Proximate analysis and *in vitro* biological assays of *Saussurea hypoleuca* Spreng. root

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Abstract: Considering the growing interest in medicinal plants having imperative phytoconstituents, a research has been steered to standardize the crude drug from "*Saussurea hypoleuca* root" by assessing its primary, secondary metabolites and to screened out in vitro biological assays of thrashed plant. Quantitative analysis was done by estimation of the primary and secondary metabolites (total proteins, total carbohydrates, total lipids, total glycosaponins, total alkaloids, total flavonoids, and total polyphenolics) in powder and extracts. The maximum value of total proteins (0.59%), total carbohydrates (53.7%), total lipids (27.12%), total glycosaponins (63.9%), total alkaloids (20.3%), total flavonoids (0.23%) and total polyphenolics (0.919%) were respectively. Antimicrobial assay was done by agar well diffusion method and Minimum Inhibitory Concentration. Haemolytic and DNA protection activity was studied by reported method. Different extracts showed various results however butanol, ethyl acetate, chloroform and methanol give promising results. The results of this present study gives an evidence for the existence of diverse primary and secondary metabolites and thus rationalizes its use in traditional medicines for the cure of different aliments owing to the safety profile on human red blood cells. The conclusions of this research work give an indication that this plant has good potential for antimicrobial activity and has possible pronounced significance as therapeutic agent.

Keywords: Primary and secondary metabolites, antimicrobial, haemolytic and DNA protection.

INTRODUCTION

Nature has been a birthplace of medicinal agents for thousands of years and a huge quantity of contemporary drugs has been insulated from natural source, mainly used is based in traditional medicines (Fawzi, 2013). Medicinal plants have been used for curing and healing of many human diseases because of presence of phytochemical compounds (Yadav *et al*., 2017). Phytoconstituents are primary and secondary metabolites which are naturally present in medicinal plants and work with fibers and nutrients to form integrated part of defensive mechanism to protect human body against various ailments and stress conditions (Daniel and Krishnakumari, 2015). The most important bioactive compounds are alkaloids, flavonoids, tannins and phenolic. Majority of phytochemicals compounds have been known to have valuable pharmacological activities such as anti-inflammatory, analgesic, anticancer, antimicrobial and antioxidant (Shamala *et al*., 2016; Kumari *et al*., 2016). Thus medicinal plants have perspective value due to the phytochemical which they enclosed.

Saussurea hypoleuca is a medicinal plant available in Quetta, Pakistan, belongs to family Asteraceae. This has great potential for commercialization because its root has been used in many folklore medicines. Mostly Asteraceae family plants are herbaceous and have gained popularity in traditional and historical medicinal systems. *Saussurea hypoleuca* is best specie of Asteraceae family. The root has been used in poly herbal formulation for liver disorders. It also has potential for different biological activities due to the presence of phytoconstituents. There are no guidelines available to standardize this crude drug for putting it into modern medicines. Thus the present work has been carried out to find out its phytoconstituents and to investigate the antimicrobial and safety profile of the root extract of *Saussurea hypoleuca* plant.

MATERIAL AND METHODS

Roots of the plant have been collected freshly from Quetta, Baluchistan, Pakistan in September 2016. The specimen was identified by taxonomist, Prof Dr. Zaheer - ul - deen, Department of Botany, GC University Lahore, Pakistan. Specimen has been kept in GC University herbarium museum, Lahore under the voucher number of GC. Herb. Bot. 3453 for further reference. The sample was dried and pulverized into powder. Methanolic extract made by maceration using rotary evaporator under reduced pressure at 45-50C°. Fractionation was performed by different solvent of increasing order of polarity n-hexane, chloroform, ethyl acetate, n-butanol and aqueous. Each fraction of these solvents was dried and preserved for evaluation of different biological activities.

Determination of primary metabolites

Total lipid Contents Determination

15grams of plant powder was subjected in thimble for 24 hours using hot extraction procedure in soxhlet apparatus. Petroleum ether used as solvent at 45-60c° temperature. After extraction and filtration, solvent was dried by rotary

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evaporator, weighed and percentages of lipids contents were determined (Jadid *et al*., 2018).

Total protein contents estimation

Estimation of total proteins contents was carried out by adopting the method (More and Chaubal, 2016). 0.5grams of plant powder was macerated in distilled water with few drops of triton -X for 10hours. Then filtered it and take 10ml of this filtrate into centrifuge machine at 2700rpm for 15minutes. After centrifuge 100µl of supernatant was taken into test tube and volume make up 1ml with distilled water. Then it was treated with three reagents A, B & C. 3ml of reagent C was added in test tube and 200µl FC reagent added. Incubate it for a half hour at room temperature and absorbance was measured at 600nm with (Bovine Serum Albumin) BSA as standard. Reagent C was prepared by 50ml of reagent A and 1ml of reagent B. Reagent B was made by taking 0.5% copper sulphate in 1% potassium tartrate while Reagent A was made by mixing of 2%sodium carbonate in 0.1N sodium hydroxide. Blank contain all the added reagents except sample. Different concentrations of BSA were made in order to plot standard curve. From this standard curve, total protein contents were estimated by using linear regression equation. The sample and the different concentrations of the standard were repeated in triplicates.

Total carbohydrate contents determination

Total carbohydrate contents were determined by applying the formula as prescribed by (Shukla *et al*., 2016). Total carbohydrate contents% = 100- (total ash contents + total moisture contents + total lipids + total proteins)

Determination of secondary metabolites

Total alkaloids, total glycosides, total flavonoids, total poly phenols and total glycosaponins were determined by using the following methods.

Total alkaloidal contents determination

For determination of total alkaloid contents,5 grams of plant powder was soaked in dilute ammonia to convert alkaloidal salt into free base.7.5ml of chloroform was mixed after 15minutes and filtered. The filtrate was taken into separating funnel with addition of 7.5ml of dilute H_2SO_4.The separating funnel shaken thoroughly for the extraction of all alkaloids in acidic layer in salt form (acidic layer). Lower layer (chloroform) was discarded and acidic layer was collected in pre weighed beaker. It was dried and percentage of alkaloidal contents were determined with reference to the sample weight and multiply this values into 100 (Amiri *et al*., 2017).

Total glycosidal contents determination

1gram of plant powder was weighed in tarred 100ml volumetric flask with 10ml of 70% of ethanol in it. Boiled it for 2minutes in water bath, filtered and filtrate was diluted with 20ml of distilled water. Afterward, 3.5ml of 10% lead acetate was added to this volumetric flask to precipitate the chlorophyll, tannins and alkaloids. Filtered it and kept the filtrate into separating funnel with 15ml of chloroform. The funnel was rotated repeatedly. Two layers were formed, lower organic layer was collected (chloroform); dried and weighed. percentage of total glycosidal contents was determined with subject to the test sample (Ugwoke *et al*., 2017).

Total glycosaponins contents determination

For determination of total glycosaponins (USP, 2005), 0.25grams of plant extract and its fractions were dissolved in 12.5ml of methanol and shake them in mechanical shaker for 30 minutes. Filtered them and excess methanol was evaporated to 2.5ml. This concentrate filtrate was added drop wise into 12.5ml of acetone in a pre-weighed Petri dishes. Saponins were appeared in the form of precipitates in Petridis. These were dried in oven at 100c˚. The percentage was calculated by dividing the weighed of saponins precipitates with reference to the sample weigh and multiplied into 100. Test was repeated in triplicate.

Total poly phenolic contents estimation

Estimation of total poly phenolic contents in plant sample and its fractions were done by applying methods as described by (Liaudanskas *et al*., 2017) with little modifications. Gallic acid was used as a standard.1mg/ml stock solutions of both standard and sample were made in methanol. 0.2ml of sample and standard dilutions were added into labeled test tube with 0.2ml of FC (Folin-Ciocalteu's reagent). Mix them completely, followed by the addition of 1ml of 15%Na_2Co_3 after 4 minutes. Methanol served as blank contains the entire reagent except sample. All sample and standard were incubated at room temperature for 2hours and test was performed at 760nm by using double bean UV spectrophotometer. Total poly phenolic contents were calculated from linear regression equation by plotting gallic acid standard curve. Values were expresses as gallic acid mg equivalents per gram of the extract. The readings were recorded in triplicate to avoid errors.

Total flavonoid contents estimation

Total flavonoids were determined from the method as explained by (Tambe and Bhambar, 2014). Quercetin was used as a standard.1mg/ml stock solutions of sample and standard were made. 0.2ml of sample and standard dilutions were taken into labeled test tubes with addition of 0.1ml of 10% aluminum nitrate solution. Afterward 0.1ml of 1M potassium acetate and 4.6ml of distilled water added into these test tubes. All test tubes were incubated at room temperature for 45 minutes and absorbance was taken at 415nm. Blank contains all the reagents except test samples. Calibration curve of quercetin was plotting in order to obtain linear regression equation for the estimation of total flavonoid. Values were expresses as quercetin mg equivalents per gram of the extract. The absorbance was measured in triplicates.

Table 1: Proximate analysis of raw powder of *Saussurea hypoleuca* root

S. No	Parameters	% values (w/w)	
		Mean	SD
1.	Total lipids	27.12	±0.16
2.	Total carbohydrates	53.7	±1.35
3.	Total protein	0.598	±0.003
4.	Total glycosides	24.4	±0.244
5.	Total alkaloids	20.3	±0.249

Table 2: Proximate analysis of various extracts of *Saussurea hypoleuca* root

S. No	Parameters	Total glycosaponins%		Total flavonoids%		Total polyphenolics%	
		Mean	SD	Mean	SD	Mean	SD
1.	Methanol	63.9	±3.53	0.095	±0.004	0.919	±0.002
2.	Chloroform	12.0	±0.15	0.167	±0.002	0.901	±0.003
3.	n-butanol	14.9	±0.064	0.194	±0.001	0.884	±0.002
4.	n-hexane	29.67	±0.5	0.207	±0.002	0.685	±0.006
5.	Ethyl acetate	12.0	±0.15	0.239	±0.005	0.882	±0.003
6.	Aqueous	15.96	±0.15	0.050	±0.001	0.661	±0.002

Table 3: Minimum inhibitory concentration (MIC) of *Saussurea hypoleuca* root

Test microorganisms	Methanol	n-hexane	Chloroform	Ethyl acetate	n-butanol	Aqueous	Powder
S.aureus	0.455	0.481	0.614	0.379	0.207	0.051	0.170
P.aeruginosa	0.301	0.218	0.425	0.310	0.180	0.089	0.142
E.coli	0.385	0.372	0.684	0.387	0.222	0.0727	0.325
S.aeruginosa	0.29	0.025	0.222	0.320	0.289	0.021	0.245
B.subtilis	0.289	0.278	0.321	0.365	0.319	0.091	0.198
C.albicans	0.243	N.D	N.D	0.190	0.201	N.D	0.045
P.notatum	0.198	0.090	N.D	N.D	0.145	0.076	0.189

N.D means not detected

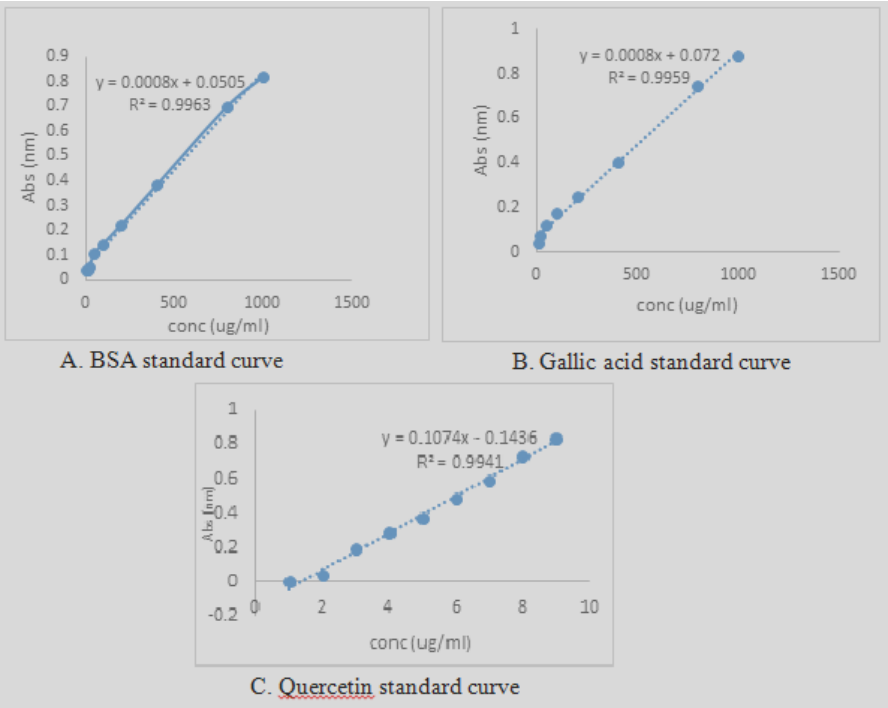

Fig. 1: (A, B & C) Standard curves of total protein, poly phenolics and flavonoids

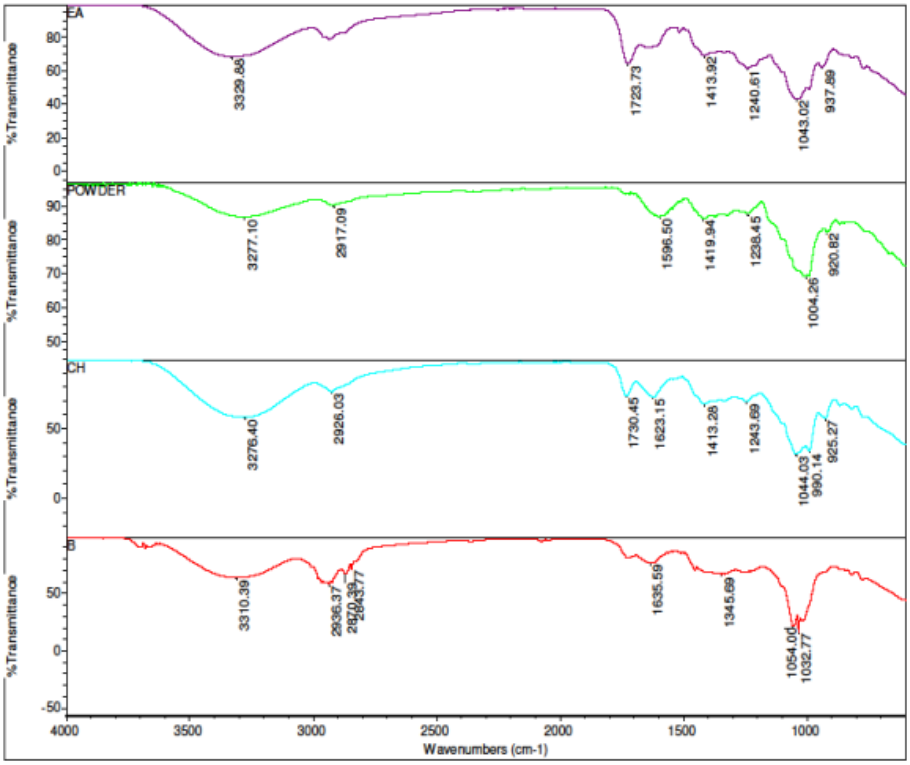

Fig. 2: FTIR spectra of ethyl acetate, powder, chloroform and butanol fractions

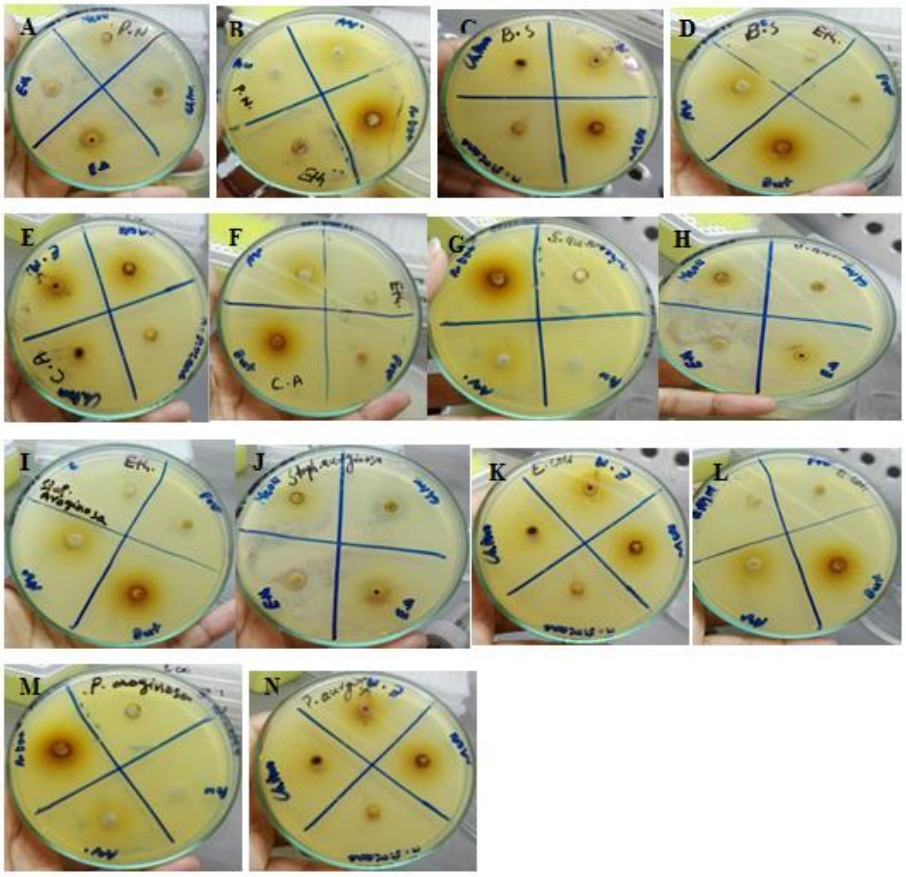

Fig. 3: (A-N) shows zones of inhibition of plant extract against *P. notatum, B. subtilis, C. albicans, S. aureus, S. aeruginosa, E. coli* and *P. aeruginosa.*

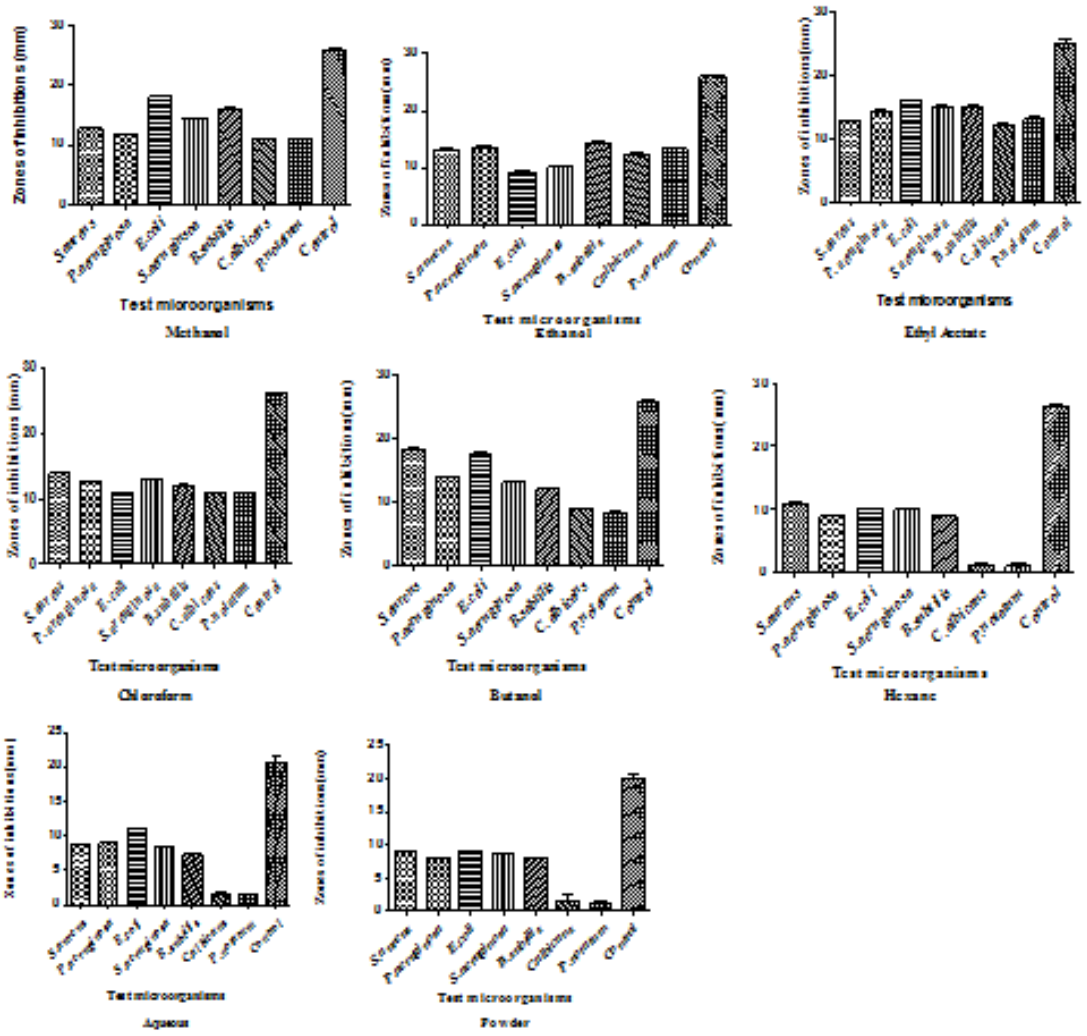

Fig. 4: (A-H) Graphical representations of zones of inhibitions

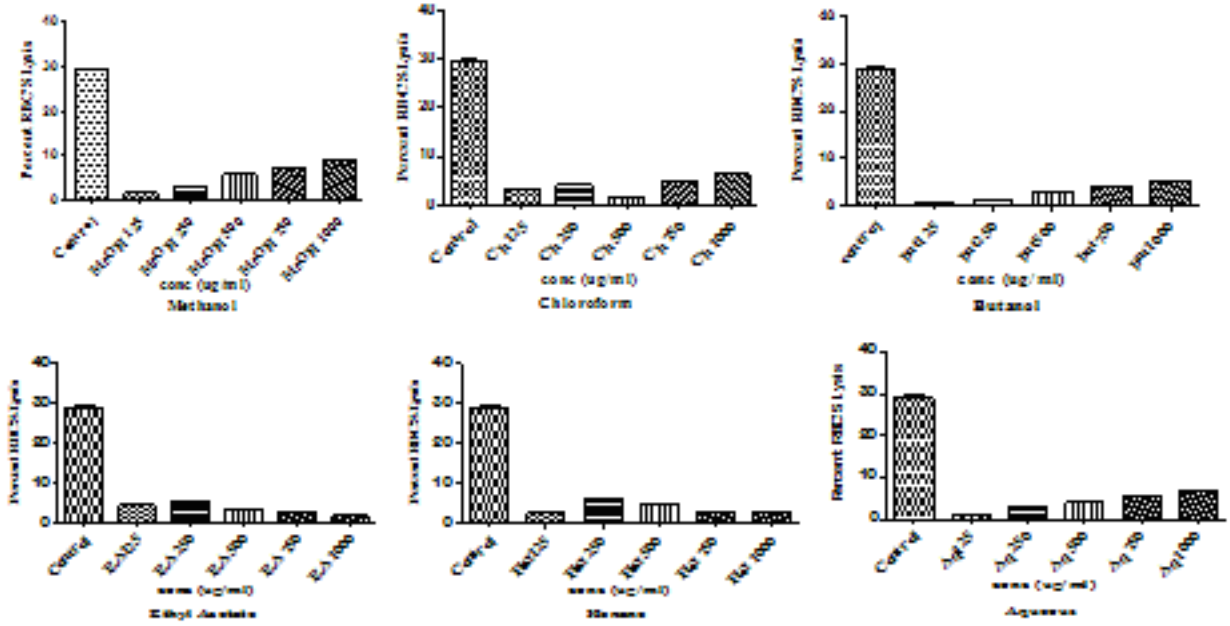

Fig. 5: Graphical representations of hemolytic activity of *Saussurea hypoleuca* root extract

FTIR spectroscopy

Powder root and fractions were analyzed in triplicates to get FTIR spectra using potassium bromide (KBr) disc.1mg crude drug and fractions were mixed with 100mg of KBr and transferred into die. The die was pressed under hydraulic press to produce the disc which were used to get the spectra in 4000-400cm^{-1} and different functional groups were observed (Sahayaraj *et al*., 2015).

Antimicrobial activity

Bacterial and fungal cultures used in this study were collected from "Fatima Memorial hospital diagnostic laboratory, Lahore" and "University Diagnostic laboratory" University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. *Escheria coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus aeruginosa*, *Bacillus stubilis*, *Candida albicans* and *Pencillium notatum.* The activity was performed by agar well diffusion method as described by (Balouiri *et al*., 2016). Mueller Hinton and Sabouraud agar media were made according to the standard protocols for antibacterial and antifungal assays respectively and sterilized them. These sterilized agar media was transferred onto petri dishes which were swabbed with freshly prepared cultured inoculum under aseptic condition. 50µl of Each sample was tipped in agar well (8mm) and kept it for 24 hour's incubation. Experiment was performed in triplicates and zone of inhibitions were calculated.

Minimum inhibitory concentration (MIC)

MIC of the methanolic extract and fractions of the selected plant samples were determined by using micro dilution 96 well plates as explained by method with slight modifications (Silva *et al*., 2016). Each sample was prepared in DMSO (10mg/1ml) and experiment was executed in sterilized polystyrene 96 well micro plate. First well of each column (1-12) was filled with sterilized nutrient broth. 50µl of plant extract was added into first well of each column 1-10 (each sample in triplicate). Serial twofold dilutions were made for consecutive each column and 50µl from the last well of column was discarded. 100µl of bacterial inoculum was added in each well. First column of the plate served as positive control and second column as negative control which have only plant extract, inoculum and nutrient broth while third column is sterility control which have only nutrient broth. Plate was incubated for 24hour's at 37C° and absorbance was logged at 630nm by using micro plate reader.

Cytotoxic studies

Haemolytic activity

Haemolytic activity of the test sample and fractions was studied by reported method (Zohra and Fawzia, 2014). 3ml of healthy human fresh blood was assorted with heparin in sterile 15ml falcon tube and centrifuge it for 5 minutes at 3000 rpm. The supernatant layer was removed and viscous RBC's pellet was washed 3 times with 5 ml of isotonic chilled 4 C° PBS (Phosphate buffer saline). RBC's were suspended in 20ml of chilled isotonic PBS to adjust the pH 7.4. The cells were counted on haemocytometer to 7.068*108 cells/ml. Different concentrations of extract and fractions were made (125, 250, 500, 750 and 1000µg/ml in DMSO). Aliquots of 20µl of extract and fractions were diluted with 180µl of RBC's in 2ml of eppendrops tubes. Incubated the tubes for 35 minutes in ice pots. 20µl 0.1%Triton X-100 was used for positive control and PBS for negative control. After incubation, tubes were centrifuged at 3000 rpm for 10 minutes. 100µl supernatant from each extract, fractions, positive as well as negative control was diluted with 900µl of chilled PBS. The tubes were placed in ice pots. 96well micro round bottom sterilized plate was marked according to the sample and plated by taking 200µl from each tube under aseptically. Test was done in triplicate and reading was taken in microplate Elisia reader at 576nm. Percentage cell lysis was calculated by using following formula:

%lysis = Abs(sample)/Abs (control)* 100

DNA protection assay

The aptitude of various concentrations of plant extract to protect genomic DNA from damaging effects of hydroxyl radicals created by Fenton's reagent was assessed by DNA protection assay as defined previously with trivial amendments. The reaction mixture contained 5µl of genomic DNA, 1µl of Fenton's reagent (30mM H_2O_2, 50mM Ascorbic acid, and 80mM $FeCl_3$) followed by the addition of different concentrations of extract (4µl of each fraction) and the final volume of the mixture was made up to 20µl using deionized water. The reaction mixture was incubated for 30min at 37°C. After incubation, 2µl bromophenol blue dye (0.25% in 50% glycerol) was added. The reaction mixture (10µl) was encumbered on 0.8% agarose gel (prepared by dissolving 0.4g of agarose in 50ml of 1 × TBE Buffer) and electrophoresis was run horizontally at 100V for 1 hour followed by 5µl ethidium bromide staining. The closed circular, linear, and relaxed forms of DNA were envisaged and measured using Gel Documentation system.

STATISTICAL ANALYSIS

Values were expressed in triplicates with mean ± SD. Statistical significance differences between values of plant sample and respective control were determined by using graph pad prism. In each case *P < 0.05, **P < 0.01; ***P < 0.001 considered significant.

RESULTS

Results of primary and secondary metabolites were given in table 1&2. Total proteins, total flavonoids and total ploy phenolic were calculated from linear regression

curve which was y = 0.0008x+0.0505 (R^2= 0.9963) fig. 1A, total flavonoids which obtained from quercetin standard curve y = 0.0008x+0.072 (R^2 = 0.9959) fig. 1B and total poly phenols calculated from Gallic acid standard curve using equation y =0.1074x-0.1436 (R^2=0.9941) fig. 1C. FTIR spectrum of ethyl acetate, powder, chloroform and butanol was showed in fig. 2.

Antimicrobial assay of *Saussurea hypoleuca* root methanolic extracts and fractions were evaluated against certain microorganisms. Among all extracts butanol, ethyl acetate, methanol, ethanol and chloroform show promising results while n-hexane, aqueous and powder of plant root showed poor activities against microbes. Graphical representation of zones of inhibition is showed in fig. 3 & 4. table 3 showed MIC of the extracts to inhibit microbial growth.

Cytotoxic assays (hemolytic and DNA protection) of *Saussurea hypoleuca* methanolic extract and fractions were presented safety profile in contradiction of RBC'S and genomic DNA. Different concentrations of the test sample were made according to the written protocols. Methanolic fraction showed 9.26% result while all other fractions exposed very minor hemolysis. Graphical representation of the results was depicted in the figs. 5-6.

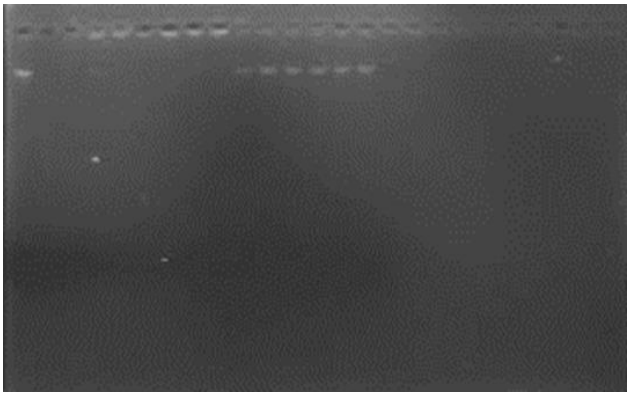

Fig. 6: Electropherogram Showing DNA Protection Effect by *Saussurea hypoleuca*

(Lane 1 = DNA + H_2O_2; Lane 2 = DNA & Fenton's reagent + H_2O_2, Lane 3 = DNA, Fenton's reagent + H_2O_2; Lane 4 = DNA treated with n-hexane extract, Fenton's reagent + H_2O_2; Lane 5 = DNA treated with chloroform fraction, Fenton's reagent + H_2O_2; Lane 6 = DNA treated with ethyl acetate fraction, Fenton's reagent + H_2O_2; Lane 7 = DNA treated with aqueous, Fenton's reagent + H_2O_2; Lane 8 = DNA treated with n-butanol, Fenton's reagent + H_2O_2; Lane 9 = DNA treated with methanol , Fenton's reagent + H_2O_2 , Lane 10 = DNA treated with n-hexane extract, without Fenton's reagent + H_2O_2; Lane 11 = DNA treated with chloroform fraction, without Fenton's reagent + H_2O_2; Lane 12 = DNA treated with ethyl acetate fraction ,without Fenton's reagent + H_2O_2; Lane 13 = DNA treated with aqueous fraction, without Fenton's reagent + H_2O_2; Lane 14 = DNA treated with n-butanol, without Fenton's reagent + H_2O_2; Lane 15 = DNA treated with methanol , without Fenton's reagent + H_2O_2)

DISCUSSION

The objective of proximate analysis and in vitro biological assays is to standardize the natural medicinal plant. Quantitative analysis of primary metabolites showed that plant consists of large number of carbohydrates, lipids and proteins. Presence of carbohydrates, protein and lipids in plant indicates that plant has rich in food value. Secondary metabolites analysis is mandatory for the separation, purification and crystallization of different phytochemical constituents presents in plant root. The plant contains a huge quantity of flavonoids, polyphenols, glycosides, alkaloids and glycosaponins which possess a wide range of pharmacological actives such as antioxidant, antimicrobial, anticancer, hepatoprotective by reducing oxidative stress (Daniel and Krishnakumari, 2015). They also known to inhibit lipid peroxidation, capillary fragility, permeability, Platelets aggregation and various lipoxygenase enzymes activities (Khalid *et al*., 2018).

Phenolic and alkaloidal compounds are very important in plant growth regulation, development and resistance against diseases. Diet rich in polyphenolic compounds protect against developing cancer, cardiovascular diseases and neurodegenerative diseases. The protective effect of plant extracts due to the presences of large amounts of flavonoids and polyphenolic constituents and it might be used to prevent cancer in future.

Antimicrobial activity conducted by using agar well diffusion method plants methanolic extract and fractions showed significant consequence against certain strains microbes. Differences in the results of the extracts in contradiction to different pathogens may be due to different chemical nature of the extracts. In agar well diffusion method lessening in microbial growth was determined from zones of inhibition. An inhibition zone of 14 mm or more has been estimated to be a substantial antimicrobial activity (Riaz *et al*., 2012). MIC was also resolute. The least MIC represent that small concentrations of the extract hold the antimicrobial activity while high MIC showed that high quantity of extract required to inhibit antimicrobial effects.

Mechanical stability of the RBC'S membrane is obligatory to reduce the cytotoxic effects of different compounds. In vitro reduction of cell lysis of different extracts is prime importance for their safety in different aliments (Mehreen *et al*., 2016). In this study safety profile of the plant extract was determined and outcomes specified them in safe range, below 10%. Cytotoxic studies direct that plant can be used in herbal medicines.

The differences in the results of these activities may be due to the phytochemicals which are produced in different species in various amounts and retains biological and pharmacological activities. Antimicrobial and cytotoxic assays reported in many medicinal plants are due to the presences of alkaloids, phenolic and flavonoids (Coronado-López *et al*., 2018). These analyzed phytochemicals in this research work might be responsible for pharmacological activities.

CONCLUSIONS

Plant derived medicines have recently gained great interest owing to their versatile benefits and minimum side effects. Medicinal plants are great resources of traditional medicines, modern medicines, pharmaceutical, nutraceutical, food, folklore medicines and chemical entities of synthetic drugs. Furthermore, the presence of active ingredients offer confidence to the use of plant for the treatment of diseases. Using plant as stimulus of innovative drugs provides infusion for novel substances. In current research work various phytoconstituents and in vitro biological assays have been screened which have a potential for the source of novel drug against aliments. The results are very auspicious but scientific authentication is necessary before putting into modern practice.

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