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HPLC, GC-MS Analysis, Hepatoprotective and Antioxidant Activities of *Saussurea hypoleuca* spreng. root

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Abstract

The current study was designed to investigate HPLC and GC-MS analysis of *Saussurea hypoleuca* root extract with hepatoprotective and antioxidant activities against paracetamol (PCM) induced liver toxicity in Wistar rats. The root methanolic and ethyl acetate (E.A) extracts (400 mg/kg) were administered orally along with standard and control groups by recommended methods for 14days. Serum biochemical tests were performed after blood sampling at 15th day which has shown that PCM intoxicated liver, drastically elevated the levels of SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamic pyruvic transaminase), ALP (alkaline phosphatase), DB (Direct bilirubin), TB (Total bilirubin), GGT (Gamma glutamyl transpeptidase), TC (Total cholesterol), LDL (low density lipoprotein), VLDL (very low density lipoprotein) and MDA (Malondialdehyde) while reduced the levels of albumin, HDL (High density lipoprotein), TP (Total protein) and GSH (Glutathione) in comparison to normal rats. Co administration of *Sausssurea* root extracts and silymarin significantly normalize these heptomarkers by reducing the drastically increased levels of SGOT (79.9 IU/mL), SGPT (29.3 IU/mL), ALP (211.9 IU/mL), DB (0.4 mg/dL), TB (0.5 mg/dL), GGT (20.2 IU/mL), TC (135.9 mg/dL), LDL (73.6 mg/dL), VLDL (24.02 mg/dL), MDA (35.2 µg/mL) and increased the levels of albumin (38.2 mg/dL), HDL (7.0 mg/dL), TP (2.7 g/L) and GSH (109.9 µg/mL) which directed that plant root has strong hepatoprotective and antioxidant activities. HPLC and GC-MS profiling indicated the presence of various phytochemicals. All these verdicts entitled that plant root holds resilient hepatoprotective and antioxidant activities.

Keywords: Hepatoprotective; Antioxidant; HPLC; GC-MS and *Saussurea hypoleuca*.

1.Introduction

Liver is a main organ of the body where detoxification, metabolism and excretion of numerous exogenous and endogenous substances occur. Liver disorders are very common worldwide while development of safer hepatoprotective agents remain an unmet need. Hepatotoxicity can be caused by the exposure to different chemicals among which paracetamol is very common. Children and pregnant women are more vulnerable to Paracetamol tempted liver toxicity. Treatment of choice for hepatic disorders are controversial because clinically available drugs are not effective significantly and produce some adverse effects [1]. Several currently existing drugs were based directly or indirectly upon natural origion. In previous years, researchers pay attention towards the scientifically proved herbal medicine to cure different aliments. Due to their effectivness, negligible adverse effects and low cost, medicinal plants are widely used although their bioactive compounds were not known [2].

Saussurea hypoleuca (Asteraceae) locally known as Qust. This plant root has been selected on its ethanotherapeutical worth and medicinal uses in native population as a liver tonic. There is no scientific data available on this root except a few studies which are done on poly herbal formulation containing *Saussurea* as a one ingredient [3]. Proximate analysis has shown that *Saussurea* root contains a lot of phytochemicals [4]. Present study was conducted to document HPLC and GC-MS analysis of *Saussurea hypoleuca* root with hepatoprotective and antioxidant activities.

2. Materials and methods

2.1. Plant collection and extraction

Saussurea root has been collected from Quetta, Baluchistan, Pakistan in September 2016 and verified

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by taxonomist, Prof Dr. Zaheer - ul - deen, Department of Botany, GC University Lahore, Pakistan. Sample has been kept under voucher number of GC. Herb. Bot. 3453 in GC University herbarium museum, Lahore. Plant root was pulverized after drying under shade. Methanolic extract was made by cold maceration using rotary evaporator under reduced pressure at 45-50 C° (Heidolph, model Laborata 4000, Schwabach, Germany). Fractionation of the methanolic extract was performed with different solvents respective to their polarity. Active fraction was selected by analytical TLC Kieselgel 60 F254 (Merck) 0.25mm thickness which was further subjected to column chromatography for isolation of pure compounds. Each fraction was dried and preserved for different biological activities [5].

2.2. Animals

Thirty Albino Wistar rats of either sex (150-200gm) were acquired from Department of Pharmacology, University College of Pharmacy, University of the Punjab, Lahore, Pakistan. They were kept in metal cages at controlled RT; 25 ± 2˚C and relative humidity; 55-65%. Each animal was free to food and water *ad libitum.* They were acclimatized in laboratory conditions for one week before the start of the experiment. Experiment was done on rats under the rules guided by institutional committee for animals handling and care under voucher no.88.

2.3. Chemicals

Silymarin was purchased from local pharmacy and PCM powder were obtained from Pacific Pharma Limited, Lahore, Pakistan. The kits for the assay of serum enzymes were provided by Sigma Aldrich.

2.4. Hepatoprotective and in vivo antioxidant activity

Wistar rats were divided into five groups having six animals in each group as described with little modifications [6]. Group I served as normal and received distilled water (DW) only. Group II was toxic and received (500 mg/kg orally administered PCM). Group III was served as standard (silymarin 100 mg/kg + PCM 500 mg/kg orally). Group IV & V were experimental (E.A 400 mg/kg + PCM 500 mg/kg and methanol 400 mg/kg + PCM 500 mg/kg orally) respectively. Treatment persists for 14 days.

2.5. Collection of blood sample

On 15th day, animals were dissected under light ether anesthesia after last dose and blood was collected by cardiac puncture. Serum was separated by centrifugation at 4000 rpm for 20 minutes.

2.6. Serum biochemical assessment

Serum was analyzed for assessment of all liver function test and lipid profile as reported by [7].

2.7. In vivo antioxidant activity

After sacrificing the rats, livers were immediately washed with normal saline and preserved in ice cold saline at -81˚C till quantification of antioxidant enzymes. 200 mg of liver tissue was detached, cut into pieces and dipped in Tris HCI buffer (pH 7.4), washed and dipped it into ice cold potassium chloride solution, again washed and dipped in phosphate buffer and then add 8 mL of EDTA. This tissue was homogenized, centrifuge it for 10 minutes at 10,000 rpm at 4C˚. Collect this supernatant in another eppendrops, centrifuge it again at 5000 rpm at 4C˚ for 10 minutes. Supernatant was collected in new clean eppendrops tubes and this supernatant was saved in chiller (-81˚C) for future experimental use. Quantification of GSH and MDA were assayed in rat liver homogenates [8,9].

2.8. Histopathological examination

Liver specimen preserved in 10% formalin solution were dehydrated, cleared, and embedded into paraffin blocks. Paraffin sections were cut and stained with hematoxylin and eosin dye to report histopathology [10].

2.9. HPLC analysis of phenolic acids and flavonoids

Methanol and active fractions (E.A and chloroform) were analyzed by HPLC using phenolic acid and flavonoid standards as reported method [11]. HPLC Agilent 1100 series furnished with Column C18 (4.6 * 2.55 mm) was used for analysis. Each sample (5mg/1mL) was prepared with HPLC grade methanol. Samples were filtered with syringe filter (0.22 µm) and 20 µL of each sample volume was injected into HPLC column at the temperature of 30˚C. Analysis was performed at flow rate 1mL/min using 60% water: 20% methanol: 20% acetonitrile mobile phase and UV- visible detector. Identification was documented by comparing the chromatogram with the known standard used in the same conditions.

2.10. GC-MS analysis

GC-MS is a common confirmation test for effective chemical analysis to provides representative spectral output of all those compounds which get separated from the test sample. It is combination of two techniques, Gas chromatography separates the components of the mixture and mass spectroscopy analyze each of the components distinctly. Agilent GC-MS 2010 with DB5 (30m* 250µm) capillary column was used for screening. Sample was injected into the injector port of GC device which vaporize the sample and separate it into various compounds.

Each compound has a specific peak which was recorded electronically and matched with standard spectrum stored in National Institute Standard and Technology (NIST) library. Compound name, retention time, molecular formula and structure were ascertained [12].

3. Statistical analysis

Results expressed in Mean ± SD; p< 0.001 considered to be significant. Data was analyzed by using student t-test followed by one-way ANOVA and Dunnett's multiple comparison test using graph pad prism version 5.01 software. Microsoft Excel 2013 was used for the calculation of all observed study data as well as for standard and regression curve analysis.

4. Results

4.1. Hepatoprotective and in-vivo antioxidant activity

Methanol and E.A were selected for hepatoprotective and *in vivo* antioxidant activity conducted for 14 days at the selected dose of 400 mg/kg based upon the results of previous acute and chronic pharmacological studies. PCM intoxicated liver, drastically elevate the levels of SGOT, SGPT, ALP, DB, TB, GGT, TC, TG, globulin, LDL, VLDL and MDA while reduced the levels of albumin, HDL, TP and GSH compared to normal rats. Administration of extracts and silymarin significantly normalizes these parameters as presented in Tables 1 & 2 and Fig. 1.

Table 1: Hepatoprotective effects of methanol and E.A extract in rat liver biochemical markers

Parameters	Normal Mean ± SD	Toxic Mean ± SD	Standard Mean ± SD	Methanol Mean ± SD	E.A Mean ± SD
SGOT(IU/mL)	67.6 ± 3.4	131.9$^{a\delta}$† ± 2.8	71.3$^{a\delta}$ ± 0.9	77.0$^{a\delta}$ ± 0.6	79.9$^{a\delta}$ ± 1.9
SGPT(IU/mL)	16.8 ± 1.0	40.8^{b}† ± 1.1	23.7$^{a\delta}$ ± 1.2	26.5$^{a\delta}$ ± 0.7	29.3$^{a\delta}$ ± 0.5
ALP(IU/mL)	168.2 ± 2.7	240.4^{a}† ± 1.5	200.8$^{a\delta}$ ± 8.1	204.8$^{a\delta}$ ± 2.1	211.9$^{b\delta}$ ± 1.2
DB (mg/dL)	0.1 ± 0.0	0.6^{a}† ± 0.0	0.3$^{a\delta}$ ± 0.0	0.3$^{a\delta}$ ± 0.0	0.4$^{b\delta}$ ± 0.0
TB (mg/dL)	0.3 ± 0.0	1.0^{a}† ± 0.0	0.4$^{a\delta}$ ± 0.0	0.5$^{b\delta}$ ± 0.0	0.5$^{b\delta}$ ± 0.0
TP (mg/dL)	7.8 ± 0.0	4.9^{a}† ± 0.0	7.5$^{a\delta}$ ± 0.0	7.2$^{a\delta}$ ± 0.0	7.0$^{a\delta}$ ± 0.0
GGT(IU/mL)	4.3 ± 0.0	35.5^{a}† ± 1.2	14.9$^{c\delta}$ ± 0.7	17.7$^{b\delta}$ ± 0.6	20.2$^{\delta}$ ± 1.2
Globulin(g/L)	164.0 ± 1.0	235.4^{a}† ± 2.1	186.0$^{a\delta}$ ± 2.4	191.9$^{a\delta}$ ± 2.5	200.1$^{a\delta}$ ± 3.4
Albumin(g/L)	3.1 ± 0.0	2.1^{b}† ± 0.1	3.0$^{a\delta}$ ± 0.0	2.8$^{a\delta}$ ± 0.0	2.7$^{a\delta}$ ± 0.0
TC (mg/dL)	105.3 ± 2.7	187.1^{a}† ± 3.5	124.8$^{a\delta}$ ± 2.1	135.8$^{b\delta}$ ± 1.7	135.9$^{b\delta}$ ± 2.9
TG (mg/dL)	57.6 ± 2.2	151.4^{a}† ± 2.1	106.7$^{c\delta}$ ± 2.3	118.7$^{\delta}$ ± 1.2	120.1$^{\delta}$ ± 2.2
HDL (mg/dL)	53.9 ± 0.7	24.3^{b}† ± 1.31	43.4$^{b\delta}$ ± 0.8	40.4$^{b\delta}$ ± 1.6	38.2$^{c\delta}$ ± 1.0
LDL (mg/dL)	39.9 ± 2.4	132.5^{a}† ± 4.282	57.1$^{a\delta}$ ± 8.5	71.3$^{c\delta}$ ± 2.9	73.6$^{\delta}$ ± 3.2
VLDL (mg/dL)	11.5 ± 0.4	30.2^{b}† ± 0.4	21.3$^{a\delta}$ ± 0.4	23.7$^{b\delta}$ ± 0.2	24.0$^{b\delta}$ ± 0.4

Results are expressed in Mean ± SD; n=6. ^{c}P<0.05, ^{b}p<0.01 & ^{a}p< 0.001 considered to be significant. One-way ANOVA variance followed by Dunnett's multiple comparison test was performed using graph pad prism soft wear. † Compared with normal group while $^{\delta}$ comparison of all groups with toxic group.

Table 2: Quantification of in-vivo antioxidant markers in rat liver

Parameters	Normal Mean ± SD	Toxic Mean ± SD	Standard Mean ± SD	Methanol Mean ± SD	E.A Mean ± SD
GSH (µg/mL)	104.251 ± 7.712	73.677^{b}† ± 4.041	105.941$^{a\delta}$ ± 2.598	108.354$^{b\delta}$ ± 4.896	109.95$^{b\delta}$ ± 2.077
MDA (µg/mL)	22.769 ± 1.3	47.59 ^{a}† ± 2.737	31.72 $^{a\delta}$ ± 1.827	37.96$^{b\delta}$ ± 2.373	35.246$^{c\delta}$ ± 0.332

Results are expressed in Mean ± SD; n=6. ^{c}P<0.05, ^{b}p<0.01 & ^{a}p< 0.001 considered to be significant. One-way ANOVA variance followed by Dunnett's multiple comparison test was performed using graph pad prism soft wear. † Compared with normal group while $^{\delta}$ comparison of all groups with toxic group.

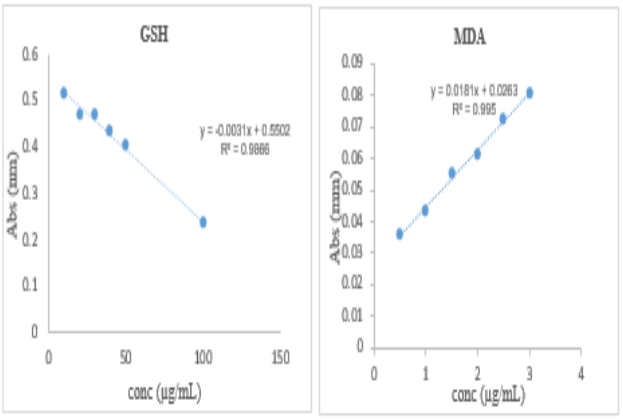

Fig. 1: Standard calibration curves of GSH & MDA markers

4.2. Histopathological observations

Histopathological observations indicate the necrosis, congested central veins, biliary obstruction and bleeding in hepatic lobes in PCM intoxicated liver (Fig, 2; panel, B) as compared to normal control group (Fig. 2; panel, A). Treatment with silymarin and experimental extracts effectively attenuated the PCM induced toxicity, significantly normalizes these biochemical markers with full recovery (Fig. 2; panel, C, D & E).

In Fig. 2: Histogram A showed normal hepatocytes and central veins, histogram B showed PCM injured tissue necrosis and degenerative changes along with dilated sinusoidal spaces. Histogram C showed full recovery treated with silymarin. Histogram D & E also showed improved recovery all congested veins and degenerative changes treated with methanol and E.A respectively.

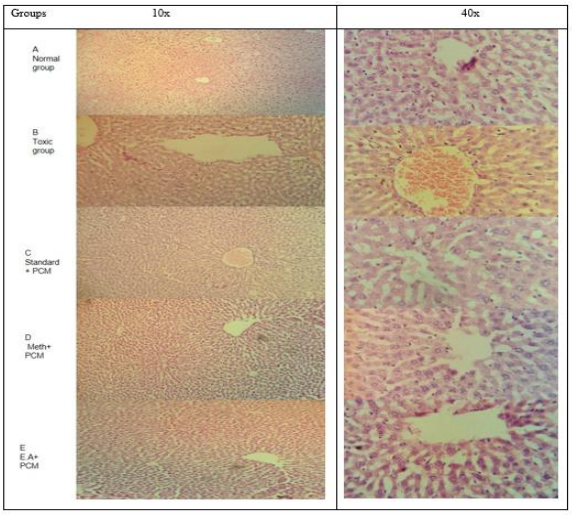

Fig. 2: Histopathological examination of rat livers at 10x & 40x (A-E)

4.3. HPLC analysis of phenolic acids and flavonoids

Root methanolic extract and active fractions were standardized by HPLC according to the protocols to ascertain the presence of phenolic acids and flavonoids against their standards as depicted in underlined chromatographs (Figs. 3 & 4) and Tables 3 & 4. Results revealed that methanol contains caffeic acid and sinapic acid while chloroform and E.A contain caffiec acid (Fig. 3). Flavonoids traced in methanol, chloroform and E.A were quercetin, myricetin and kaempferol (Fig. 4).

Table 3: RT and wavelength of HPLC phenolic acids standards

Sr. No.	Peak Name	Retention Time (RT) (min)	Wavelength (nm)
1.	Caffeic acid	1.9	280
2.	Gallic acid	11.4	280
3.	Sinapic acid	13.3	280

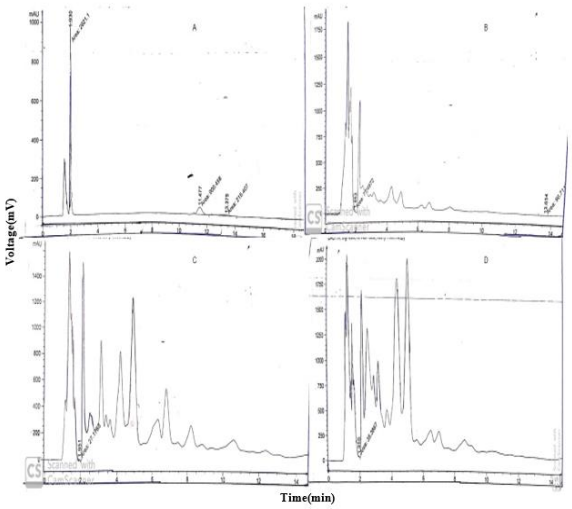

Fig. 3: Chromatographs of phenolic acids standard (A), methanol (B), chloroform (C) & E.A (D) of Saussurea root.

Table 4: RT and wavelength of HPLC flavonoids standards

Sr. No.	Peak Name	Retention Time (RT) (min)	Wavelength (nm)
1.	Quercetin	4.3	360
2.	Myricetin	8.1	360
3.	Kaempferol	14.5	360

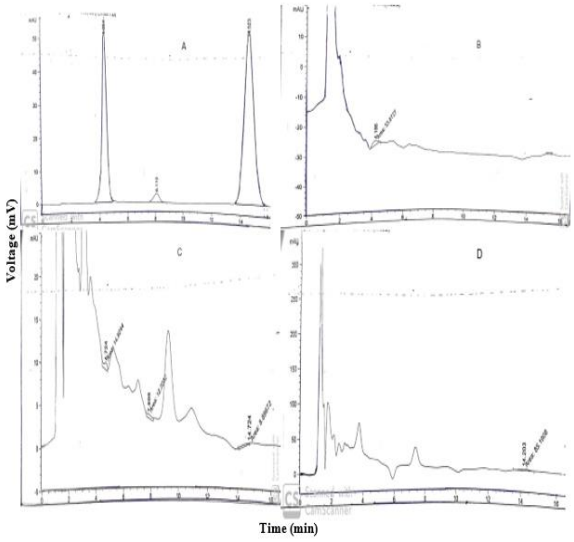

Fig. 4: Chromatographs of flavonoids standard (A), methanol (B), chloroform (C) & E.A (D) of *Saussurea* root

4.4 GC-MS Screening

Components separated with mobile phase n-hexane: chloroform (40:60, 50:50 & 90:10) from column chromatography were analyzed by using GC-MS apparatus are illustrated in Tables 5-7 and in Fig5-7.

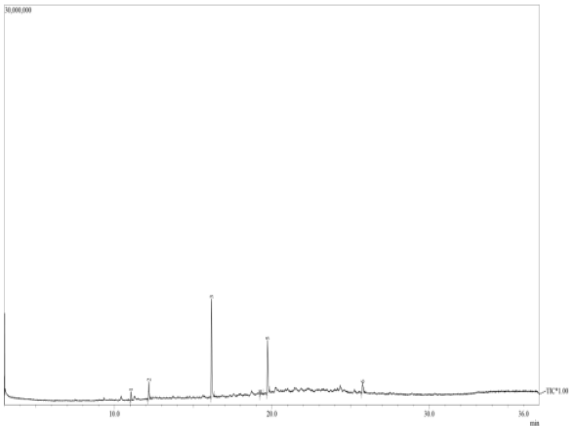

Fig. 5: GC-MS screening of (40:60) n-HF

Table 5: Components detected in (40:60) n-HF by GC-MS screening

Sr. No.	SI	R.Time	Mol.Wt	Formula	Compound Name	Structure
1.	92	11.042	270	$C_{17}H_{34}O_2$	Pentadecanoic acid	
2.	91	12.192	296	$C_{19}H_{36}O_2$	11-Octadecenoic acid	
3.	95	16.167	390	$C_{24}H_{38}O_4$	Di-n-octyl phthalate	
4.	43	19.258	394	$C_{28}H_{42}O$	3-alpha,5-alpha-Cyclo-ergosta-7,9(11),22t-triene-6-beta-ol	
5.	88	19.733	426	$C_{26}H_{50}O_4$	Decanedioic acid	
6.	80	25/767	412	$C_{30}H_{52}$	Tetracosapentaene	

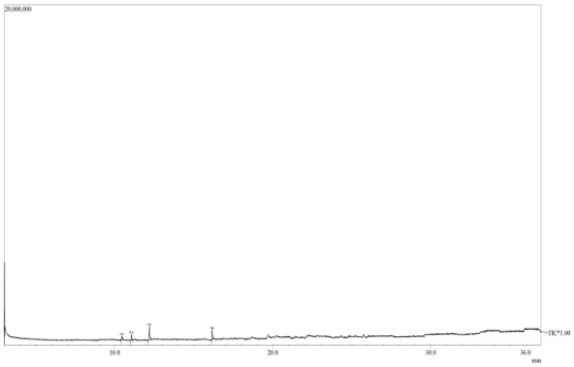

Fig. 6: GC-MS screening of (50:50) n-HF

Table 6: Components detected in (50:50) n-HF by GC-MS screening

Sr. No.	SI	R.Time	Mol.Wt	Formula	Compound Name	Structure
1.	87	10.442	270	$C_{17}H_{34}O_2$	Isopropyl Myristate	
2.	88	11.058	270	$C_{17}H_{34}O_2$	Pentadecanoic acid	
3.	92	12.192	296	$C_{19}H_{36}O_2$	10-Octadecenoic acid	
4.	92	16.167	390	$C_{24}H_{38}O_4$	Di-n-octyl phthalate	

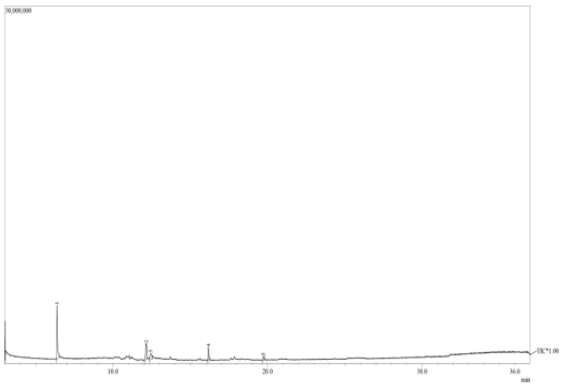

Fig. 7: GC-MS screening of (90:10) n-HF

Table 7: Components detected in (90:10) n-HF by GC-MS screening

Sr. No.	SI	R.Time	Mol.Wt	Formula	Compound Name	Structure
1.	90	6.383	185	$C_{10}H_{19}NO_2$	3 4-hexanedione 2 2 5 5 tetramethyl monoxime	
2.	85	12.158	298	$C_{18}H_{31}ClO$	9,12-Octadecadienoyl chloride	
3.	87	12.450	282	$C_{18}H_{34}O_2$	Oleic Acid	
4.	94	16.175	390	$C_{24}H_{38}O_4$	Di-n-octyl phthalate	
5.	81	19.733	426	$C_{26}H_{50}O_4$	Decanedioic acid	

5. Discussion

Herbs are important sources of innovative medicines because many drugs are indirectly produced from medicinal plants. Plant constitutes a wide collection of chemical compounds with invincible pharmacological activity. But for their complete exploitation intensive efforts need to be taken. Studying of medicinal plants also facilitates to comprehend plant toxicity and also helps to protect human and animals from natural poisons. Hence the present study was undertaken to find out HPLC and GC-MS screening of *Saussurea hypoleuca* root with hepatoprotective and antioxidant activities.

Acetaminophen is commonly used as antipyretic and analgesic agent. Numerous studies were reported on the hepatotoxicity induced by toxic doses of PCM in human and experimental rats [13]. PCM induced cellular damage or necrosis has been used as reliable and most appropriate method for the investigation of hepatoprotective activity of herbal medicines. It is normally metabolized in liver and excreted in the form of sulfate and glucuronide conjugate. When a part of PCM is activated by P-450 cytochrome into toxic metabolites N-acetyl-p-benzoquinoneimine, hepatotoxicity occurs [14]. These toxic metabolites can alkylate and oxidized the intracellular GSH leading to the depletion of liver GSH and repletion of MDA by withdrawing hydrogen from poly unsaturated fatty acids, leading to the hepatic damage. These reactive metabolites were also cause cell stress with reduction of cellular GSH, or binding to enzymes, lipids and further other cellular organelles [15]. SGOT and SGPT specifically located in mitochondria of hepatocytes, so they are good parameter for the evaluation of hepatic damage. Serum level of ALP and bilirubin was also related to

the hepatic injury. PCM intoxicated liver, drastically elevated the levels of SGOT, SGPT, ALP, DB, TB, GGT, TC, TG, globulin, LDL, VLDL and MDA while reduced the levels of albumin, HDL, TP and GSH in comparison to normal rats (Tables 1 & 2). The elevated level of MDA and depletion of GSH in PCM intoxicated liver may be due to the increased mechanism of lipid peroxidation leading to development of oxidative stress and failure of antioxidant defensive system. Decreased level of GSH in liver mitochondria might be the conjugation of GSH with toxic metabolites to form mercapturic acid. Decreased level of MDA and increased level of GSH with extracts treated liver represented that plant root has strong antioxidant potential through regulatory mechanisms on cellular permeability, stability and in reducing oxidative stress. This antioxidant potential may be due to the abundance of phytoconstituents in plant root.

Methanol, E.A and chloroform were evaluated by HPLC against phenolic acids and flavonoids standards as marker compounds (Tables 3 & 4). The components at RT 1.930, 11.477 and 13.375 in phenolic acid standard chromatograph were caffeic acid, gallic acid and sinapic acid respectively while the components at 4.364, 8.110 and 14.523 are quercetin, myricetin and kaempferol respectively in flavonoids standard chromatograph. Methanol, E.A and chloroform were compared with standard chromatographs which have shown that methanol contained caffeic acid, sinapic acid and quercetin, chloroform has caffeic acid, quercetin, myricetin and kaempferol while E.A represented the caffeic acid and kameferol (Figs. 3 & 4). Several studies have reported that the presence of phenolic acids and flavonoids in plant crude extract play vital role in performing pharmacological activities [16]. Phenolic acids having - OH group kill free radicals and act as antioxidant in body when utilized in dietary form [17,18].

GC-MS analysis has shown different components from ethyl acetate fraction of column chromatography (figs.5-7) with n-hexane and chloroform mobile phase. The eluted components reside biological properties. GC-MS of plant extract gives a clear understanding the medicinal value of this medicinal plant root which would be helpful for further detailed investigation. In this work components identified are pentadecanoic acid, 10-octadecenoic acid, 11-octadecenoic acid, di-n-octyl phthalate, 3- alpha, 5-alpha -cyclo -ergosta -7,9 (11), 22t-triene-6-beta-ol, decanedioic acid, tetracosapentaene, isopropyl myristate, 3,4-hexanedione, 2, 2, 5, 5 tetramethyl monoxime, 9,12-octadecadienoyl chloride and oleic acid (Tables 5-7). In current study all these verdicts were accorded with the documented literature [12].

6. Conclusion

Saussurea hypoleuca root holds strong hepatoprotective and antioxidant properties owing to presence of phytochemicals.

Conflict of interest

Authors have no conflict of interest.

Acknowledgement

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