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In vitro phytochemical and anticancer activity of *Misopates orontium* L. and *Dicliptera bupleuroides* Nees

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Abstract: In the present study phytochemical analysis and anticancer activity of *Misopates orontium* L. and *Dicliptera bupleuroides* Nees was carried out. Methanolic extracts of *M. orontium* and *D. bupleuroides* were selected for phytochemical analysis. The present analysis showed the presence of phytochemical such as carbohydrates, proteins, tannins, glycosides, alkaloids, saponins, phenols and flavonoids in *M. orontium* and *D. bupleuroides.* Anticancer assays including MTT, Alamar Blue (AB), Neutral Red (NR) and lactate dehydrogenase (LDH) were employed on whole herb methanolic extract and all other fractions of both plants to calculate the % age of cell viability and cell cytotoxicity. The percentage of cell viability was highly significant in all anticancer assays for all fractions. Therefore, ethyl acetate and aqueous fractions showed the excellent profile in evaluation of cytotoxicity in each assay. All above findings indicated that the whole herb of both selected plants have strong anticancer activity.

Keywords: Phytochemical, anticancer activity, MTT, alamar blue (AB), Neutral Red (NR), LDH.

INTRODUCTION

Ethnobotanical studies of natural plants have great importance being utilized by native individuals for therapeutic purposes (Ghimeray *et al*., 2009; Sultana *et al*., 2007). Different tribes are engaged in folklore use of these meditative herbs, shrubs and trees species in treating the ailments in the United State. In past, several active compounds were isolated from natural meditative flora (Aburjai *et al*., 2007). It was the need of time that how the people of a particular culture make the use of different indigenous plants for the sake of benefit of human kind in respect of collection of data of their native verities and plant diversity in the surroundings (Kesarker *et al*., 2009; Buer *et al*., 2010). Around extensive variety of therapeutic herbal types due to various variety of herbal geographic and situations of climatic in Pakistan. Approximately six thousand kinds of plants are existing in Pakistan (Abbasi *et al*., 2009). In Pakistan a huge amount of floras nearly four thousand herbal types are kept to hilly areas such as North West Frontier Province (NWFP) and Hindu Kush-Himalayas northern region.

Cancer could be a cluster of diseases caused by loss of cell cycle management (Vijayakumar *et al.*, 2019). Uncontrolled cell growth is associated with cancer. External factors which cause cancer are tobacco, chemicals, radiations and infectious organisms while internal factors are mutations, hormonal imbalance, immune condition and other metabolic changes. It is a world widely problem due to limited resources of early detection methods, poor prognosis diagnosed in later stages and frequent exposure to radiation and also intake of unhealthy diet on global scale (Luke *et al*., 2020).

Pakistan is an agricultural land and blessed with a wide variety of medicinal as well as economically important plant species. Two medicinal plants were selected from hilly areas of Azad Kashmir, Pakistan. Scrophulariaceae family is commonly known as figwort family, comprised of large number of herbs and small number of shrubs. Acanthaceae is well famous family by local people, especially in expressions of healing purposes. For the treatment of various disorders these plant species commonly used. Most of the plants reported to have various ethanomedicinal importance like antimicrobial, antidiabetic, anticancer, hepato-protective and anti-inflammatory (Sher *et al.,* 2011).

Misopates orontium is commonly called snapdragon used as fodder, in medicinal preparations, as fiber, as fuel wood, as timber, in tanning industry and in preparation of gum (Jabeen *et al*., 2009). Traditionally it is used as diuretic, for scurvey, in liver disorder and in tumors also. Leaves and flowers were used as antiphathologistic, resolvent and all kinds of inflammation (Al-Snafi 2015). *M. orontium* is claimed to be used for a number of therapeutically assiduities such as, it has bitter and stimulant properties, and the whole plant has been employed for the treatment of tumors and ulcers (Lönnig *et al*., 2007).

Dicliptera bupleuroides Nees. of family Acanthaceae is a perennial herb commonly known in Urdu as Kaali booti (Ajaib *et al*., 2015; Singh *et al*., 2014). This species is

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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* possessed 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; Luo *et al*., 2002). The objective of the current study was to evaluate the anticancer activities of both plant species on human hepatic carcinoma cell lines for their effective and safe use.

MATERIALS AND METHODS

Plant material collection

The plants were collected from Bhimber, Kotli, Azad Kashmir and authenticated by Dr. Uzma Hanif, Department of Botany, Government College University, Lahore, Pakistan. Specimen of both plants *M. orontium* and *D. bupleuroides* were deposited in the herbarium of Government College University under voucher No.: GC. Herb. Bot. 3458, voucher No: GC. Herb.Bot.3402 respectively. The plants were dried under shade and powdered the whole herb.

Extraction and fractionation

The powdered herbs were socked in methanol for 7 days, filtered it and evaporated by using rotary evaporator (Heidolph, model Laborata 4000, Schwabach, Germany). After extraction, fractionation was done using different solvents (n-hexane, Chloroform, Ethyl acetate and Butanol) according to polarity. Active fractions were separated by using small column chromatography, preparative TLC (Li *et al*., 2008).

Phytochemical analysis

Phytochemical analysis was conducted on both selected plant species *M. orontium* and *D. bupleuroides.* They were screened to determine the presence of carbohydrates, proteins, steroids, fats, alkaloids, tannins, glycosides, phenols and flavonoids (Wagner and Ulrich-Merzenich, 2009; Costa *et al*., 2010).

In vitro anticancer activity

Anticancer potential of both plants fractions were investigated by employing various assays i.e. MTT, AB, NR and LDH assays.

Preparation of cell culture

T-75 flask were used in anticancer activity for cell culture preparation, this flask covered with 70-80% cells. 5% FBS mixed with 0.5% pen strept solution, added into DMEM and this medium used to sustain cell culture in incubator. Incubator was set at 37˚C and relative humidity 95% (Myint *et al*., 2019).

Preparation of sample

Raw crude extract all other fractions were used for preparing the stock solutions (1mg/mL) in DMSO, sterilized them and finally filtered by passing through syringe filter of 0.22µm. More concentrations (3.12, 6.25, 12.5, 25, 50, 100 & 200µg/mL) of stock solutions made in sterilized media. Stored at -20˚C in sterilized sample vials according to the manufacturer's procedures.

Tetrazolium reduction assay (MTT assay)

This assay was used to determine the anticancer potential of crude extracts and various fractions of both the plants by the method of Mosmann, 1983 with certain modifications. Took 96 well microplate and seeded 200µL suspension of cells, incubate it at 37 ˚C in CO_2 (5%) environment for 24 hrs. at 95% humidity. After incubation, cells were observed under microscope without disturbing the cells, settle down at the bottom of microplates. Then these cells were exposed with different concentrations of each sample, 200µL of each stock solution added in labelled microplate well and incubate again for 24 hrs. on similar conditions. After incubation, 80% content of labelled well was removed and added 20µL 0f MTT reagent (5mg/mL in DMSO), incubate it again for 4 hrs. 150µL of DMSO added in each well after incubation and wait for dissolving formazan crystal which give purple color with MTT reagent. Incubate for 20 mint, after incubation absorbance was recorded at 600nm at ELISA reader in comparison with positive as well as negative control (Malar *et al*., 2020). Percentage viability of cell was calculated as:

$$\%\,\text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of controlled cells}} \times 100$$

Alamar blue assay (AB assay)

Aqua blue assay was used to determine cell viability. Reducing environment of living cells monitored by this assay. Rampersad described the formation of pink color fluorescent compound indicated the active metabolism of living cells which reduced resazurine into resorifin. It was also carried out as MTT assay by incubating the suspension of 200µL cells into 96 well plate for 24 hrs. in addition with 20µL resazurine reagent (0.1% W/V solution 5mg/mL in DMSO). After incubation, excitation and emission wavelength at 544nm and 590nm respectively detected on fluorescent plate reader (Specian *et al*., 2016; Silva *et al*., 2019). Percentage of cell viability of cell was calculated as:

$$\%\,\text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of controlled cells}} \times 100$$

Neutral red assay (NR assay)

Cell viability was determined for NR assay according to the method described by Repetto *et al*., 2008. Principle of this assay based on the passive diffusion of neutral dye into lysosomes which were weekly cationic in nature interact with anionic and phosphate group of lysosomal

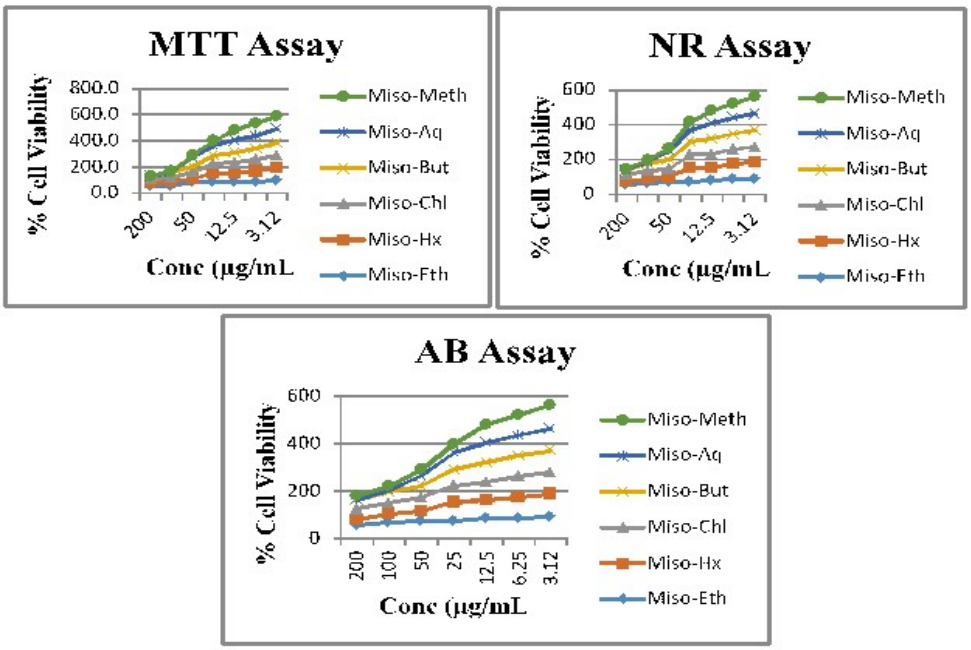

Fig. 1: % age Cell viability of MTT, NR, AB assays of crude extract and fractions of *Misopates orontium* L.

Table 1: Preliminary phytochemical analysis of crude methanolic extracts of *M. orontium* and *D. bupleuroides*

Phytochemical group	Identification test	*M. orontium*	*D. bupleuroides*
Terpenoids	Salkowaski test	+ +	+ + + +
	Liebermann's test	+ +	+ + + +
Tannins	Ferric Chloride test	+	+ + +
	Bromine water test	+ +	+ + +
Glycosides	Keller killani test	+ + +	+
	Legal 's test	+	+ +
Flavonoids	Alkaline reagent test	+ +	+ + +
	Lead acetate test	+ +	+ + +
Alkaloids	Mayer 'test	+ + +	+ +
	Wagner 'test	+ + +	+ +
	Hager 's test	+ + +	+ +
	Dragendroff 's test	+ + +	+
Proteins	Millon 's test	+ +	+ +
	Ninhydrin test	+	+ +
Carbohydrates	Molisch 's test	+ +	+ + +
	Benedicts 's test	+ + +	+ + +
Saponins	Foam test	+ +	+
Fats and Fixed oil	Spot test	+	+ +

+ = Present; - =Absent

matrix. Integrity of the membrane is directly proportional to the extent of dye defused into cells was measured by spectrometer. Incubate the sample similar to MTT assay, 100µL of neutral red was added into each valve and incubate at 37˚C for 3 hrs. Absorbance was recorded at 540nm after incubation, taking DMSO as negative control and DXR as positive control. Doxorubicin act as DNA intercalating agent which block the synthesis of DNA and RNA in mammalian cells. It also inhibits DNA topoisomerase II.

Lactate dehydrogenase assay (LDH assay)

Conversion of pyruvate into lactate catalyzed by lactate dehydrogenase in the presence of NADH. LDH released into extracellular environment due to impaired cell membrane. Activity of LDH in the specimen is measured in proportional to oxidation of NADH and absorbance also increased at 340nm due to this oxidation process. Incubation process is similar as in MTT assay under similar conditions. After incubation, 50µL of LDH was added to each valve, microplate incubate for 30 min and

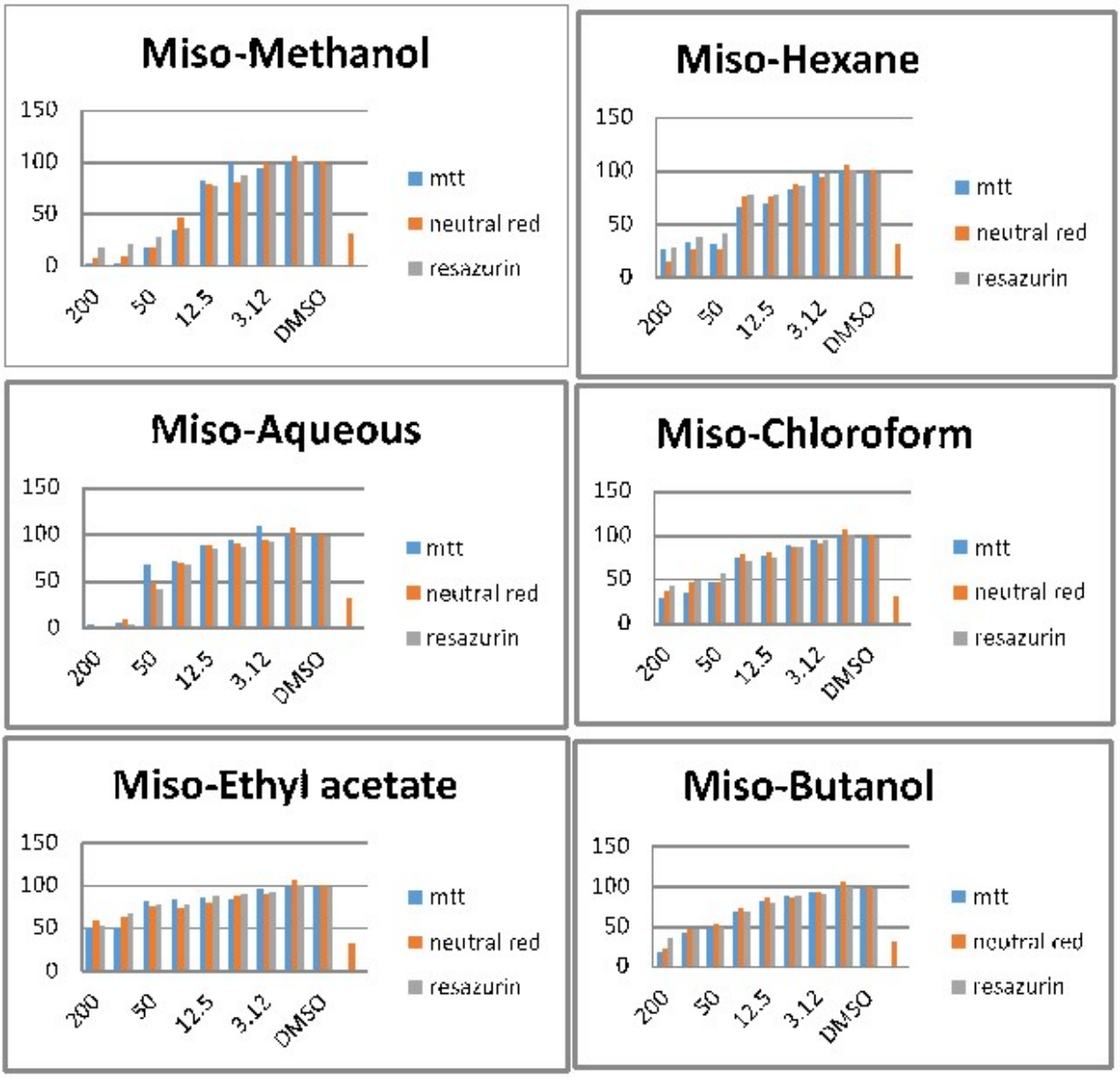

Fig. 2: Comparison of % age cell cytotoxicity of all Anticancer Assays (MTT, AB & NR) of *Misopates orontium* L.

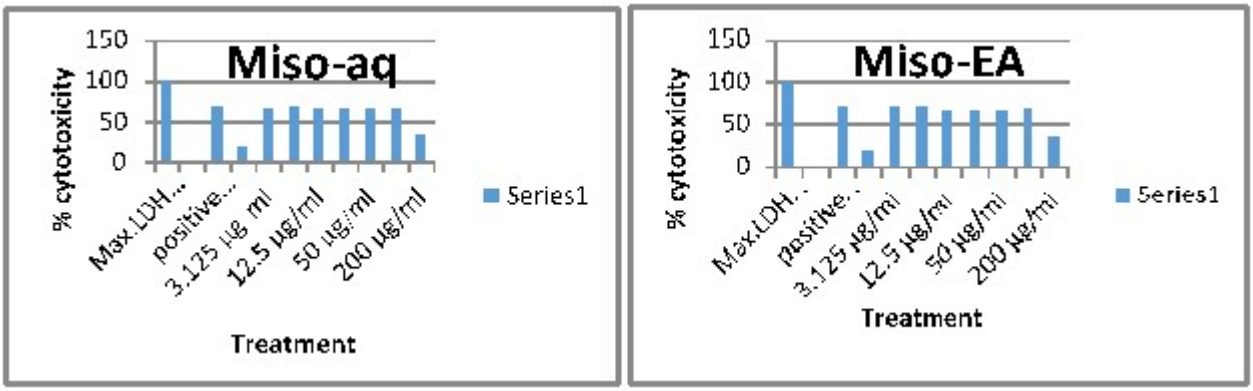

Fig. 3: LDH assay of *Misopates orontium* L

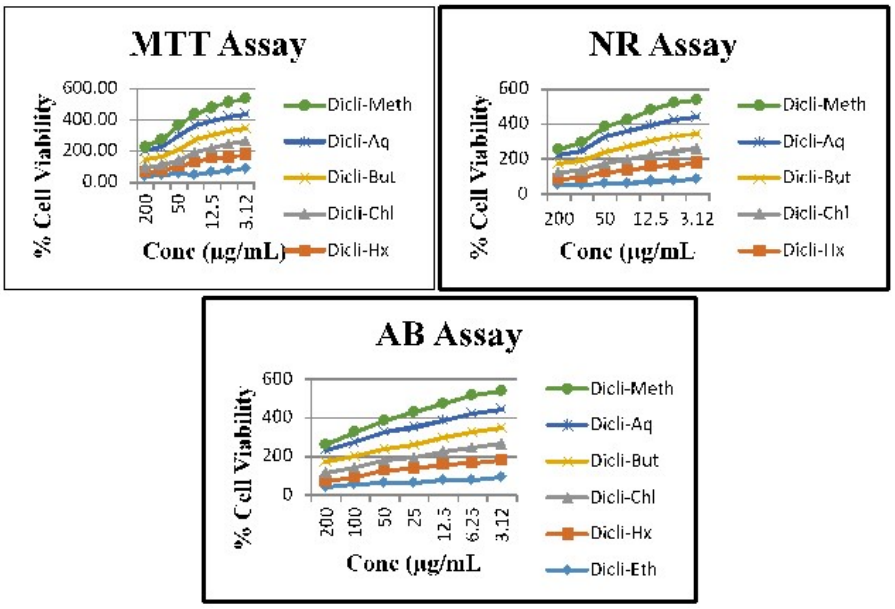

Fig. 4: % age Cell viability of MTT, NR, AB Assays of Crude Extract and Fractions of *Dicliptera bupleuroides* Nees.

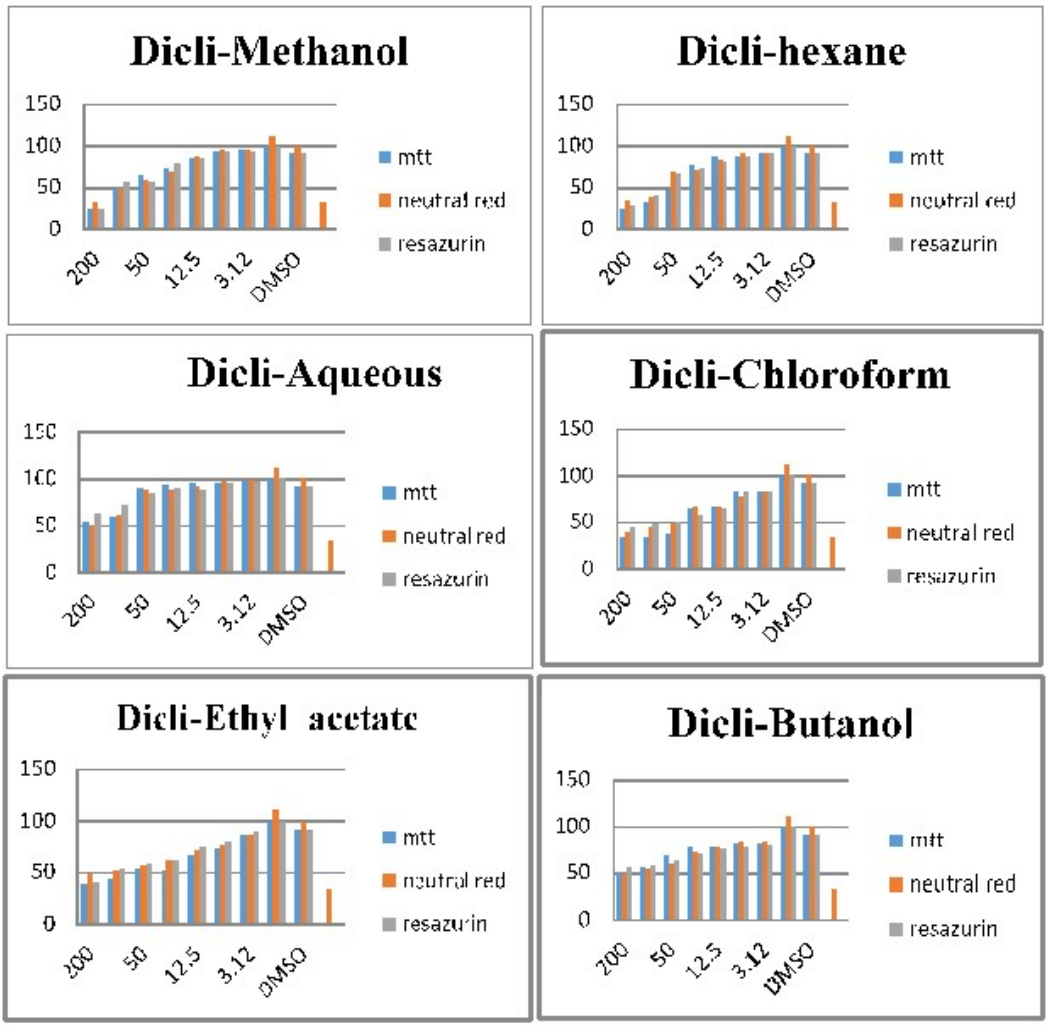

Fig. 5: % age Cell Cytotoxicity of all Anticancer Assays (MTT, AB & NR) of *Dicliptera bupleuroides* Nees.

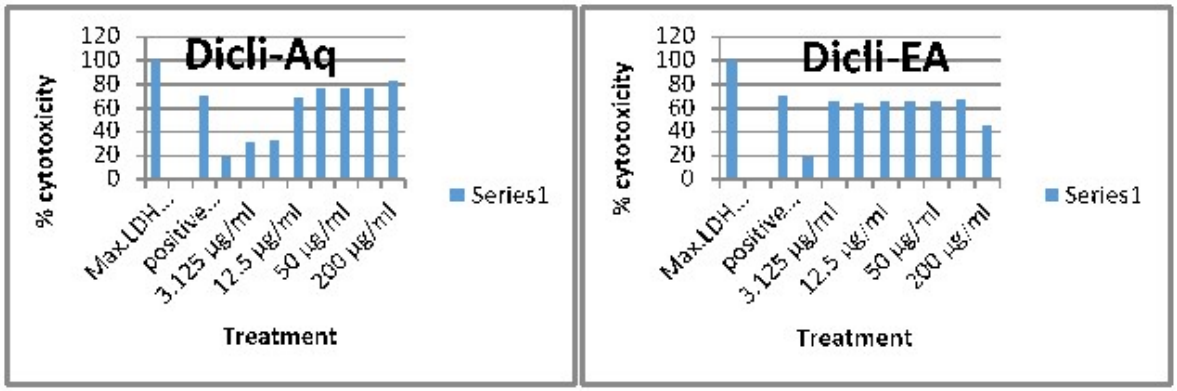

Fig. 6: LDH assay of *Dicliptera bupleuroides* Nees.

measured absorbance at 490nm on ELISA reader. Percentage of LDH release from cells was evaluated (Fotakis and Timbrell, 2006).

STATISTICAL ANALYSIS

All results were expressed as Mean ± SD. To determine the level of significant difference between various calculations one-way variance (ANOVA) between groups and column data were employed using graph pad prism version 5.01 software. Value of $p<0.05$ considered to be significant.

RESULTS

Phytochemical analysis

In the present study phytochemical screening of crude methanolic extracts of both plants *M. orontium* and *D. bupleuroides* revealed the presence of different phyto-constituents (carbohydrates, proteins, lipids, steroids, triterpenes, glycosides, alkaloids, flavonoids, saponins and phenols) given in table 1.

Anticancer activity

Anticancer assay including MTT, AB, NR and LDH were recorded on crude methanolic extract and all other fractions of the both the plant species to investigate the anticancer potential of these plants. Results of all assay displayed that all fractions have anticancer potential at lowest concentrations as compared to positive and negative controls while aqueous and ethyl acetate fraction have highest potential when compared to other fractions. On the basis of results, LDH assays were performed on aqueous and ethyl acetate fraction (fig. 3, 6). Results are depicted in percentage of cell viability and cell cytotoxicity which are inversely proportional at same concentrations as given in fig. 1, 2 and 4, 5 respectively.

DISCUSSION

Phytochemical analysis showed the presence of various phytochemicals which are responsible for therapeutic activity of medicinal plants (Padmaharish and Lakshmi, 2017). These constituents responsible for antioxidant activity of these plants. Due to this antioxidant potential *M. orontium* showed strong hepatoprotective agent and *D. bupleuroides* also proved it (Akbar and Ishtiaq, 2020; Akbar *et al*., 2020). Antioxidants have potential to inhibit cancer cells by xenobiotic metabolizing enzymes that change the metabolic activation of potential carcinogen and prevent the development of cancer. In case of plant extracts and its fractions were used for anticancer activity evaluations showed different kinds of results because of complex nature of plant constituents. For this investigation various types of assays such as MTT, AB, NR and LDH assays were used for investigation of anticancer potential (Abdullha *et al*., 2014). MTT assay is most common and more extensively used and quantitative colorimetric method. In this assay all plant fractions were evaluated for anticancer potential against Hep G2 carcinoma cell lines at different concentrations. The results were very promising which revealed that all fractions have high percentage of cell viability at lower concentration. Increased inhibition of cancer cell growth was observed with minimal of percentage of cell viability (fig. 1, 4). In the aqua bluer assay, Resazurin is converted into fluorescent resorufin. Percentage of cell viability is significantly high in all fractions of both plants (fig.1, 4). Results of this assay indicated that both plants have strong potential against anticancer activity. NR assay is more simple, economical and sensitive technique.

It based upon the principle of ability of viable dye to cross the plasma membrane of the integrated lysozyme of the viable cell as described by the method of (Repetto *et al*., 2008). Results revealed that all fractions showed anticancer ability. All fractions showed comparable results with reference drug (figs. 1, 2 4, 5). Membrane integrity is also in important parameter for the determination of LDH assay. LDH is an enzyme catalyzes the lactate into pyruvate. LDH present in the plasma of cells indicated the cell death and cellular membrane leakage. Two fraction aqueous and ethyl acetate fraction of both plants were used for LDH assay on the basis of

better result. Results obtained are depicted in (fig. 3, 6) that there is no significant level of LDH was present in culture medium after 24 hrs. It was observed in all assays that cell viability decreased significantly by increasing the concentration of the test sample. Different assays yield different results as have different functions. However, all results indicated the excellence performance in evaluating the anticancer profile of each assay (Specian *et al*., 2016). All above findings concluded that all fractions hold strong anticancer activity which need to chemically proven for future prospective.

CONCLUSION

The potential anticancer activity of *Misopates orontium* L and *Dicliptera bupleuroides* Nees was analyzed. The present finding showed the presence of phytochemical constituents such as carbohydrates, flavonoids, phenols, alkaloids, glycosides, saponins, tannins in crude methanolic extracts of both the plants. Anticancer assays of both plants indicated that these two medicinal plants can be effectively used as anticancer agents.

REFERENCES

Abbasi AM, Khan MA, Ahmad M, Zafar M, Khan H, Muhammad N and Sultana S (2009). Medicinal plants used for the treatment of jaundice and hepatitis based on socio-economic documentation. *Afr. J. Biotechnol*., **8**(8): 7-13.

Abdullah AH, Mohammed AS, Abdullha R, Mirghani MES and Al-Qubasisi M (2014). Cytotoxic effects of *Mangifera indica* L. kernel extract on human breast cancer (MCF-7 and MDA-MB-231 cell lines) and bioactive constituents in the crude extract. *J. Altern. Complement. Med.,* **14**(1): 199.

Aburjai T, Hudaib M, Tayyem R, Yousef M and Qishawi M (2007). Ethnopharmacological survey of medicinal herbs in Jordan, the Ajloun Heights region. *J. of Ethnopharm*., **110**(2): 294-304.

Ahmad B, Khan MR, Shah NA and Khan RA (2013). In vitro antioxidant potential of *Dicliptera roxburghiana*. *BMC Complement Altern Med*., **13**(1): 1.

Ajaib M, Ali T and Siddiqui MF (2015). A survey of ethnobotanically important herbaceous plants of tehsil Jatoi, district Muzaffargarh, Punjab, Pakistan. *Int. J. Biol. Res*., **3**(2): 87-92.

Akbar S and Ishtiaq S (2020). Hepatoprotective effect of *Misopates orontium* in rats. *Bangladesh J. Pharmacol*., **15**(3): 75-81.

Akbar S, Ishtiaq S, Hussain K, Ghayas S and Bushra R (2020). Hepatoprotective and antioxidant activity of *Dicliptera bupleuroides* Nees. extracts on paracetamol induced hepatotoxicity in rats. *Pak. J. Pharm. Sci*., **33**(4): 1899-1906.

Al-Snafi AE (2015). The pharmacological Importance of *Antirrhinum majus*-A review. *Asian J. of Pharm. Sci. & Tech*., **5**: 313-320.

Bahuguna RP, Jangwan JS, Kaiya T and Sakakibara J (1987). Flavonoids and fatty acids of *Dicliptera roxburghiana*. *Int. J. Crude Drug Res*., **25**(3): 177-178.

Buer CS, Imin N and Djordjevic MA (2010). Flavonoids: new roles for old molecules. *J. Integr. Plant. Biol*., **52**(1): 98-111.

Costa DA, Chaves MH, Silva WCS and Costa CLS (2010). Constituintes químicos, fenóis totais e atividade antioxidante de Sterculiastriata St Hil. et Naudin. *Acta Am*., **40**(1): 207-212.

Fotakis G and Timbrell JA (2006). *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, **160**(2): 171-177.

Ghimeray AK, Jin CW, Ghimire BK and Cho DH (2009). Antioxidant activity quantitative estimation of azadirachtin and nimbin in *Azadirachta indica*. A. Juss grown in foothills of Nepal. *African J. Biotechnol*., **8**(33): 3084-3091.

Jabeen A, Khan MA, Ahmad M, Zafar M and Ahmad F (2009). Indigenous uses of economically important flora of Margallah Hills National Park, Islamabad, Pakistan. *Afr. J. Biotechnol*., **8**(5): 763-784.

Kesarkar S, Bhandage A, Deshmukh S, Shevkar K and Abhyankar M (2009). Flavonoids: An overview. *J. Pharm. Res*., **2**(6): 1148-1154.

Li S, Han Q, Qiao C, Song J, Cheng CL and Xu H (2008). Chemical markers for the quality control of herbal medicines: an overview. *Chin Med*., **3**(1): 1-18.

Luke AM, Patnaik R, Kuriadom ST, Jaber M and Mathew S (2020). An *in vitro* study of *Ocimum sanctum* as a chemotherapeutic agent on oral cancer cell-line. *Saudi J. Biol. Sci*., **28**(1): 887-890.

Luo Y, Feng C, Tian Y and Zhang G (2002). Glycosides from *Diclipte rariparia*. *J. Phytochem*., **61**(4): 449-454.

Lönnig WE, Stüber K, Saedler H and Kim JH (2007). Biodiversity and Dollo's Law: To what extent can the phenotypic differences between *Misopates orontium* and *Antirrhinum majus* be bridged by mutagenesis? *Biorem. Biodiv. Bioavail*., **1**: 1-30.

Malar TJ, Antonyswamy J, Vijayaraghavan P, Kim YO, Al-Ghamdi AA, Elshikh MS and Kim HJ (2020). *In-vitro* phytochemical and pharmacological bio-efficacy studies on *Azadirachta indica* A. Juss and *Melia azedarach* Linn for anticancer activity. *Saudi J. Biol. Sci*., **27**(2): 682-688.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**(1-2): 55-63.

Myint PP, Dao TT and Kim YS (2019). Anticancer activity of *Smallanthus sonchifolius* methanol extract against human hepatocellular carcinoma cells. *Molecules*, **24**(17): 3054.

Padmaharish V and Lakshmi T (2017). Anticancer

activities of medicinal plants: An update. *Int. J. Pharm. Sci. Res*., **9**(4): 432.

Panigrahi G and Dubey AK (1983). *Dicliptera bupleuroides* var. *roxburghiana*, a new name for D. roxburghiana CB Clarke, non Nees (Acanthaceae). *Taxon*., **32**(2): 286-288.

Rampersad SN (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors*, **12**(9): 12347-12360.

Repetto G, Del Peso A and Zurita JL (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, **3**(7): 1125.

Sher Z, Khan Z and Hussain F (2011). Ethnobotanical studies of some plants of Chagharzai valley, district Buner, Pakistan. *Pak. J. Bot*., **43**(3): 1445-1452.

Silva AM, Silva SC, Soares JP, Martins-Gomes C, Teixeira JP, Leal F and Gaivao I (2019). *Ginkgo biloba* L. leaf extract protects HepG2 cells against paraquat induced oxidative DNA damage. *Plants*, **8**(12): 556.

Singh H, Husain T, Agnihotri P, Pande PC and Khatoon S (2014). An ethnobotanical study of medicinal plants used in sacred groves of Kumaon Himalaya, Uttarakhand, *India. J. Ethnopharmacol*., **154**(1): 98-108.

Specian AFL, Serpeloni JM, Tuttis K, Ribeiro DL, Cilião HL, Varanda EA, Sannomiya M, Martinez-Lopez W, Vilegas W and Colus IM (2016). LDH, proliferation curves and cell cycle analysis are the most suitable assays to identify and characterize new phytotherapeutic compounds. *Cytotechnology*, **68**(6): 2729-2744.

Sultana B, Anwar F and Przybylski R (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam. trees. *Food Chem*., **104**(3): 1106-1114.

Vijayakumar S, Malaikozhundan B, Saravanakumar S, Duran-Lara EF, Wang MH and Vaseeharan B (2019). Garlic clove extract assisted silver nanoparticle-Antibacterial, antibiofilm, antihelminthic, anti-inflammatory, anticancer and ecotoxicity assessment. *J. Photochem. Photobiol. B. Bio.,* **198**: 111558.

Wagner H and Ulrich-Merzenich G (2009). Synergy research: Approaching a new generation of phytopharmaceuticals. *Phytomedicine*, **16**(2-3): 97-110.