ser236	3.04761 2.93877 2.813 2.62467
Grandiflorenic acid	80.5565	O2-Cys194 O2-Arg193	2.79087 2.53575
Isocinnamic acid	78.4851	O2-Tyr483	2.20933s
Salicylaldehyde	77.1603	O2-Tyr483 O1-Asn364	2.05992 2.79446
4-methoxy benzaldehyde	74.88	O1-Trp188 O2-Tyr483	2.98373 1.95683
4-methyl benzaldehyde	69.4798	O1-Trp188	2.88663
Benzyl alcohol	64.1606	O1-Trp188	2.85338
Borneol	41.0623	H14-Tyr485	2.47619

Table 12: Binding interactions of the identified compounds with COX-2 enzyme

Compounds	Docking score	Binding residue	H-bond distance

	(Kcal/mol)		(Å)
Glucocaffeic acid	120.627	H25-Ser354 H27-Leu353	2.70836 2.04016
Ivalin	107.517	H19-Gln204 O3-His208 H26-Tyr386	2.86138 2.46392 2.19277
Ellagic acid	107.367	H26-Ala200 H24-Ala200	2.79437 2.52698
4-methoxy catechol	84.2429	H28-Ala200	2.29784
Ethyl salicylate	81.053	H13-Ala203	2.48799
Methyl salicylate	74.554	H12-Ala203	2.87587
Isocinnamic acid	74.0148	O2-His208 H13-Tyr386	2.84423 2.71489
22-hydroxy-4 methoxybenzaldehyde	73.9859	H12-Ala203 H16-Thr207 H17-Trp388	2.35835 2.46128 2.42617
vanillin	73.2434	O3-His208 H16-His208 H18-Trp388	2.96946 2.52053 2.83921
4-methoxy benzaldehyde	70.5049	O2-His208	2.78538
4-methyl benzaldehyde	65.1323	O1-His208	2.97401
Salicylaldehyde	63.5422	H10-Ser531 O1-Tyr386	1.91683 2.28193
Benzyl alcohol	60.8285	H10-Ser351	2.66287

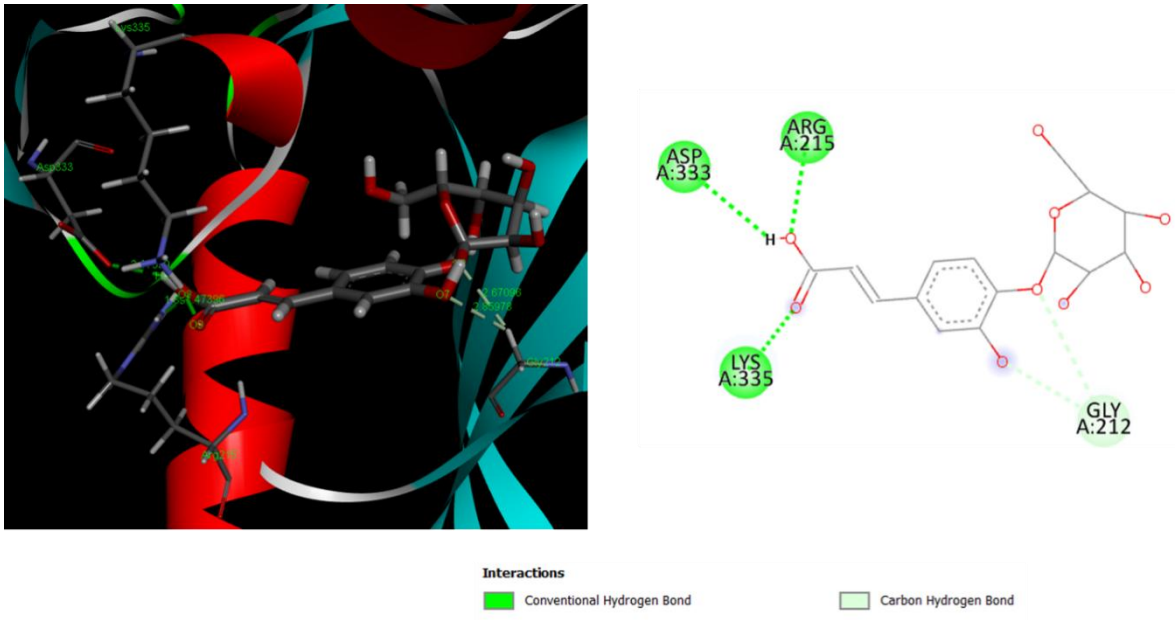

Fig10:3D and 2D posed shows binding interactions of Glucocaffeic acid with TGF-βreceptor

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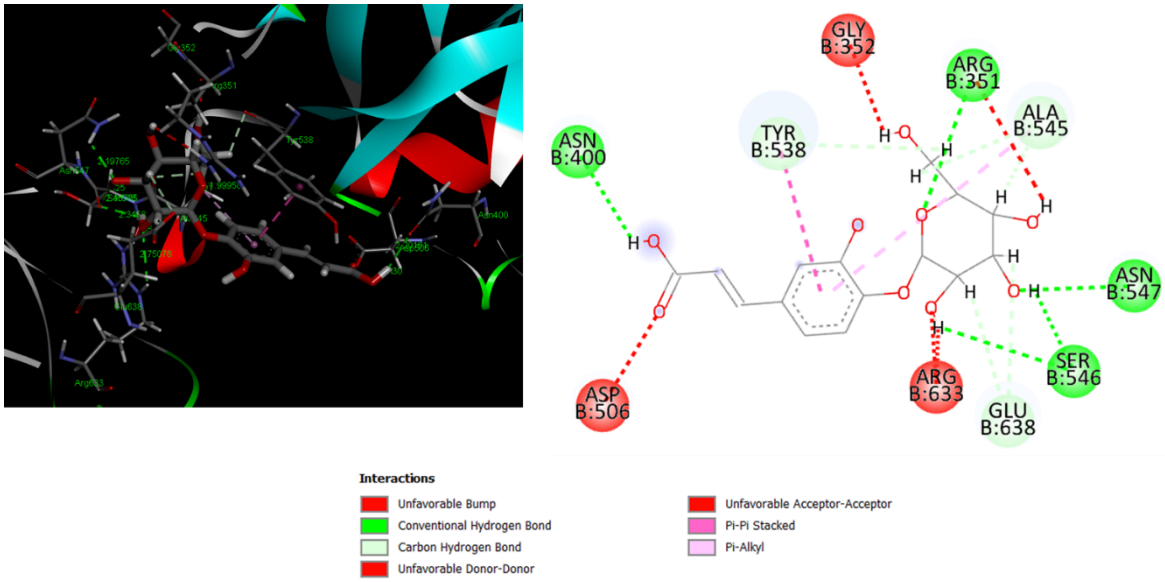

Fig 11: 3D and 2D posed shows binding interactions of Glucocaffeic acid with NF-κ β receptor

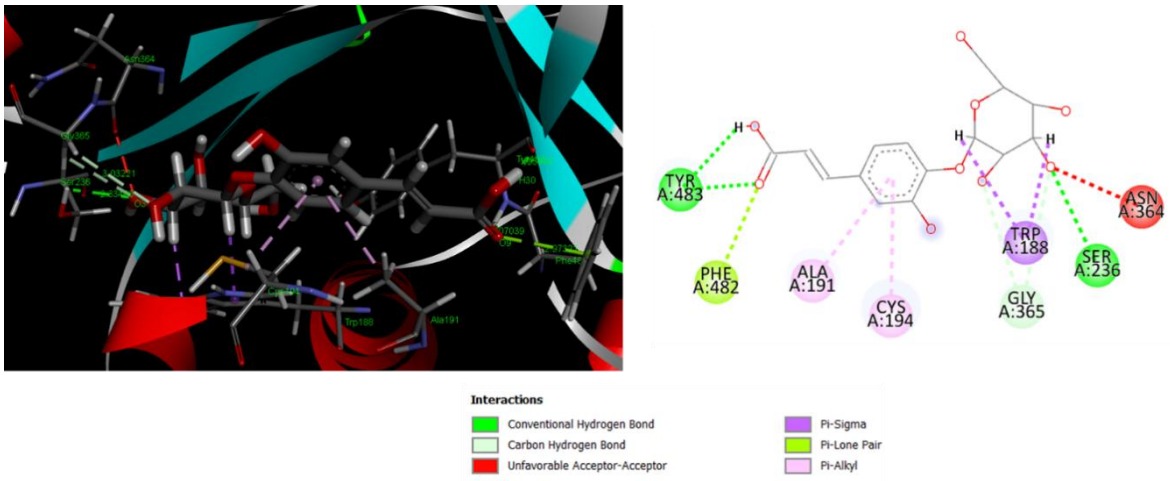

Fig 12: 3D and 2D posed shows binding interactions of GlucocaffeicacidwithiNOS enzyme

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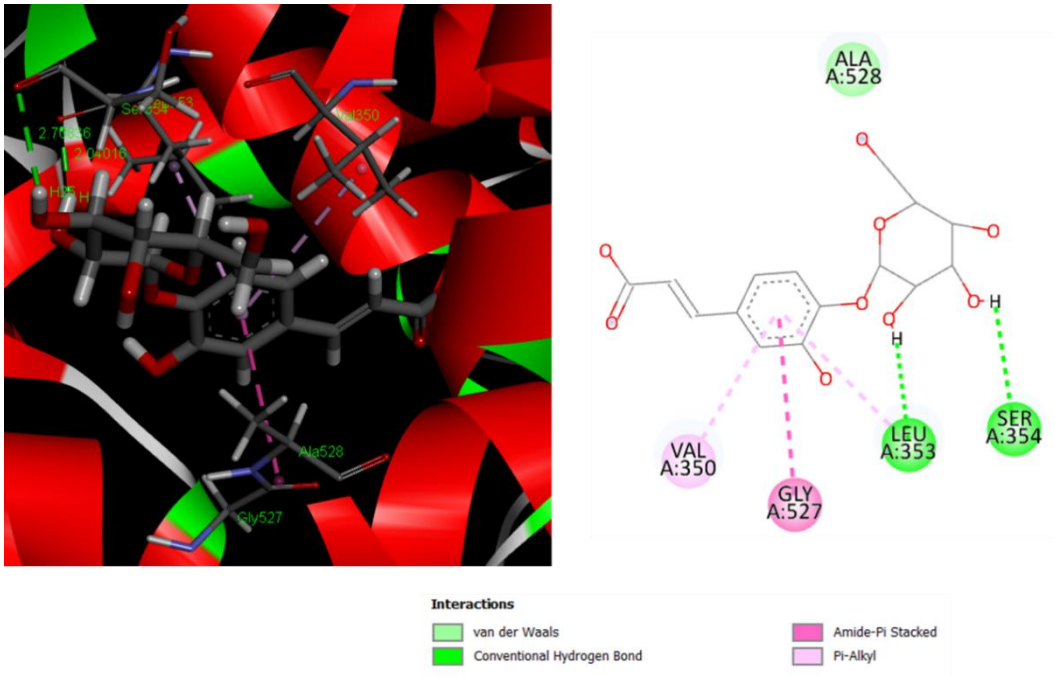

Fig 13: 3D and 2D posed shows binding interactions of Glucocaffeic acid with COX-2 enzymes

4. Discussion:

D. Hamiltonii has shown to have Hepatoprotective activities in traditional medicine [24,25]. In ayurvedic system the roots are being used to relieve pain, flatulence reliever and as tonic [26], Vitalizer and blood purifier, skin disease, fever, nutritional disorder [28] epilepsy treatment, CNS disorders [27], Rejuvenate the body [28,29], infusion for wound healing and bronchial asthma[31], diuretic property [32]. Consequently, efforts have been made to substantiate the conventional statements made on *D. Hamiltonii* pertaining to its utilization in the management of liver cirrhosis. It is well known that several compounds from various classes can be found in plants or their extracts. The majority of them, including flavonoids, steroids, triterpenoids, and their glycosides and alkaloids, are significant from a pharmacological perspective. The extract HADHE was therefore subjected to a preliminary phytochemical screening. According to a preliminary analysis of the HADHE, it contains steroidal/triterpenoidal and flavonoidal glycosides, saponins, and phenolic compounds, among other chemical elements of pharmacological value.200 g/ml was discovered to be the C50 value. As a result, the portion concentration of 50,100,200 g/ml was chosen. It was determined that 200 g/ml of DHHAE was the 50% inhibitory concentration. Further cytoprotective effects of the extracts (50, 100, and 200 g/ml) were determined using CCl4 induced cell toxicity. The metabolic process for CCl4 is carried out by liver alcohol dehydrogenase. Liver alcohol dehydrogenase is responsible for CCl4 metabolism. The

probable mechanism for hepatoprotective of DH might be ability to inhibit lipid peroxidation. Nevertheless, the microsomal electron transfer system also takes part in CCl4 oxidation via cytochrome P 450 (2E1, 1A2, and 3A4 isoforms), which generates free radicals causing cell damage, under conditions such as extract have some functions in preserving the structural integrity of cellular membrane alcohol abuse. Numerous natural plants/plant products have been shown in HepG2 cells to be hepatoprotective against CCl4-induced toxicity[20,28,29] Cell viability was drastically reduced (from 100% to 53.06%) after treatment with 100 mM CCl4. However, more research into the active phytoconstituents and biochemical mechanisms underlying *D. hamiltonii* hepatoprotective effect is required.

5. Conclusion

The butanol fraction of *D. Hamiltonii* demonstrated significant free radical scavenging activity and protect against oxidative injury. The mechanism underlying such a requirement might be owing to the existence of a high phenolic content. So, more research is being conducted by isolating pure phenolic compounds from butanol fractions and rescreening there in vivo hepatoprotective potential in animal models.

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Declaration of Competing Interest

The authors declares that they have no known financial or interpersonal conflicts that might have looked to have influenced the research presented in this study.

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