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PROXIMATE ANALYSIS AND *IN VITRO* BIOLOGICAL EVALUATIONS OF *DICLIPTERA BUPLEUROIDES* NEES

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ABSTRACT

Proximate analysis plays an important role in assessing the appropriateness of medicinal plants or their extracts orally taken by the marginal communities. It assessing its primary, secondary metabolites and to screened out in vitro biological assays of medicinally active plant. Quantitative analysis of *Dicliptera bupleuroides* was done by estimation of the primary and secondary metabolites (total carbohydrates, total starch, total proteins, total amino acids, total lipids, total glycosaponins, total alkaloids, total polyphenolics and total flavonoids) in plant powder. The maximum value of total carbohydrates (10.4 mg), total starch (4.81mg), total protein (15.78mg), total amino acids (7.87mg), total lipids (2.65mg)/100mL, total glycosaponins (11%), total alkaloids (5.51%), total polyphenolics (25.40%) and total flavonoids (3.58%) were respectively. Haemolytic, DNA protection assay and sun protection assay were determined according to the standard procedures. Different extracts showed various results however ethyl acetate fraction show best results in all assays while different fractions show diverse results. Results of the present study give an evidence for the existence of diverse primary and secondary metabolites and thus rationalizes its use in traditional medicines for the curement of different aliments owing to the safety profile on human red blood cells. There is a positive indication in safe use of this plant.

Keywords: *Dicliptera bupleuroides*, primary and secondary metabolites, Haemolytic, DNA protection, SPF.

INTRODUCTION

Herbal medicine have been used for the ailment of different diseases since ancient times. Every part of the world has established its own herbal list based on traditional knowledge of the ingredient and through experience (Al-Snafi 2015). Phytochemicals are found in almost every part of the plant; leaves, roots, stem and vegetables. They are classified as primary and secondary constituents. Primary constituents include chlorophyll, sugars and proteins while alkaloids terpenoids and phenolic substances are secondary compounds. Plants are the rich source of medicinally active organic compounds like tannins, steroids, terpenoids, flavonoids, carbohydrates and alkaloids. The most important bioactive compounds are alkaloids, flavonoids, tannins and phenolics. 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). All these ingredients possess definite pharmacological actions.

Dicliptera bupleuroides Nees. (family Acanthaceae) is a perennial plant commonly known in Urdu as Kaali booti (Ajaib *et al.,* 2015). It is found in the planes of Pakistan and Afghanistan. It is an erect herb, height up to 90cm, bushy, twigs are hairy and leaves ovate or elliptic acute (Singh *et al*., 2014). This species is also arrogated to be used in traditional medicines for applying on wound of snake bite, in fever, in stomach troubles and also used in bone fracture (Panigrahi and Dubey 1983). *Dicliptera bupleuroides* possesses antioxidant, hepatoprotective, antimicrobial and other biological activities. It contains phenols, flavonoids, ascorbic acid, lipids, starch, glycosides and many other compounds (Ahmad *et al.,* 2013, Bahuguna *et al.,* 1987).

MATERIALS AND METHODS

Collection of plant material, authentication and extraction

The plant was collected from Bhimber (Bandiala), Kotli, Azad Kashmir and got authenticated by Dr. Uzma Hanif, Department of Botany, Government College University (GCU), Lahore, Pakistan. A specimen of the plant was deposited in herbarium of GCU under voucher No: GC. Herb. Bot. 3402. The plant whole herb was dried under shade for about 15 days and then pulverized. Powder was stored in amber colored bottles at dry place. Methanolic extract made by maceration using rotary evaporator under reduced pressure at 45-50 ˚C. Fractionation was

performed by different solvent of increasing order of polarity n-hexane, chloroform, ethyl acetate, n-butanol and aqueous. Each fractions of these solvents was dried and preserved for evaluation of different biological activities.

DETERMINATION OF PRIMARY METABOLITES

Determination of carbohydrate content

Carbohydrates contents were determined by the method followed by Chandran *et al*., (2013) with certain modification.

Determination of starch content

One hundred mg of sample powder was weighed to determine its starch contents by the method followed by Chandran *et al*., (2012).

Determination of protein content

Protein content in the given plant sample was determined by the method given in Shukla *et al.,* (2016).

Determination of amino acid content

One hundred mg of sample powder was added to 10mL of 80% ethanol. It was centrifuged at 3000rpm, supernatant was collected and made up to 10 mL with distilled water and used for the estimation. Working standard solution marked as S_1, S_2, S_3, S_4, S_5 and test sample solutions as T_1, T_2 respectively. One another test tube labelled as Blank (1mL distilled water). Add 1mL of Ninhydrin reagents in all test tubes, vortex them and then added 5mL of diluent in each tube. Absorbance measured at 570nm and draw a standard curve (More and Chaubal, 2016).

Determination of total lipids

Fifteen gram of powder was subjected in thimble for extraction procedure in soxhlet apparatus and percentages of lipids contents were determined following Jadid *et al*., (2018).

DETERMINATION OF SECONDARY METABOLITES

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

Determination of saponin content

Saponin quantitative determination was carried out using the method reported by Ejikeme *et al.* (2014). The saponin content was calculated as a percentage:

$$\%\, Saponin = \frac{Weight\ of\ saponin}{Weight\ of\ sample} \times 100$$

Determination of glycoside content

One gram of powder was added in 100mL volumetric flask then add 10mL of 70% ethanol in it. Boiled it, filtered and filtrate was diluted with distilled water. Then add 3.5mL of 10% lead acetate. Filtered it and kept the filtrate into separating funnel with 15mL of chloroform. Two layers were formed, lower organic layer was collected (chloroform); dried and weighed. Percentage of total glycosidal contents was determined (Ugwoke *et al*., 2017).

Determination of alkaloidal content

Quantitative determination of alkaloid was done according to the method of Amiri *et al.* (2017). The percentage of alkaloid is expressed as:

$$\%\, Alkaloid = \frac{Weight\ of\ alkaloid}{Weight\ of\ sample} \times 100$$

Total phenolic content

Estimation of total poly phenolic contents in plant sample was 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.

Total flavonoid content

Flavonoid determination was by the method reported by Ejikeme *et al.* (2014). The percentage of flavonoid was calculated.

DETERMINATION OF SOLAR PROTECTION FACTOR (SPF)

Different fractions of *D. bupleuroides* were dissolved in distilled water in such a way that concentration of each solution is 1mg/mL. These solutions were diluted in distilled water to get two concentrations (200µg/mL and 400µg/mL). Then spectrophotometer readings of these solutions were taken in wavelength ranging from 290 to 320 at 5nm interval and readings were noted. SPF was calculated by using following formula;(Suva, 2014)

$$SPF = CF \times \sum_{290}^{320} EE \times I \times Abs$$

Here; EE= Erythemal effect spectrum, I =Solar intensity spectrum,
Abs = Absorbance of sample, CF=Correction factor (=10)

The values of EE×I are predetermined as shown in Table 1.

Table 1. Values of EE×I used in the calculation of Solar Protection Factor (SPF).

Wavelength(nm)	**EE×I**
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	1

SPF calculated in triplicates and then average was taken.

CYTOTOXIC STUDIES

***In Vitro* Haemolytic Activity**

Fresh human blood (3mL) was taken in EDTA vial and centrifuged for 5min at 850 rpm. Clear supernatant was decanted and sedimented pellets were washed three times with chilled sterilized isotonic phosphate buffer saline (PBS) followed by the formation of suspension in 20mL of chilled sterilized PBS. Cells were counted using Haemocytometer. Triton-X was used as positive control and PBS served as negative control. Reaction mixture contain 20µL of plant extract and 180µL of blood cells suspension. They were incubated at 37°C for 40min. After incubation tubes were placed in ice cold PBS for 5min and then centrifuged at 1500 rpm for 5min. Collect carefully collected 100 µL of supernatant in eppondrof tubes and diluted with 900 µL of chilled sterilized PBS. All these samples including positive and negative control (200 µL) were loaded into 96well plate. By using ELISA microplate reader took the absorbance at 630nm (Zubair *et al*., 2017).

$$\% \text{ Hemolysis} = \frac{Abs(Sample\ absorbance)}{Abs\ (control\ absorbance)} \times 100$$

DNA Damage Protection Assay

DNA was isolated from human blood using DNA isolation kit. Isolated DNA was quantified using nano drop technique. The ability of different fractions of plant extract to protect genomic DNA was determined using the previously described method with slight modifications (Kaur *et al.*, 2019). In this method, DNA damage was induced by hydroxyl radicals generated from Fenton's reagent. Reaction mixture contained 4 µL of genomic DNA, 3µL of Fenton's reagent and 4µL of different fractions of plant and make up the volume upto 20µL using deionized water. Positive and negative controls were also prepared. Reaction mixtures were incubated at 37°C for 30 min. Bromophenol dye was added in each sample after incubation. The reaction mixture (10 µL) was loaded in the wells of 1% Agarose gel and electrophoresis was allowed to run horizontally for 1h at 100V followed by staining with ethidium bromide. DNA was under Gel documentation system

FTIR Spectroscopy

Whole plant powder was analyzed in triplicates to get FTIR spectra using potassium bromide (KBr) disc. 1mg of sample powder was 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 (Fig. 2) (Sahayaraj *et al*., 2015).

Statistical Analysis

Values were expressed as mean ± SD for triplicate observation. In each case *P < 0.05, **P < 0.01; ***P < 0.001 considered significant. Statistical analysis used one way (ANOVA) and graphical representation used graph pad prism 6.

RESULTS

Results of quantitative analysis of primary and secondary metabolites were summarized in Table 2 & 3. Total polyphenols and total flavonoid content were calculated from linear regression curves (Fig. 1). SPF values presented in Table 4 & 5 at 200µg/mL, 400µg/mL conc. While presence of different chemical groups given in FTIR spectra (Fig. 2). In cytotoxicity hemolysis of RBCs at different concentration given in Fig. 3 and DNA protection presented in Fig 4.

Table 2. Proximate analysis of primary metabolites of *D. bupleuroides* Nees.

Sr.no	**Parameters**	**mg/100mL values (w/w)**	
		Mean	SD
1.	Total carbohydrates	10.4	± 0.20
2.	Total starch	4.81	± 0.05
3.	Total protein	15.78	± 0.03
4.	Total amino acids	7.87	± 0.02
5.	Total lipids	2.65	± 0.041

Table 3. Proximate analyses of secondary metabolites of *D. bupleuroides* Nees.

Sr.no	**Parameters**	**% values (w/w)**	
		Mean	SD
1.	Total glycosaponin	11	± 0.01
2.	Total alkaloid	5.51	± 0.02
3.	Total phenols	25.40	± 1.02
4.	Total flavonoid	3.58	± 0.77

Table 4. Absorbance of different fractions of *D. bupleuroides* at concentration of 200µg/mL

Wavelength (λnm)	EE*I (Normalize)	Absorbance					
		Methanolic	n-Hexane	Aqueous	Chloroform	Ethyl acetate	n-Butanol
290	0.0150	0.632±0.02	0.105±0.00	0.142±0.00	0.514±0.00	0.879±0.01	0.602±0.22
295	0.0817	0.650±0.01	0.068±0.00	0.005±0.00	0.290±0.01	0.636±0.01	0.681±0.00
300	0.2874	0.650±0.04	0.065±0.00	0.053±0.02	0.245±0.00	0.54±0.00	0.461±0.00
305	0.3278	0.648±0.02	0.079±0.00	0.031±0.00	0.216±0.00	0.468±0.00	0.440±0.00
310	0.1864	0.644±0.01	0.106±0.00	0.062±0.00	0.174±0.00	0.392±0.00	0.412±0.00
315	0.0839	0.629±0.11	0.095±0.00	0.040±0.00	0.154±0.00	0.345±0.00	0.414±0.00
320	0.0180	0.621±0.03	0.103±0.00	0.062±0.00	0.126±0.00	0.299±0.00	0.386±0.00

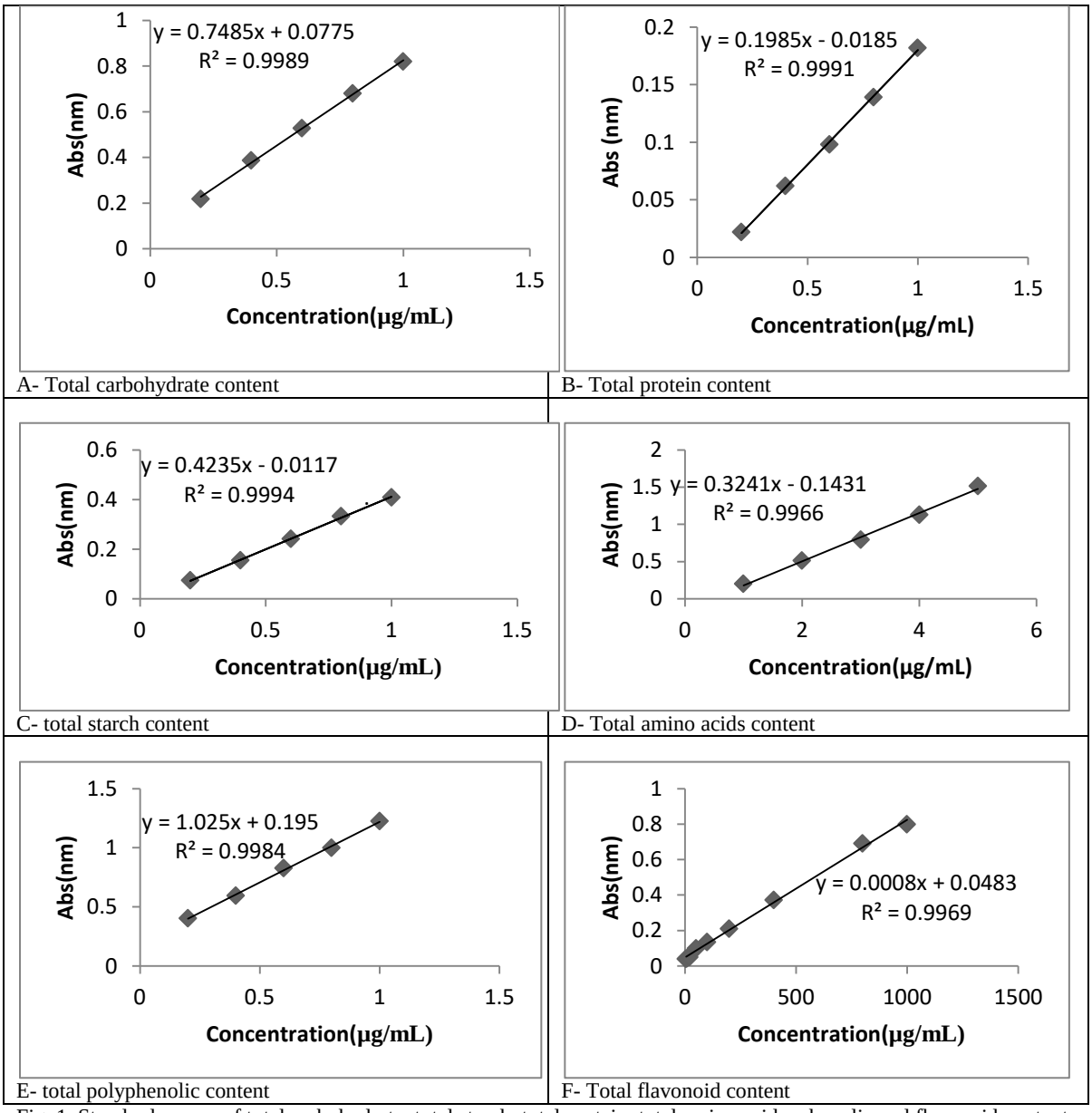

Fig. 1. Standard curves of total carbohydrate, total starch, total protein, total amino acids, phenolic and flavonoid content.

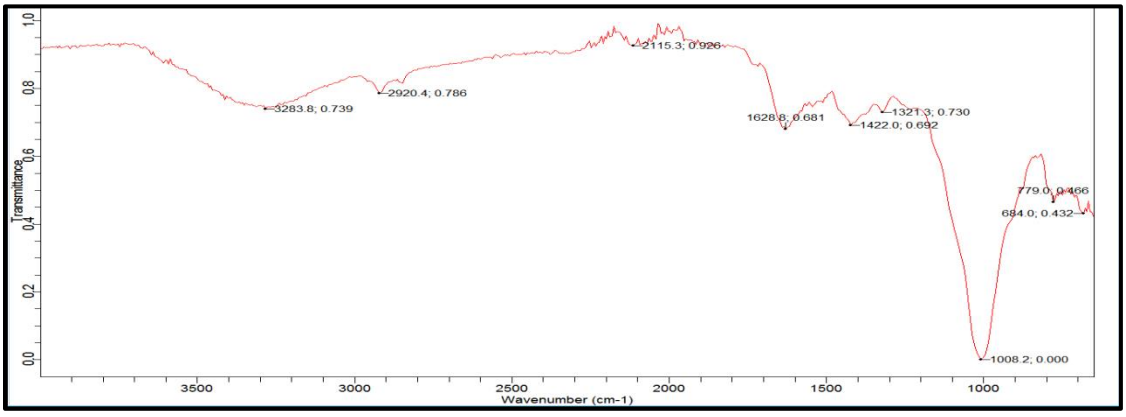

Fig. 2. FTIR analysis of whole herb powder of *D. bupleuroides* Nees.

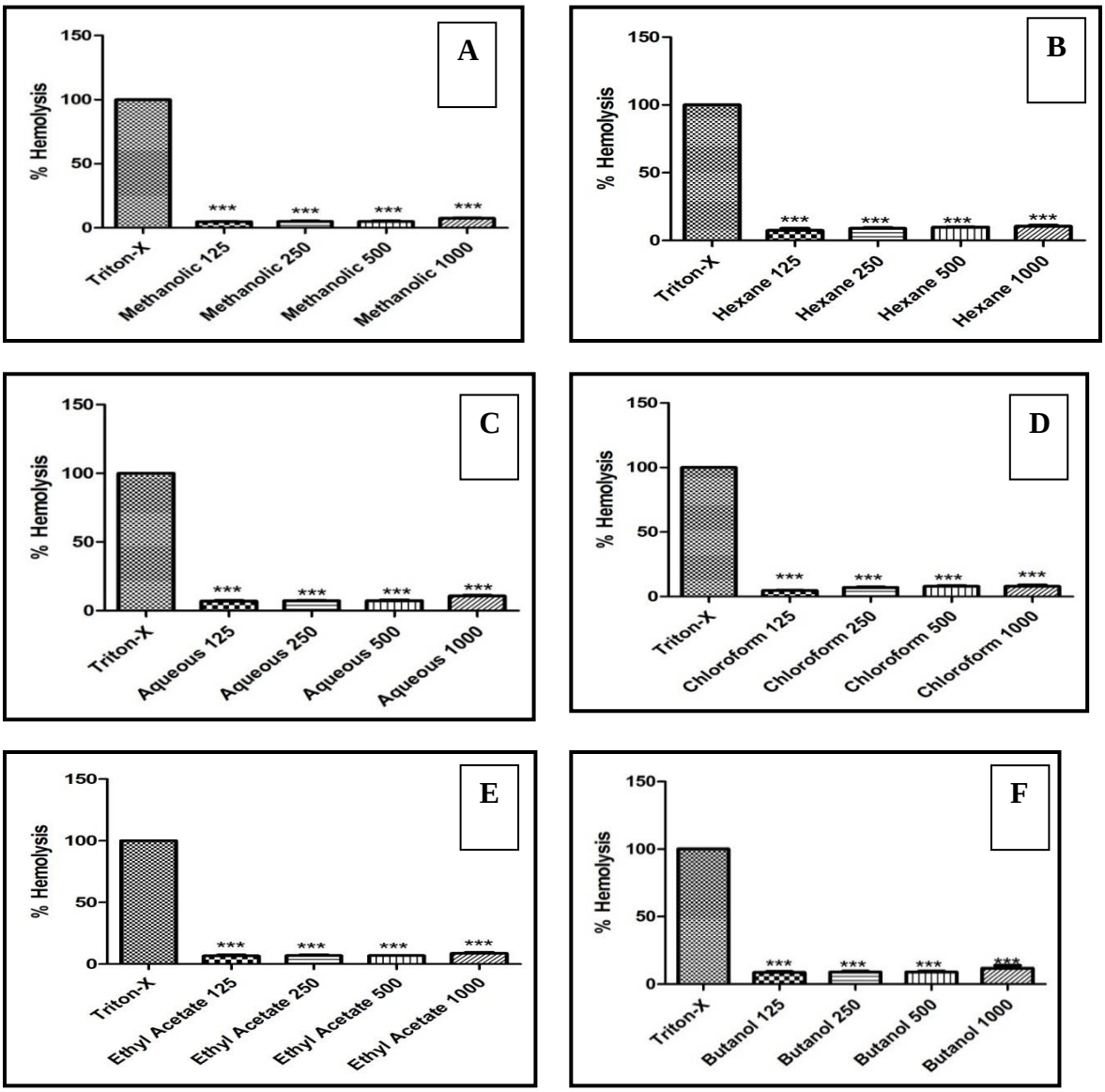

Fig. 3. Graphical representation of percentage hemolysis (*P < 0.05, **P < 0.01; ***P < 0.001 showed level of significance) of different fraction of *D. bupleuroides* Nees.

Table 5. Absorbance of different fractions of *D. bupleuroides* at concentration of 400µg/mL.

S. No.	Wave length (λnm)	EE*I (Normalize)	Absorbance					
			Methanolic	n-Hexane	Aqueous	Chloroform	Ethyl acetate	n-Butanol
1.	290	0.0150	0.932±0.04	0.256±0.01	0.256±0.00	1.078±0.00	1.845±0.03	1.603±0.00
2.	295	0.0817	1.033±0.08	0.122±0.00	0.092±0.00	0.798±0.00	1.540±0.00	1.334±0.00
3.	300	0.2874	1.039±0.05	0.092±0.00	0.068±0.00	0.734±0.01	1.377±0.00	1.217±0.00
4.	305	0.3278	1.023±0.02	0.092±0.00	0.051±0.00	0.656±0.00	1.227±0.00	1.139±0.01
5.	310	0.1864	1.016±0.04	0.075±0.00	0.028±0.00	0.593±0.00	1.093±0.00	1.081±0.00
6.	315	0.0839	1.00±0.02	0.065±0.00	0.027±0.00	0.546±0.00	0.955±0.02	1.043±0.00
7.	320	0.0180	0.974±0.10	0.067±0.00	0.013±0.00	0.484±0.00	0.872±0.00	1.019±0.01

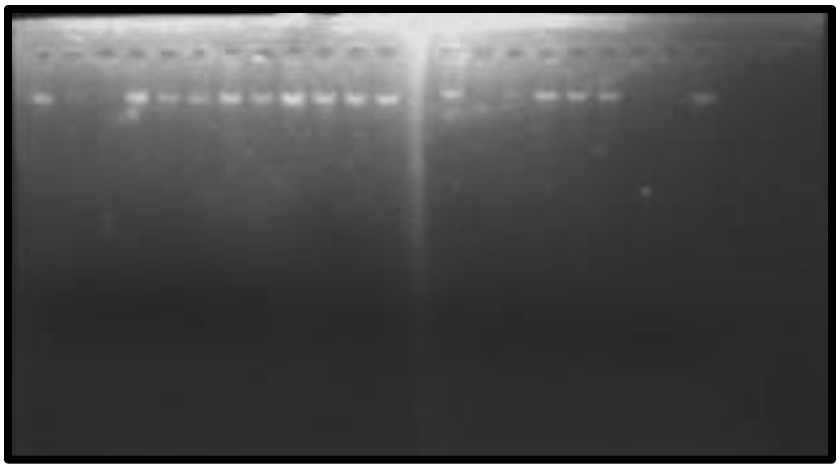

Fig. 4. DNA damage protection activity of *D. bupleuroides*. **Lane 1**: 4µl DNA+16µl DW, **Lane 2**: 3µl FR+17µl DW, **Lane 3**: 4µl DNA+3µl FR+13µl DW, **Lane 4**: 4µl DNA+4µl Butanol extract (50µg/µl) +3µl FR+9µl DW, **Lane 5**: 4µl DNA+4µl Butanol extract (100µg/µl) +3µl FR+9µl DW, **Lane 6**: 4µl DNA+4µl Butanol extract (200µg/µl) +3µl FR+9µl DW, **Lane 7:** 4µl DNA+4µl Hexane extract (50µg/µl) +3µl FR+9µl DW, **Lane 8**: 4µl DNA+4µl Hexane extract (100µg/µl) +3µl FR+9µl DW, **Lane 9:** 4µl DNA+4µl Hexane extract (200µg/µl) +3µl FR+9µl DW, **Lane 10**: 4µl DNA+4µl Methanol extract (50µg/µl) +3µl FR+9µl DW, **Lane 11**: 4µl DNA+4µl Methanol extract (100µg/µl) +3µl FR+9µl DW, **Lane 12**: 4µl DNA+4µl Methanol extract (200µg/µl) +3µl FR+9µl DW, **Lane 13**: 4µl DNA+4µl Ethyl acetate extract (50µg/µl) +3µl FR+9µl DW, **Lane 14**: 4µl DNA+4µl Ethyl acetate extract (100µg/µl) +3µl FR+9µl DW, **Lane 15**: 4µl DNA+4µl Ethyl acetate extract (200µg/µl) +3µl FR+9µl DW, **Lane 16**: 4µl DNA+4µl Chloroform extract (50µg/µl) +3µl FR+9µl DW, **Lane 17**: 4µl DNA+4µl Chloroform extract (100µg/µl) +3µl FR+9µl DW, **Lane 18:** 4µl DNA+4µl Chloroform extract (200µg/µl) +3µl FR+9µl DW, **Lane 19**: 4µl DNA+4µl Aqueous extract (50µg/µl)+3µl FR+9µl DW, **Lane 20**: 4µl DNA+4µl Aqueous extract (100µg/µl)+3µl FR+9µl DW, **Lane 21**: 4µl DNA+4µl Aqueous extract (200µg/µl)+3µl FR+9µl DW.

Aqueous and ethyl acetate fraction showed 9.82% and 9.61% haemolysis of RBCs at higher concentration while all other fractions showed least haemolysis even at higher concentration. The result of all fractions were shown in (Fig. 3)

Hydroxyl radicals generated by Fenton's reagent causes DNA damage as DNA is absent in 3rd lane and only smear of degraded DNA can be observed. Methanolic and ethyl acetate extracts were found to be most effective, Hexane extract show little activity at high concentration, however, Aqueous and Butanol extract showed no activity at these concentrations (Fig. 4).

DISCUSSION

Phytochemicals present in plants based foods and herbs improve the quality of health. These phytochemicals possess various pharmacological and biochemical actions when used by animals (Usunobun *et al.*, 2015). *D. bupleuroides* was found to be a rich source of primary metabolites (carbohydrates, lipids, protein). Plant carbohydrates can be used as artificial sweeteners and help to rebuild the body of diabetic patients. Presence of carbohydrates, starch, proteins, free amino acids and lipids is an indication that plant has high nutritional values. Quantification of secondary metabolites is essential for extraction, purification, separation, crystallization and identification of different phytochemicals (Daniel and Krishnakumari, 2015). Plant contains a number of secondary metabolites including phenols, alkaloids, saponins, glycosides. Alkaloids contribute towards analgesic, antispasmodic and antimicrobial effect (Shukla *et al.*, 2015).

Flavonoids are potent antioxidants that prevents the cell damage induced by oxidative stress and are used as anti-inflammatory agent, anticancer, in heart and skin diseases (Riaz *et al*., 2012). Saponins are antimicrobial agents as well as regulate the blood lipids and glucose and also decrease risk of cancer (Achi *et al.*, 2017). Tannins have reported various medicinal properties i.e. antimicrobial, anti-inflammatory and wound healing (Daniel and Krishnakumari, 2015).

FTIR analysis of *D. bupleuroides* powder shows broad band centered at 3283.8cm^{-1} due to OH absorption. Broadening of this band is related to OH stretching vibration with intramolecular hydrogen bonded at OH. This is an indication that alcoholic or phenolic group is present in plant powder or it might be complex polyhydroxy group of tannins. The narrow band centered at 2920.4 cm^{-1} represents the CH stretching vibration of methyl, methylene and methoxy group. Weak band at 2115.3cm^{-1} due to C≡C stretching indicating the presence of monosubstituted alkyne.

Medium band at 1628.8 cm^{-1} is due to C=C stretching indicating the presence of conjugated alkene or the cyclic alkenes or aromatic ring in powder plant material or it might be due to N-H bending showing the presence of amines . Peak at 1422.0 cm^{-1} and 1321.3 cm^{-1} is due to OH bending showing the presence of alcohols or carboxylic acid group or phenols. Strong peak at 1008.2 is due to C=C bending confirming the presence of unsaturation in compound. The present data have showed the presence of methyl, aryl, carboxylic acid, amine, along with some -OH group in the form alcohols or phenols.

Red blood cells (RBCs) are the major cells in circulation and easy to isolate from blood. Their membrane resembles with structure of other membranes in the body and has complex structure that maintains the morphology, elasticity and deformability of cells. Exposure to toxic agents can change the membrane structure that results in hemolysis of RBCs. Anything that causes hemolysis is cytotoxic to RBCs and other body cells. Hence, to assess the cytotoxicity of molecules erythrocytes are widely used as biological model (Farag and Alagawany, 2018).

DNA maintains the growth and repair by different metabolic reactions. Various factors like radiations, chemicals, hydroxyl radicals, and oxidative stress can damage DNA. Oxidative stress is one of the major causes of DNA damage in Human. This damage to DNA consequently onset different diseases including early age diabetes, cancer, Alzheimer disease, Parkinson's disease etc. Medicinal plants are considered to be the rich source of bioactive components. These bioactive components may have DNA damage protection activity. Hence they played an important role in providing better health to living beings (Kaur *et al*., 2019). In the present study, DNA damage protection activity of different fractions of *Dicliptera* was previously analyzed using Fenton's reagent as damaging agent. All the extracts lessen the oxidative stress and protect the DNA from hydroxyl radicals generated by Fenton's reaction. Butanol extract was comparatively found to be the most effective to protect DNA bands followed by n-hexane, aqueous and methanol extract. Butanol and aqueous extract showed dose dependent protection. DNA damage protection activity of these fractions is due to phytochemicals like phenolic and flavonoids present in these fractions. These phytochemicals scavenge free radicals and protect DNA from damaging effects of these radicals. Butanol and hexane fractions significant activity shown by extract is attributed to their inability to quench hydroxyl radicals.

UV radiations are one of major cause of skin damage. They are responsible for sunburns, wrinkles, early aging and allergies. UV radiation penetrate into skin cell and causes gene mutation first stage of development of skin cancer. Therefore everybody requires protection from damaging effects of UV radiation. Synthetic agents are no more suitable to use as photo protective agents due to their toxicity in humans. Natural substances have absorbance in UV region and are good antioxidants ,therefore, they can be used as photo protective agents (Korać and Khambholja, 2011; Mukherjee *et al.*, 2011). After obtaining significant DNA damage protection results from different fraction of Misopates, they were analyzed for photoprotective potential. The results revealed that Misopates fractions can be used in skin formulation as they have good sun protection factor value. Flavonoids present in plants are responsible for their solar protection factor (Ebrahimzadeh *et al.*, 2014). Therefore photo protective potential of *D. bupleuroides* might be due to their high flavonoids content

CONCLUSIONS

D. bupleuroides has a rich source of primary and secondary metabolites. All the fractions have acceptable safety profile against human red blood cells. Butanol and Ethyl acetate fractions have more potential to be used in cosmetics as it protects the cells and DNA effectively against the damaging effect of free radicals and UV radiation. However, further studies are needed to isolate the phytochemicals responsible for its biological effects.

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CONFLICT OF INTEREST

It is stated that author has no conflict of interest.

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