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Article

Extraction optimization, Total Phenolic-Flavonoids content, HPLC-DAD finger printing, antimicrobial, antioxidant and cytotoxic potentials of Chinese folklore *Ephedra intermedia* Schrenk & C. A. Mey

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Plants from genus *Ephedra* are commonly used by the Chinese people as folk medicine for treatment of various diseases. The current study was designed to explore the ethno-pharmacological based pharmacological potentials of *Ephedra intermedia* Schrenk & C.A. Mey. (*E. intermedia*). Plant aerial parts were extracted using ten solvent systems with increasing order of polarity. Samples were analyzed for total phenolic and flavonoid contents, HPLC-DAD analysis, antibacterial, antifungal, HepG2 cell line cytotoxicity, hemolysis and antioxidant potentials following standard procedures. Highest percent extract recovery was observed in Eth+WT (25.55 % w/w) solvent system. Flavonoid and phenolic contents were higher in chloroform and Met+WT fractions respectively. Considerable antibacterial activity was shown by Eth+Met extract against *B. subtilis* and *K. pneumonia* (MIC of 11.1µg/mL for each). Eth extract exhibited high antifungal activity against *A. fumigates* (15±0.31 mm DIZ). Met+WT extract showed significant cytotoxicity against HepG2 cell lines with IC_{50} of 13.51+0.69 µg/mL. Substantial free radical scavenging activity (74.9%) was observed for Met+Eth extract. In the current study, several solvent systems were used for more effective extraction of fractions and can be useful in the isolation of phytochemicals. Various fractions exhibited considerable antimicrobial, antioxidant and cytotoxic potentials. Biological potentials of *E. intermedia* signify its potential uses in microbial, cancer and degenerative disorders and thus warrant further detailed studies.

Keywords: *Ephedra intermedia*. Hemolytic assay. Antibacterial activity. Cancer. HepG2 cell lines.

INTRODUCTION

The use of medicinal plants in drug discovery is known to human beings from time immemorial-immortal (Farnsworth, 2008; Ayaz *et al.*, 2017a). Currently, plant based drugs are getting more attention in complementary and alternative medicine and as source of novel bioactive compounds (Ayaz *et al.*, 2017b; Ullah *et al.*, 2018). Owing to the diversity of secondary metabolites, safety and biodegradable nature, herbal drugs may be useful alternative to the currently available drugs (Ayaz *et al.*, 2019a). *Ephedra intermedia* Schrenk & C. A. Mey belongs to the family Ephedraceae. It mainly distributes in Baluchistan, North-West Himalayas, Kashmir, western Tibet and Afghanistan (Mehra, 1950). Plants from genus *Ephedra* are commonly used by the Chinese people as folk medicine for treatment of allergies, kidney

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problems, bronchial asthma, chills, diuretic, gastric and intestine inflation, fever, diaphoretic, headache, edema, flu, cold, cough, and nasal congestion (Dehkordi *et al*., 2015).

E. intermedia is a gymnosperm shrub, belonging to the family Ephedraceae and order Gnetales. It is distributed in Balochistan, North-West Himalayas, Kashmir, western Tibet and Afghanistan (Mehra, 1950). The genus *Ephedra* became research focus shortly after the isolation of important drug ephedrine (Abourashed *et al*., 2003). Ephedrine is an alkaloid and valuable in the treatment of asthma, nasal decongestant, anti-asthmatic, promoting weight loss, cardiovascular diseases, glaucoma, narcolepsy, urinary incontinence, depression, diabetes and hyperthyroidism (Abourashed *et al*., 2003; Bissa, 2015). The genus *Ephedra* contains different species which are good source of medicinally important phytochemicals as shown in Figure 1 (Andraws, Chawla, Brown, 2005). Pseudoephedrine and ephedroxane, which have anti-inflammatory potential, have been extracted from the methanolic extract of *E. intermedia* and *Ephedra* herbs (Figure 1) (Hikino *et al*., 1980). Potential hypotensive compound flavano-flavonolephedrannin has been isolated from the roots of *Ephedra* plants (Hikino, Takahashi, Konno, 1982) a hypotensive principle of Ephedra roots. Cyclopropyl-a-amino acids have been isolated from the stem of *Ephedra altissima* and *Ephedra foeminea* (Starratt, Caveney, 1995). *Ephedra* species such as *E. procera* (Dehkordi *et al*., 2015), *E. sarcocarpa* (Rustaiyan *et al*., 2011), *Ephedra foliate* (Bissa, 2015), *E. pachyclada* and *E. strobilacea* (Parsaeimehr, Sargsyan, Javidnia, 2010) antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra have been evaluated for their antimicrobial potential. Different species of genus *Ephedra* have already been evaluated for their antifungal (Wanlong, Lizhen, 1995; Bagheri-Gavkosh *et al*., 2009; Mewari, Kumar, 2011) and anticancer potential (Lee *et al*., 2000; Nam *et al*., 2003a; Nam *et al*., 2003b; Takara *et al*., 2005).

Although several species of Ephedraceae family are phytochemically and pharmacologicaly explored, detailed studies on *E. intermedia* have not been performed yet. Consequently, this detailed study was designed to evaluate the phytochemical, antioxidant, antibacterial, antifungal, hemolytic and anticancer potential of *E. intermedia*. Solvents with different polarities have been used to find the best one for each type of activity. Previous studies have used one or two solvent extracts, which probably do not contain all the phytochemicals. Herein, we for the first time optimized solvent extraction system for the plant, analyzed its phytochemistry and appraised various samples for their antioxidant, anti-microbial, anti-tumor potentials and hemocompatibility behavior.

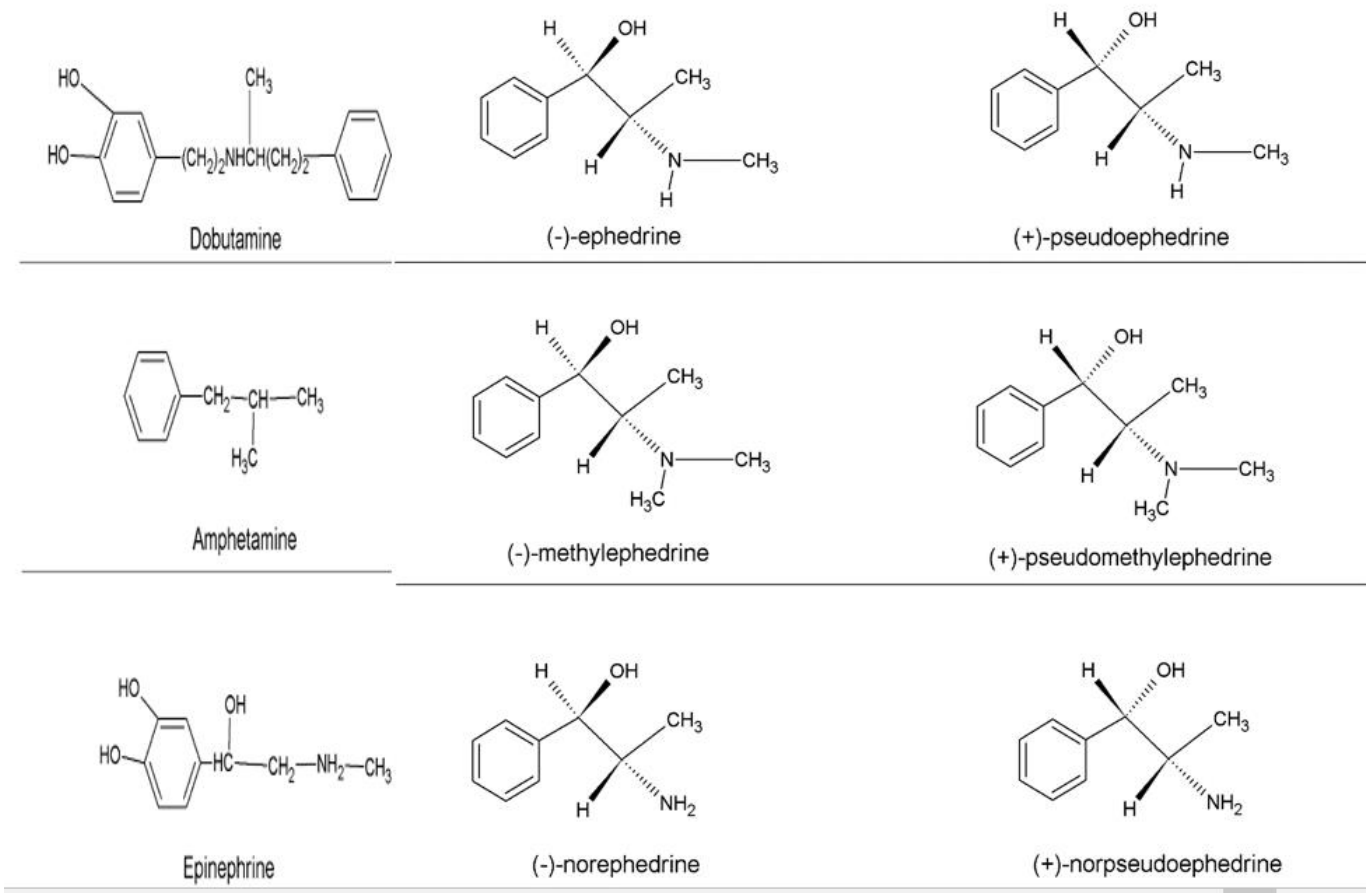

FIGURE 1 - Reported medicinally important phytochemicals extracted from different species of *Ephedra*.

MATERIAL AND METHODS

"The Minimum Standards of Reporting Checklist contains details of the experimental design, statistics, and resources used in this study."

Chemicals and Reagents

Analytical grade solvents were used for extraction. The solvents comprised: dimethyl sulfoxide, methanol, ethyl acetate, acetone, ethanol, chloroform, and n-hexane. The solvents and reagents like dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ferric chloride, trichloroacetic acid, ammonium molybdate, potassium ferricyanide, sulphuric acid, aluminium chloride, gallic acid, quercetin, ascorbic acid, potassium acetate, were obtained from Merck, Darmstadt, Germany, Tween-20 was bought from Merck, 2, 2-diphenyl-1-picryhydrazyl (DPPH), Folin-Ciocalteu reagent were bought from (Sigma–Aldrich) while phosphate buffer saline (PBS), nutrient agar, Sabouraud dextrose (SDA), trypton soy broth (TSB), sterile normal saline solution (0.9%), sea salt, 0.5% triton x-100,cefixime, vincristine were obtained from Sigma (Sigma Aldrich, USA) and amphotericin-B form Caisson Laboratories Hungary.

Microbial Cultures and cell line

Fungal strains include *Mucor* species (FCBP 0300), *Aspergillus flavus (A. flavus)* (FCBP 0064), *Aspergillus fumigatus (A. fumigatus)* (FCBP 66) and *Aspergillus niger (A.niger)* (FCBP 0198). The bacterial strains used as test strains for antibacterial assay were *Klebsiella pneumoniae* ATCC- 1705 (*K. pneumoniae*), *Staphylococcus aureus* ATCC-6538 (*S. aureus)*, *Pseudomonas aeruginosa* ATCC-9721

(*P. aeruginosa*), *Staphylococcus epidermidis ATCC-12228* (*S. epidermidis*), *Escherichia coli* ATCC-25922 (*E. coli*) and *Bacillus subtilis* ATCC-6633 (*B. subtilis*). HepG2 cancer cell lines (RBRC-RCB1648) were used to determine the anticancer potential. All standard strains were provided by Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan. HepG2 cell line contamination was analysed following NCBI database for contamination of cell lines (Ayaz *et al*., 2015).

Collection of Plants sample

Aerial parts of *Ephedra intermedia* were collected in July 2016 from Kawas village of District Ziarat, Balochistan, Pakistan. Plant was identified by taxonomist Professor Dr. Zabta Khan Shinwari, and herbarium sheet under voucher no: 348 was deposited at Department of Biotechnology, Molecular Systematic and Applied Ethnobotany Lab of Quaid-i-Azam University, Islamabad, Pakistan.

Drying and extraction

The plant was shade dried for one week and ground to fine powder. Powder weighing 30 g was macerated in 150 mL of seven single solvents (WT: water, Met: methanol, Eth: ethanol, Eacet: ethyl acetate, Acet: acetone, Chlo: chloroform and NH: n-Hexane,) and three solvents in combinations (Met + WT; methanol: water, Eth + WT; ethanol: water and Met + Eth; methanol: ethanol). The ratios of methanol: water, ethanol: water and methanol: ethanol was kept as 1:1 (75 mL each) for all. The plant powder was socked for three days in shaking incubator (IRMECO Germany) and was sonicated in ultrasonic bath (Elma Schmidbauer GmbH · Gottlieb-Daimler-Str. 17 · D-78224 Singen) at 35°C for five minutes on daily basis. After three days, the solution was passed through muslin cloth to remove large debris and then filtered through Whatman No.1 filter paper. The solvent was evaporated completely from the filtrate at 40 °C in a drying oven. The crude powder extract obtained was stored at 25°C (Ayaz *et al.* 2017c; Kamal *et al.*, 2017). For further experimentation, the extract was completely dissolved in 1% DMSO or the respective media used for culturing the microbes or cell lines.

Percent extract recovery of extract

The percentage extract recovery after extraction was done according to formula given below: $\left(\frac{x}{y}\right)$ x 100

Where (x) and (y) represent the weight of extract recovered and weight of plant powder used respectively (Zohra *et al.*, 2019a).

Phytochemical analysis

Estimation of Total Flavonoid contents

Total flavonoid content was estimated according to the protocol reported by (Ul-Haq *et al.*, 2012). 20 µL of each solvent extract (4 mg/ml DMSO dilution) was taken in 96 well plate. Then 10 µL of $AlCl_3$ (10% w/v in H_2O) and 10 µl of 1.0 M CH_3CO_2K were added. At the end, distilled water (160 µl) was added to each well to make the final volume 200 µL and it was incubated for 30 min at 37°C. Different concentrations (1.56 to 250 µg/mL) of quercetin as standard were used to obtain calibration curve ($y = 0.0058x + 0.0538$, $R^2 = 0.9925$) and the results were presented as µg QE/mg plant extract (Zohra *et al.*, 2019b).

Estimation of Total Phenolic contents

Estimation of TPC was done by the protocol reported by (Clarke *et al.*, 2013). Stock solutions (4 mg/mL DMSO) of each solvent extracts were prepared and 20 µL of each solvent extract was added to 96 well plate followed by addition of 90µL Folin-Ciocalteu reagent (FCR). After 5 min 90 µL Na_2CO_3 (7.5% w/v in H_2O) was added to each well and the plate was incubated for 1 h. The absorbance was measured at 760 nm using microplate reader (Bioteck, USA, Elx 800). Gallic acid and DMSO were used as positive and negative control respectively. Different concentrations of Gallic acid (1.56 –100 µg/mL) were used to obtain calibration curve ($y = 0.0427x + 0.1448$, $R^2 = 0.9826$) and the results were presented as µg GAE/mg plant extract (Nasar *et al.*, 2019).

High performance liquid chromatography-Diode Array detection (HPLC-DAD) analysis

HPLC-DAD analysis was performed as per our previously reported procedure from our Laboratory (Ovais *et al.,* 2018a; Ahmad *et al*., 2020).

Antioxidant Assays

Total antioxidant capacity

Phosphomolybdenum protocol was used to determine antioxidant potential of solvent extracts according to previously reported protocol (Clarke *et al*., 2013) with some modifications. 100µL of each solvent extract (4 mg/mL in DMSO) was taken in eppendorf tubes and 900 µL of reagent (0.6 MH_2SO_4, 28 mM $NaH_2PO_4(H_2O)$ and 4 mM ammonium molybdate solution in H_2O) was added. Ascorbic acid (4 mg/mL) was used as positive control and negative control contained 900 µL of reagent solution and 100 µL of DMSO without extract. All eppendorf tubes were kept at 95 °C for 90 min in water bath and then left to cool down to room temperature. After cooling, 200 µL of that reaction mixture was taken into 96 well plate and absorbance was monitor at wavelength of 630 nm by micro plate reader. Different concentrations of ascorbic acid (100, 50, 25, 12.5, 6.25, 3.12 and 1.562 µg/mL) were used to obtained calibration curve ($y = 0.0021x + 0.099$, $R^2 = 0.9802$).

DPPH free radical scavenging assay

Each solvent extracts were evaluated for their free radical scavenging potential according to previously reported procedures (Clarke *et al.*, 2013; Qasim *et al*., 2019). DPPH solution (4.6 mg/50 mL in methanol) was prepared and 180 µL of DPPH solution was mixed with 20µL of four different dilutions of each solvent extract with final concentrations of 200, 66.66, 22.22 and 7.41 µg/mL. The reaction was incubated for 30 min at 37 °C and absorbance was recorded at 515 nm with the help of micro plate reader (Bioteck, USA, Elx 800). Ascorbic acid was used as positive control. Percent free radicals scavenging effect was determined by using the formula:

$$\%FRSA = (1\text{-}Ab_s/Ab_c) \times 100$$

Where Ab_s represents extract samples and Ab_c represents DMSO only as a negative control.

Total Reducing power assay

Total reducing power of each solvent extract was estimated by potassium ferricyanide colorimetric assay previously reported by (Jafri *et al*., 2017). 400 µL of phosphate buffer (pH 7,0.2 mol/L) and potassium ferricyanide (1% w/v in H_2O) was mixed with 200 µL of each solvent extracts (4 mg/mL DMSO) and incubated for 20 min at 50°C. After incubation, 400 µL of trichloroacetic acid (10% w/v in H_2O) was added to the mixture and centrifuged at 3000 rpm at room temperature for 10 mins. A volume of 500 µL was taken carefully from upper layer of the reaction mixture and mixed with 500 µL distilled water and 100 µL of $FeCl_3$ (0.1% w/v in H_2O). After that, 200 µL was taken from that reaction mixture into 96 well plate and absorbance was measured at 630 nm. 200 µL DMSO was used as blank for the said reaction mixture instead of extract. Different concentrations of ascorbic acid (100, 50, 25, 12.5, 6.25, 3.12 and 1.562 µg/mL) were used to obtained a calibration curve ($y = 0.0236x + 0.0996$, $R^2 = 0.9661$) and the results of reducing power were expressed as µg AAE/mg extract.

Antibacterial assay

Well diffusion method was used for the antibacterial activity of each solvent extract according to protocol previously reported (Valgas *et al*., 2007; Ayaz *et al*., 2016). The bacterial strains were cultured in nutrient broth and incubated in incubator (Memmert GmbH, Schwabach Germany) at 37°C for 24 h. After 24hrs, the turbidity of each inoculum was adjusted with (0.5 McFarland) standard solution. The refreshed inoculums of each strain (100 µL) was swabbed onto Tryptic soy agar plates. Each solvent extract measuring 10 µL (20 mg/ml DMSO) was added to the wells (6 mm). Cefixime and DMSO were used as positive and negative controls respectively. The plates were incubated for 24 h at 37°C. After the incubation

period, the zone of clearance was observed and measured in mm with help of Vernier caliper.

Those extracts which produced inhibition zone ≥ 10 mm were consider as active and further processed to determine minimum inhibitory concentrations (MICs) by three-fold micro-broth dilution methodology (Fatima *et al*., 2015). Different concentrations (100 µg/mL to 3.70 µg/mL) of active solvent extracts were prepared in 96-well microtiter plate. A stock solution of each active extract was serially diluted with Mueller Hinton broth. A standardized inoculums (5×10^4 CFU/ml) for each bacterial strain was prepared and put in 96-well microtiter plate. The plates were then kept at 37°C for an overnight incubation. The lowest concentrations inhabiting the growth were considered as MIC by measuring the OD at 600 nm and the assay was performed three times.

Antifungal assay

Disc diffusion method was used for evaluation of antifungal activity of each solvent extracts according to protocol previously described (Ihsan-Ul-Haq *et al*., 2012; Zohra *et al*., 2019). The fungal strains (*A. niger* (FCBP-01981), *A. fumigatus* (FCBP- 66), *Mucor species* (FCBP-0300), and *A. flavus* (FCBP-0064) were obtained from fungal culture bank of Pakistan and grown on SDA media (Sabouraud Dextrose agar). The spores of each fungal strain were suspended in 0.02% Tween 20 solution and their turbidity was set according to standard (0.5 McFarland) solution. SDA plates were prepared as swabbed with 100 µL of harvested spores. Filter paper discs engrossed with 5 µL of extract (100 µg/disc) as well as 5 µL standard antifungal drug amphotericin B (250 µg/mL) and DMSO were placed on seeded SDA plates. The plates were incubated at 28°C and growth inhibition zones were measured after 24–48 h with the help of Vernier caliper.

Anti-tumoral activity

Evaluation of anticancer potential of each solvent extract against HepG2 liver cancer cell line was done according to previously reported protocol and precautions regarding the use of botanicals (Butterweck, Nahrstedt 2012; Ahmad *et al*., 2016). HepG2 cell line was cultured in Dulbecco's Modified Eagle's Medium (Lonza Bangkok 10110 Thailand) (pH=7.2) containing 10% FBS (Fetal Bovine Serum (Corning USA) in a humidified carbon dioxide incubator (37°C, 5% CO_2) to attain a confluent culture. The cells (1×10^4 cells per well) were then seeded in a 96 well micro plate and incubated to adhere the cell with the walls of the wells. Thereafter, the old media was removed and fresh media (190 µL) were added into it, and 10 µL (final concentration 100 µg/mL) of each extract was added to each well. The extract solutions in final diluted form were filtered through syringe filter before adding to each well. The cells were then incubated at 37°C for 72 hr in humidified CO_2 (5%) incubator. After incubation, 10 µL of sterilized MTT (Sigma Aldrich Germany) solution (1 mg/mL in distilled H_2O) was added and plate was incubated again at 37°C for 4 hrs in humidified CO_2 (5%) incubator. Then, 100 µL of DMSO (Sigma Aldrich Germany) was added to each well and mixed with the cells thoroughly for the complete dissolution of formazan crystals. DMSO (1%) in PBS (phosphate buffer saline) (Corning USA) and serial dilutions of vincristine was used as negative and positive control respectively. The absorbance was measured at 540 nm using a micro plate reader.

Hemolytic assay

Erythrocytes were obtained from fresh human blood by centrifuging (Memmet Germany) 1 mL of blood at 14000 rpm for 5 min. After centrifugation, the supernatant was discarded, 200 µL of precipitated erythrocytes were taken in a falcon tube and 9.8 mL of PBS was added to it. The resulting mixture was centrifuged for 10 min at 2000 rpm for washing. After centrifugation, the supernatant was discarded and washing step was repeated three times. Further, 100 µL of red blood cell suspension in PBS (4% volume) was placed in 96 well plate and 10 µL of plant extract (200 µg/mL) was added to each well. The volume was adjusted to 200 µL by adding 90 µL PBS, and the plates were incubated for one hour at 5°C. Triton X-100 (0.5%) was taken as positive control while the solvent DMSO and PBS were used as negative controls. The hemoglobin release was observed and reading was taken at 540 nm using microplate ELISA (BioTek Instruments, United States) reader. Percent hemolysis was calculated by using formula:

$$\textbf{Percent hemolysis} = \left(\frac{\textbf{Abs}(\textbf{T}) - \textbf{Abs}(\textbf{B})}{\textbf{Abs}\,(\textbf{P}) - \textbf{Abs}(\textbf{B})}\right) \times$$

Here: is the absorbance of extract sample, is absorbance of PBS (negative control) and is the absorbance of Triton X-100 (0.5%) (positive control)

Statistical analysis

Each experiment was performed at least in triplicates; presented data are as mean ± SD (standard deviation). Origin Pro 8 and GraphPad softwares were used to present data graphically.

RESULTS

Percent extract recovery of extract

Extraction of *E. intermedia* aerial parts was done using solvents with different polarity and the maximum % extract recovery was found in Eth + WT extract (25.55 % w/w) followed by Met extract (20.75%w/w) and Met + WT (19.65%w/w) as shown in Table I. While Chlo and Eacet extracts exhibited minimum yield (0.45%w/w and 0.80%w/w) respectively.

TABLE I - Percent extract recovery of *E. intermedia* using different solvents

Samples	*% Extract Recovery*
WT	5.10
Met	20.75
Eth	10.10
Eacet	0.80
Acet	3.65
Chlo	0.45
NH	1.10
Met + WT	19.65
Eth + WT	*25.55*
Eth + Met	15.75

Values obtained are as mean from three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol

Total Flavonoid contents

The Highest TFC was found in Chloroform and Eacet extract with flavonoid contents of 204 and 197 **µ**g QE/mg extract respectively as shown in Figure 2.

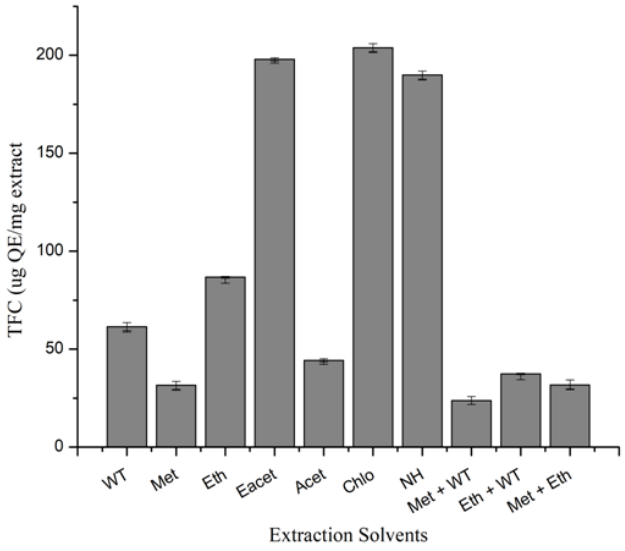

FIGURE 2 - Total Flavonoid content µg/mg extract quercetin equivalent (QE) of *E. intermedia*. Values obtained are as mean ± Standard error from three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol.

Total phenolic contents

Figure 3 shows the total phenolic contents of different solvents extract. The highest phenolic contents (137, 136 and 135 **µ**g GAE/mg extract) was found in Met + WT, Acet and Met+Eth extracts respectively.

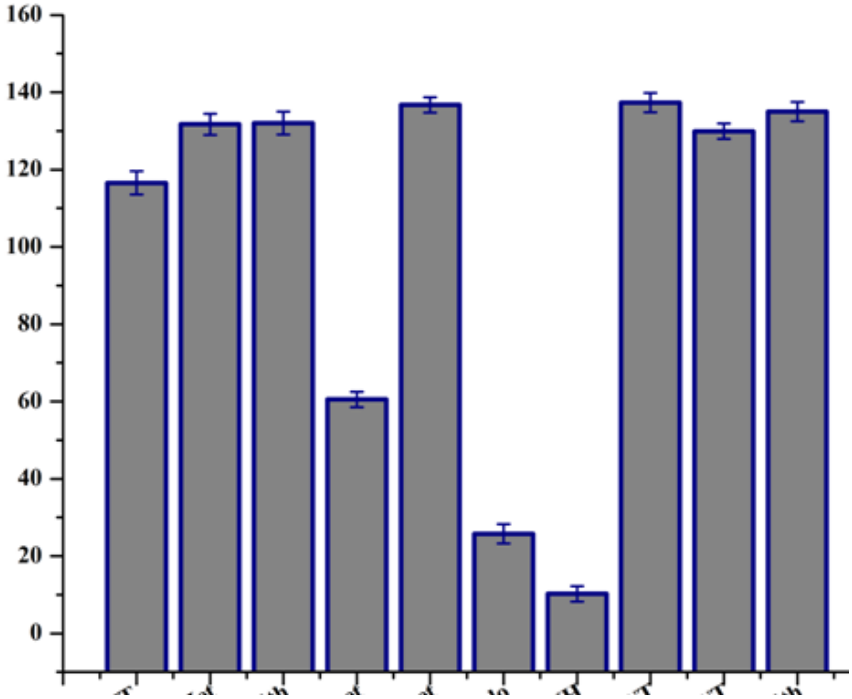

FIGURE 3 - Total phenolic content **µ**g/mg extract gallic Acid equivalent (GAE).

Values obtained are as mean ± Standard error from three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol.

HPLC-DAD fingerprinting

High performance liquid chromatography-Diode Array detection (HPLC-DAD) analysis of methanolic, chloroform, ethylacetate and n-hexane fractions was performed (Figure 4 A, B, C, D). In case of methanolic fraction, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 34.0 (Rosmarinic acid), 31.0 (HHDP-galloyl-glucose), 27.8 (Chicoric acid) min, which were 58.6, 15.0, 7.8 and 6.47 % respectively. In case of chloroform fraction, highest concentrated signals were observed at retention times of 32.9 (Quercetin), 33.7 (Phloroglucinol), 30.9 (Quercetin), 29.6 (Caffeic acid derivative) min, which were 46.8, 14.2, 8.8 and 8.2 % respectively. In case of ethylacetate fraction, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 34.0 (Rosmarinic acid), 31.1 (Quercetin-3-(caffeoyldiglucoside)-7-glucoside), 26.9 (p-Coumaric acid) min, which were 58.4, 16.7, 8.3 and 5.8 % respectively while for n-hexane, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 30.1 (Mandalic acid), 22.8 (Proanthocyanidin B1), 31.0 (HHDP-galloyl-glucose) min, which were 38.4, 13.3, 12.5 and 7.4 % respectively. The compounds were identified by comparing absorption spectra of the sample with the standard compounds or from the values reported in the literature and our previously published papers (Aaby, Ekeberg, Skrede 2007; Mradu *et al.*, 2012; Ibrahim *et al.*, 2015; Ovais *et al.*, 2018b). The current pharmacological effects of solvent extracts can be attributed to the presence of these compounds.

Antioxidant Assays

Total antioxidant capacity (Phosphomolybdenum method)

Figure 5 shows the total antioxidant capacity of different solvent extracts. Among all extracts, highest TAC was shown by Met+Eth extract (392µg AAE /mg

extract) followed by Acet extract (301 µg AAE /mg extrac) and Met (275 µg AAE /mg extract). The lowest antioxidant activity was shown by Eacet (63 µg AAE / mg extract) and NH (17 µg AAE /mg extract).

DPPH free radical scavenging

In case of DPPH free radical scavenging, maximum scavenging potential was observed in Met+Eth extract (74.9%) followed by Acet (74.5%) and Met (74%) extracts as shown in Figure 6. The minimum activity was shown by NH extract (10%).

Reducing power

The highest reducing power was detected in Eth extract (271 µg AAE/mg extract) followed by Acet extract (236 µg AAE/mg extract) as shown in Figure 7.

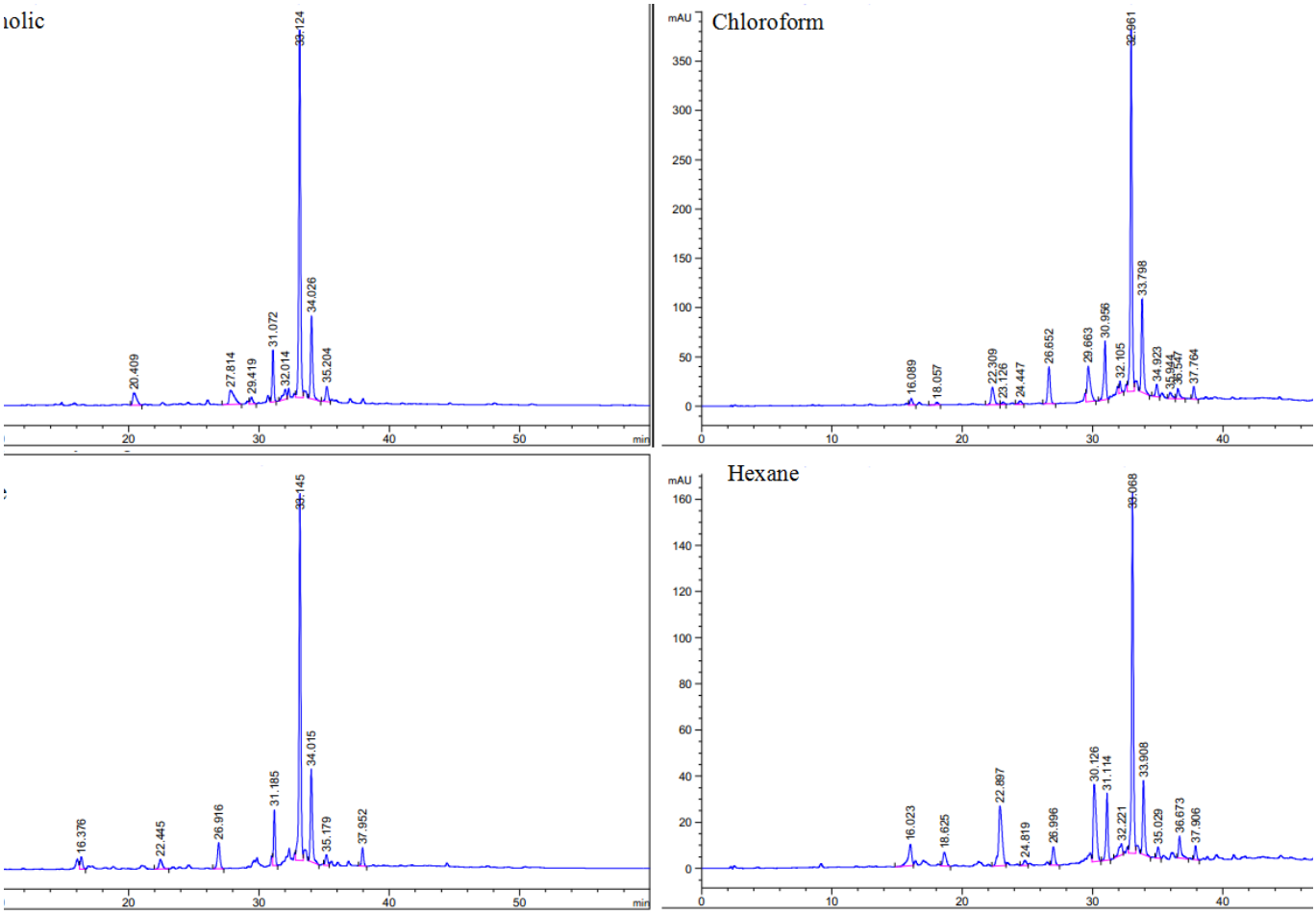

FIGURE 4 - HPLC-DAD finger printing of **A)** methanolic fraction, **B)** chloroform fraction **C)** Ethylacetate fraction and **D)** *n*-Hexane fraction.

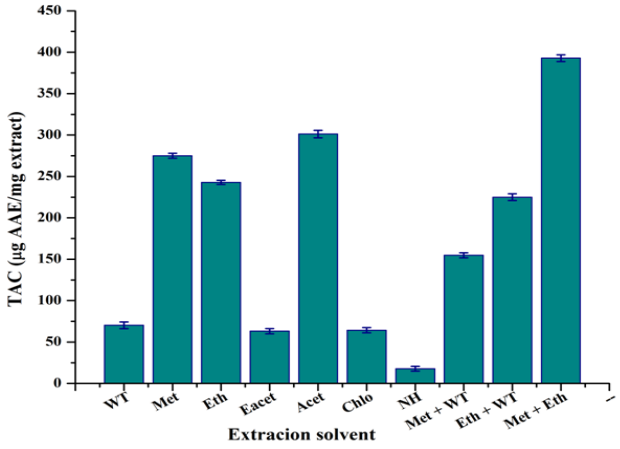

FIGURE 5 - Total antioxidant capacity (µg/mg extract ascorbic acid equivalent (AAE) of different solvent extracts of *E. intermedia.*

Values obtained are mean ± Standard error from three independent experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol

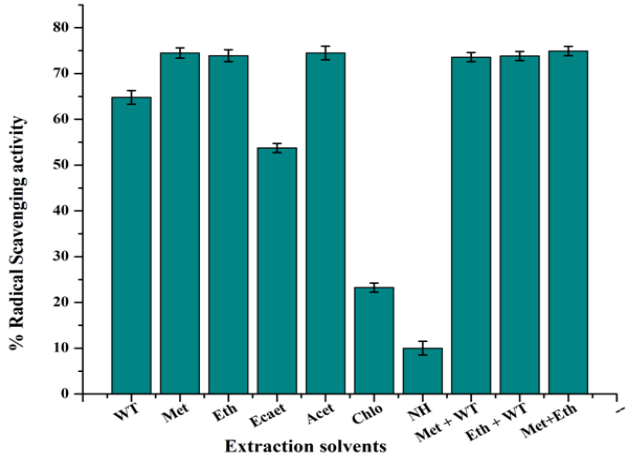

FIGURE 6 - Percent free radical scavenging activity of different solvent extracts of *E. intermedia.*

Values obtained are as mean ± Standard error from three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met+WT;** methanol+water, **Eth+WT;** ethanol + water, **Met + Eth;** methanol + ethanol.

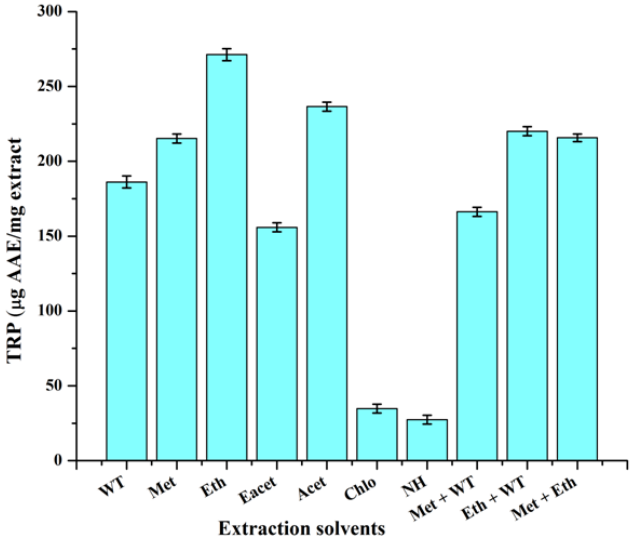

FIGURE 7 - Total reducing power of different solvent extracts of *E. intermedia*.

Values obtained are as mean ± Standard error from three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, NH: n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol.

Antibacterial activity

The antibacterial potential of each solvent extracts of *E. intermedia* is shown in Table II. Growth inhibitory zone was calculated through agar well diffusion method and MIC was determined by broth micro dilution method. Among all solvents extract, Eth+Met extract was found effective against *B. subtilis, K. pneumoniae* and *S. epidermidis,* producing zones of inhibition of 15 ± 0.04, 15 ± 0.72 and 14 ± 0.33 mm with MIC of 11.1, 11.1 and 33.3 µg/mL respectively. Eth extract was found active against *B. subtilis, E.coli, P. aeruginosa* and *K. pneumonia*. Met extract showed significant activity against (MIC 33.3 µg/mL) against *P. aeruginosa.*

TABLE II - Antibacterial activities of different solvent extract of *E. intermedia*

Sample 10 µL (20 mg/mL) DMSO)	Zone of growth inhibition (mm ± SD) and MIC (µg/mL)											
	B. subtilis	**MIC (µg/mL)**	***S. aureus***	**MIC (µg/mL)**	***E. coli***	**MIC (µg/mL)**	***P. aeruginosa***	**MIC (µg/mL)**	***K. pneumoniae***	**MIC (µg/mL)**	***S. epidermidis***	**MIC (µg/mL)**
WT	8.3 ±0.04	>100	7.1±0.54	>100	9.1 ± 0. 11	>100	9.1 ± 0.30	>100	9.6 ± 0. 11	>100	10 ± 0.12	100
Met	12.1±0.06	100	7.2± 0.71	>100	9.3 ± 0.21	>100	14 ± 0.62	33.3	11.2 ± 0.63	100	--	--
Eth	13 ± 0.16	33.3	9 ± 0.81	100	14 ± 0.10	33.3	14 ± 0.81	33.3	13 ± 0.53	33.3	8.1 ± 0.12	>100
Eacet	7.2 ± 0.04	>100	--	--	9.1 ± 0.10	>100	--	--	--	--	9 ± 0.23	>100
Acet	--	--	8.1± 0.21	>100	10 ± 0.91	100	--	--	8.3 ± 0.83	>100	--	--
Chlo	7.1± 0.98	>100	--	--	--	--	8 ± 0.81	>100	--	--	13 ± 0.12	33.3
NH	--	--	7.2± 0.77	>100	9 ± 0.61	>100	--	--	--	--	7.2 ± 0.32	>100
Met+ WT	9 ± 0.08	>100	--	--	7 ± 0.21	>100	8 ± 0.21	>100	7 ± 0.52	>100	--	--
Eth + WT	7 ± 0.18	>100	8 ± 0.32	100	8.1 ± 0.38	>100	13 ± 0.72	33.3	7 ± 0.22	>100	8 ± 0.21	>100
Eth + Met	15 ± 0.04	11.1	8 ± 0.54	>100	--	--	8.2 ± 0.73	>100	15 ± 0.72	11.1	14 ± 0.33	33.3

Values obtained are as mean ± standard error from three separate experiments. -- = No activity, sandard drug cefixime gave growth inhibition zones of 19 ± 0.9 mm: *B. subtilis*, 14 ± 0.7mm: *S. aureus*, 25 ± 1.09 mm: *E.coli,*17 ± 0.21 mm: *P. aeruginosa*,19 ± 1.12 mm: *K. pneumonia* and 22±0.91 mm; *S. epidermidis*

Antifungal activity

In case of antifungal assay, the highest zone of inhibition (15 ± 0.31 mm) was shown by Eth extract against *A. fumigatus* followed by Met + WT extract (14 ±0.66 mm) against *A. niger* shown in Table III. Eth + Met extract was found to be active against *Mucor* spp. only and Met+WT gave highest zone of inhibition (11 ± 0.69 mm) among all extracts against *A. flavus*.

TABLE III - Antifungal activities of different solvents extracts of *E. intermedia*

Sample	Diameter of growth Inhibition Zone (DIZ) (mm ± SD at 100 µg/Disc (extract)			
	A. fumigates	***A. flavus***	***A. niger***	***Mucor* spp**
WT	9 ± 0.51	10 ± 0.77	7 ± 0.71	--
Met	8 ± 0.05	8 ± 0.43	--	--
Eth	15 ± 0.31	8 ± 0.31	7 ± 0.51	7 ± 0.31
Eacet	--	10 ± 0.44	--	8 ± 0.64
Acet	12 ± 0.25	10 ± 0.44	--	8 ± 0.68
Chlo	10 ± 0.39	--	10 0.67	9 ± 0.16
NH	--	9 ± 0.67	--	--
Met + WT	13 ± 0.12	11 ± 0.69	14 ±0.66	--
Eth + WT	9 ± 0.57	--	8 ± 0.26	--
Eth + Met	14 ± 0.69	7 ± 0.19	13 ± 0.41	14 ± 0.46

Values obtained are as mean ± Standard error from three separate experiments. Disc's diameter (5 mm), each disc contain (5 µL) of concentration 100 µg/disc in disc diffusion assay. **–:** no activity

Percent hemolysis

The lowest % hemolysis was shown by Eth extract (1.98%) followed by Met, Acet and Met + Eth with % hemolysis of 2, 3 and 3% respectively as shown in Figure 8. The highest % hemolysis was shown by NH and Chlo extracts i.e. 43.56 and 30.81% respectively.

Anti-tumoral potential

Each solvent extracts were appraised for their anticancer potential against HepG2 liver cancer cell line as shown in Figure 8. Among all extracts, Met + WT, Eth + Met and Eth extract exhibited significant anticancer activities with IC_{50} values of 13.51, 16.12 and 19.23 µg/mL respectively while standard drug vincristine showed IC_{50} of 7.3 µg/mL (Figure 9).

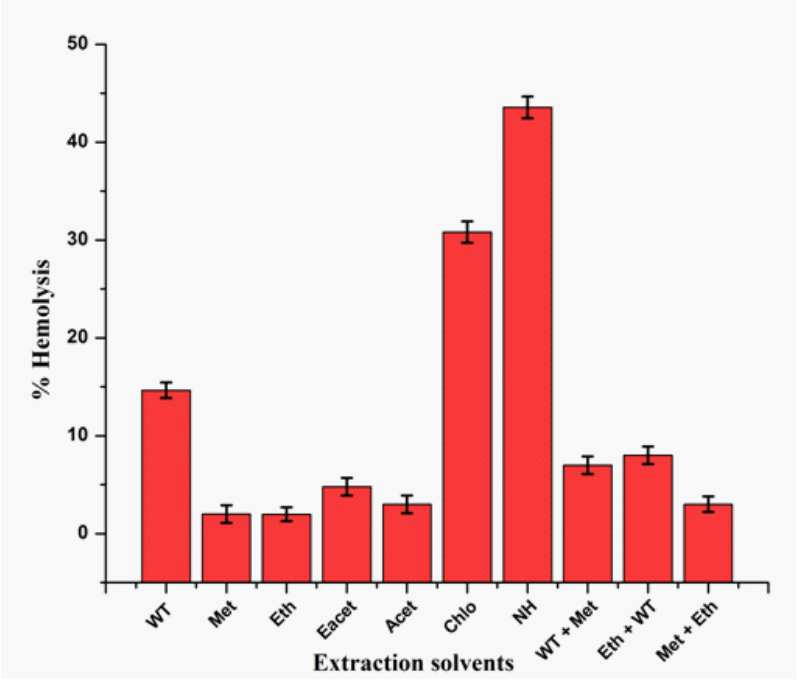

FIGURE 8 - Effect of different solvent extracts of *E. intermedia* on human erythrocytes.

Data is represented as mean ± standard error of three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol.

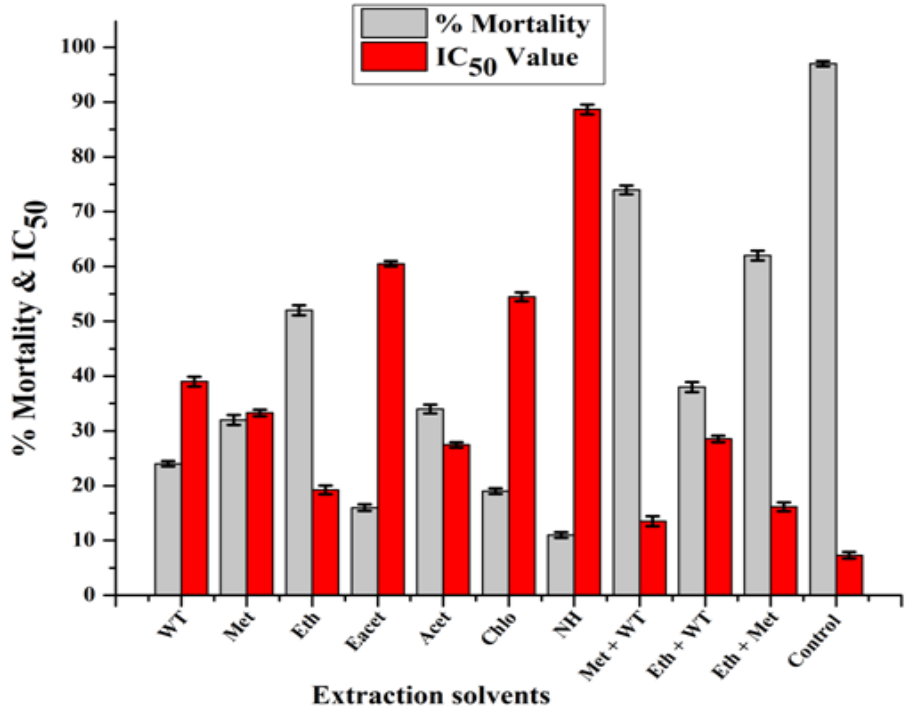

FIGURE 9 - Cytotoxic effects and IC_{50} of different solvent extracts of E. *intermedia* on HepG2 liver cancer cell lines.

Data is represented as mean ± standard error of three separate experiments. **WT:** water, **Met:** methanol, **Eth:** ethanol, **Eacet:** ethyl acetate, **Acet:** acetone, **Chlo:** chloroform, **NH:** n-Hexane, **Met + WT;** methanol + water, **Eth + WT;** ethanol + water, **Met + Eth;** methanol + ethanol, Slandered drug was vincristine.

DISCUSSION

As the extraction procedure involves the solubility of some phytochemicals on polarity basis, it is significant to investigate the correlation between the extraction method applied and their physiochemical properties (Tabassum *et al*., 2017). Chloroform and Eacet extract were found to have high flavonid content. Different flavonoids such as C-glycosyl flavones, flavonol-3-O-glycosides and proanthocyanidins have been reported for different species of *Ephedra* (Wallace *et al.* 1982; Boulos, 1983; Nawwar, El-Sissi HI, Barakat, 1984; Hussein *et al*., 1997).

Both edible and non-edible plants contains different phenolic compounds (Kumaran, Karunakaran, 2007). As phenolic compounds are very responsive towards free radicals, so the quantification of these compounds correlate to antioxidant activity (Loizzo *et al*., 2012). The genus *Ephedra* is reported to contain different phenolic compounds (Parsaeimehr, Sargsyan, Javidnia, 2010; Jaradat, Hussen, Al Ali, 2015). It is reported that methanolic extract of *E. procera* showed high phenolic contents (Parsaeimehr, Sargsyan, Javidnia, 2010). Nebrodenside A and nebrodenside B are phenolic glycosides isolated from the aerial parts of *E. nebrodensis* (Cottiglia *et al*., 2005). Different species of genus *Ephedra* such as *E. major*, *E. distachya subsp. helvetica, E. fragilis E. major*, *E. distachya sub sp. E. fragilis*, *E. foeminea, E. altissima, E. alata,* and *E. foliate* have already been evaluated for their flavonoids and phenolic contents (Ibragic, Sofić, 2015; Aghdasi *et al*., 2016). In HPLC-DAD analysis, in case of methanolic fraction, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 34.0 (Rosmarinic acid), 31.0 (Galloyl-Hexahydroxydiphenoyl-galloyl-glucose), 27.8 (Chicoric acid) min, which were 58.6, 15.0, 7.8 and 6.47 % respectively. In case of chloroform fraction, highest concentrated signals were observed at retention times of 32.9 (Quercetin), 33.7 (Phloroglucinol), 30.9 (Quercetin), 29.6 (Caffeic acid derivative) min, which were 46.8, 14.2, 8.8 and 8.2 % respectively. In case of ethylacetate fraction, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 34.0 (Rosmarinic acid), 31.1 (Quercetin-3-(caffeoyldiglucoside)-7-glucoside), 26.9 (p-Coumaric acid) min, which were 58.4, 16.7, 8.3 and 5.8 % respectively while for n-hexane, highest concentrated signals were observed at retention times of 33.1 (Quercetin-3-O-glucoside), 30.1 (Mandalic acid), 22.8 (Proanthocyanidin B1), 31.0 (Galloyl-Hexahydroxydiphenoyl-galloyl-glucose) min, which were 38.4, 13.3, 12.5 and 7.4 % respectively.

Among all different solvent extracts, the highest and lowest total antioxidant capacity was observed in Met+Eth and NH extracts respectively. Phenolic compounds such as gallic acid, rutin and catechin are responsible for antioxidant activity as these compounds donate hydrogen atom or chelate metals which results in interruption of chain oxidation reactions. The high amounts of phenolic compounds are responsible for high antioxidant activity (Viuda-Martos *et al*., 2010).

The ability of antioxidants present in extracts to decolorize 2, 2-diphenyl-2- picryl-hydrazyl (DPPH) is the basic principle of free radical scavenging assay. Highest free radical scavenging potential was observed in Met+Eth extract while lowest free radical scavenging potential was observed in NH extract. The results of free radical scavenging potential and total phenolic contents were found to have positive correlation. It is reported that phenolic compounds possess free radical scavenging properties (Tabassum *et al*., 2017). *E. nebrodensis* has been also reported for its antioxidant potential (Ballero *et al*., 2010).

Reducing power of the extract is one of the prominent indicator that the extract possesses antioxidant potential. The Eth extract was found to have maximum reducing power. The results show that total phenolic content and reducing potential have positive correlation. Ephedrine and pseudoephedrine are its two primary active ingredients, which possess a variety of biological activities. However, the active components are still unknown and future research is required to isolate and characterize compounds responsible for their biological activities (Mir *et al*., 2019; Saleem *et al*., 2021).

Antibiotic resistance is becoming global issue and there is urgent need to search novel antibacterial agents (Ovais *et al*., 2018c; Ovais *et al*., 2019). Plants are one of the potential source of innovative antibacterial agents as they contain many bioactive compounds (Bissa, 2015; Ayaz, *et al.* 2017d). Chlo extract showed activity against *S. epidermidis* only while *S. aureus* strain was found

resistant against all solvent extracts. The significant antibacterial activities of Eth + Met, Eth and Met extracts against *B. subtilis, K. pneumonia, S. epidermidis*, *P. aeruginosa* and *E.coli* may be due to phenolic compounds like protocatechuic acids, caffeic acid, p-hydroxybenzoic, p-coumaric acids, oleuropein and vanillic present in the extracts (Aziz *et al.*, 1998). The antimicrobial potential of medicinal plants extract is mostly based upon solvent used for extraction, tested organism and part of the plant used (Tabassum *et al.*, 2017). Different species of *Ephedra* have been already evaluated for the antibacterial potential (Dashtdar, Dashtdar, Dashtdar, 2013; Khan *et al.*, 2017) Castanea sativa, Ephedra sinica stapf and shilajita mumiyo Against Gram Positive and Gram Negative Bacteria In Vitro Antioxidant and Antimicrobial Activities of Ephedra gerardiana (Root and Stem. Methanol extract of *E. sarcocarpa* growing in Iran showed good activity against *E. coli* and *P. aeruginosa* (Rustaiyan *et al.* 2011). Petroleum ether extract of *Ephedra foliate* stem and leaves showed maximum activity against *Klebsiella pneumoniae* and *Enterobacter aerogenes* respectively (Bissa, 2015). Methanol extract of *Ephedra alataalenda* has high antibacterial activity *against Enterococcus facealis* and *Baccillus subtilus* (Jerbi *et al.*, 2016)++++++.

The tested fungal strains (*A. niger*, *A. fumigatus*, *Mucor species* and *A. flavus*) have been reported to cause different human diseases (Waxman *et al.*, 1987; Gugnani *et al.*, 1989; Pai, Platt, 1995). In our study, Eth extract showed high antifungal activity against *A. fumigatus* while Met + WT extract showed high activity against *A. niger.* Our results can be justified by previous studies (Bonjar, 2004; Parsaeimehr, Sargsyan, Javidnia, 2010)antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra. Other species of genus *Ephedra* such as *E. major* Host (Bagheri-Gavkosh *et al.*, 2009), *E. sinica* (Wanlong, Lizhen, 1995) and *Ephedra foliata* Bois (Mewari, Kumar, 2011) have been reported for their antifungal potential.

The *in vitro* cytotoxity on the red cells was evaluated by using different solvent extracts. There are many phytochemicals in medicinal plants which can disrupt the biological membranes therefore it is necessary to check the effect of extract on red blood cells (Sharma, Sharma, 2001). Evaluation of phytotherapeutic products must be routinely considered before the product is marketed as the use of these products is increasing globally and can cause serious public health problems (Secco, 1990; Willcox, Ash, Catignani, 2004; de Freitas *et al.*, 2008). RBCs provide a good platform and experimental sample for the study of membrane stability. After lysis of RBCs, hemoglobin is released and quantified through spectrophotometer. In our study, Eth and Met extract showed minimum % hemolysis while maximum % hemolysis was shown by NH and Chlo extracts. Our results are supported by previous work (Erdem *et al.*, 2013),"even_P¾A, which reported that chloroform extract possess high hemolytic activity.

In our study, it was found that Met + WT and Eth + Met extracts showed high anticancer potentials. The anticancer potential might be due to the active compounds present in the extract that are responsible for changing the signal transduction pathways; kinase inhibitor, involved in cell cycle arrest (Ovais *et al.*, 2017; Ovais *et al.*, 2018c; Ayaz *et al.*, 2019b), inhibitory effect of flavonoids on protein kinases, different transcriptional factors (Miranda *et al.*, 1999) and extract may contain molecules which inhibit the interaction of (MDM2) murine double minute and p53. MDM2 is mostly over expressed in cancerous cells and it results in inactivation of p53 (Riaz *et al.*, 2017).

CONCLUSION

In present study, different solvent extracts of *E. intermedia* were evaluated for their phytochemical analysis and *in vitro* biological activities. We have reported the use of different solvent system for the comprehensive phytochemical as well as biological analysis of subjected plant. The results showed that the diverse biological activities of extracts depend on the organic solvent in which the extraction is done. Polar solvent extracts may contain phytochemicals responsible for good antioxidant potentials as compared to non-polar solvent extracts. This plant can be used for isolation of antioxidants, antimicrobial agents and anticancer compounds. Moreover, all the reported activities need isolation of bioactive compounds through HPLC, GCMS and NMR based on the reported results of biological activities.

DECLARATIONS

Approval of Ethical Committee

The study was approved by Research Ethics Committee, Department of Biotechnology, Quaid-i-Azam University Islamabad, Pakistan, Dated 15-1-2017 via reference No: DREC/BIO/20170600/07.

Consent to publish

Not applicable in this section

Availability of data and materials

The data presented in this manuscript belong to research work of Muhammad Qasim Nasar and has not been deposited in any repository yet. However, the materials are available to the researchers upon request.

Competing Interest

The authors declare that they have no competing interest

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AUTHORS' CONTRIBUTIONS

MQN carried out all experimental work, data collection, evaluation, literature search and manuscript preparation. TZ, MA, ATK, MO, MZ, EO and IU facilitated in execution of experiments, data analysis and manuscript write up. ZKS supervised research work, helped in study design and drafted the final version of the manuscript. All authors read and approved the final manuscript for publication.

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