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ORIGINAL ARTICLE

Flavonoids from *Heliotropium subulatum* exudate and their evaluation for antioxidant, antineoplastic and cytotoxic activities II

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Abstract The flavonoids are the largest group of phenolic compounds isolated from a wide range of higher plants. These compounds work as antimicrobials, anti-insect agents and protect plants from other types of biotic and abiotic stresses. Various researchers have suggested that flavonoids possessed antioxidant, antineoplastic and cytotoxic activities. The main objective of this study was to test dichloromethane fraction of resinous exudate of *Heliotropium subulatum* for their antioxidant, antineoplastic and cytotoxic activities, as well as to search new antioxidant and antineoplastic agents for pharmaceutical formulations. Five flavonoids were isolated from resinous exudate of this plant species and screened for their in vitro and in vivo antioxidant models (DPPH radical scavenging, reducing power, superoxide anion scavenging, metal chelating scavenging systems, catalase and lipid peroxidation), antineoplastic (Sarcoma 180), and cytotoxic (Chinese hamster V79 cells) activities. Tricetin demonstrated maximum antioxidant activity against both in vitro and in vivo experimental systems while galangin exhibited maximum inhibition (78.35%) at a dose of 10 μg/kg/day against Sarcoma 180. Similarly, it was found that galangin also showed highest activity (21.1 ± 0.15%) at a concentration of 70 μg/ml to Chinese hamster V79 cells. The observed results suggest that tricetin has a potential to scavenge free radicals in both in vitro and in vivo models while the galangin could be considered as antitumor and cytotoxic agent.

Keywords *Heliotropium subulatum* · Antioxidant · Antineoplastic and cytotoxic activity · Flavonoids

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Introduction

Heliotropium subulatum is an annual, erect, scabrous and hispid herb and can grow up to height of 50–60 cm. This species is inhabited in North-Western India and Deccan Peninsula (Pullaiah and Naidu 2003; Nasir and Ali 2005). The plant is recommended for the treatment of wound caused by scorpion-stings and the leaves combined with *Indigofera pulchra* are prepared as poultice and applied on sore breasts. In African countries, the aerial parts are used as bitter tonic, stimulant and applied as paste for the cure of boils (Burkill 1985). The decoction of leaves is being drunk as remedy for relief from postpartum disease in women as well as treatment of the ulcers, throat infections and snake bites (Ruffo et al. 2002; Schmelzer and Gurib-Fakim 2008; Aswal et al. 1984; Jain and Defilipps 1991). The resinous exudate of glandular trichomes in *Heliotropium* species increases

tolerance against abiotic stresses and is rich in terpenoids and flavonoids (Torres et al. 1996; Modak et al. 2009; Singh and Sharma 2015). Similarly, the resinous exudate of several other species of *Heliotropium* have been investigated for antioxidant (Campos et al. 2012), antiproliferative (Modak et al. 2011a, b), and cytotoxic activities (Modak et al. 2004).

As per available literature about the biological and pharmacological activities of *H. subulatum*, attempts were made to investigate the presence of flavonoids and their screening for the antioxidant, antineoplastic and cytotoxic activities. The reactive oxygen species (ROS) are chemically reactive molecules having oxygen itself and are produced as byproducts during oxygen metabolism. ROS damage the carbohydrates, lipids, proteins and nucleic acids due to which several types of diseases occurr in humans (Ebadi 2006). The chemically synthesized antioxidant drugs are available in the market but they leave negative effects on human health so, for their replacement, scientists are searching for new natural secondary metabolites to minimize health risks (Anagnostopoulou et al. 2006).

Cancer is the most dreaded disease and it involves abnormal cell growth with capability to spread to other parts of the body (Mohanty and Sahu 2010). Chemotherapy, surgery and radiotherapy strategies are being used for the treatment of cancers in current times but these measures of treatment are not effective against advanced stages of cancers (Feng and Chien 2003). To maximize the chemotherapeutic effects against advanced stages of cancer as well as to minimize the adverse effects of chemotherapeutic agents, there is an urgent need to develop new therapeutic modalities in modern times. Search for new chemotherapeutic agents remains a thrust area along with drug discovery mechanisms. Therefore, analyses of antioxidant, antineoplastic and cytotoxic activities were carried out by using DPPH and other scavenging systems, using Sarcoma 180 and Chinese hamster V79 cells to determine the possible uses of flavonoids from *H. subulatum*.

Materials and methods

Plant material

The plant material of *Heliotropium subulatum* Hochst. ex DC (syn. *H. zeylanicum* Clarke; Fam.—Boraginaceae) was collected (June, 2013) from the fields of the Agricultural Research Station, Durgapura (Research Station of SKN Agriculture University, Jobner), Jaipur. The authentication of plant material was done by Professor R. S. Mishra, Department of Botany, University of Rajasthan, Jaipur, India (Herbarium sheet no. RUBL—20493).

General experimental conditions

The Capillary Toshniwal melting point apparatus (Vadodara, Gujarat, India) was used for the recording of melting points of isolated compounds. The IR spectra were obtained on a Nicolet-Avtar 330 spectrometer (Madison, WI, USA). ^{1}H NMR (400 MHz) and ^{13}C NMR (300, 200, 100 and 75 MHz) were measured in $CDCl_3$ on a Bruker AM 400 spectrometer (Mumbai, India) with tetramethylsilane as internal standard. MS spectra were recorded with a Hewlett Packard HP 5930 A instrument (Bangalore, India). The UV absorbance was recorded on Perkin-Elmer 200 spectrophotometer (Hyderabad, India). The HPLC data were determined on HPLC-JASCO (Tokyo, Japan) instrument. The DPPH was purchased from the Sigma-Aldrich (St. Louis, MO, USA). The above facilities were provided by the Central Facility, Department of Chemistry, University of Rajasthan (Jaipur, India). All the chemicals and solvents were used of analytical grade. The Silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany) was used for column chromatography while silica gel G for thin layer chromatography. The authentic samples were obtained from the Natural Products Laboratory, Department of Chemistry, University of Rajasthan (Jaipur, India).

Extraction and characterization

Fresh plant material (2.5 kg) was immersed in dichloromethane (2.5 l) for 30 s, filtered and concentrated at room temperature (20.127 g). The concentrated extract of exudate was used for separation of fractions by column chromatography. Using column chromatography, in total 10 fractions (1–10) were collected. The five compounds were isolated (galangin, naringenin, pinocembrin, tricetin and eriodictyol) and purified by preparative thin layer chromatography (PTLC) over silica gel G.

Galangin (I) and naringenin (II)

Collected fractions (1 and 2) were pooled together (6.667 g). These two compounds were purified by

preparative thin layer chromatography (PTLC-20x20 cm diameter glass plates, wet thickness 0.5 mm). The TLC plates were developed using the following solvent system - Toluene: EtOAc: AcOH (32-14-4.0, v/v). The compound was detected on TLC plates by spraying Naturstoff reagent ($R_f \sim 0.63$) and identified as galangin (mp 213-215 °C, $C_{15}H_{10}O_5$). The isolated compound was positive in the Shinoda test. Similarly, the 3 and 4 fractions were combined together (5.823 g) and the second compound was purified by PTLC (Toluene: EtOAc: Formic acid; 5.0-4.0-1.0; v/v). The TLC plates were sprayed with the Naturstoff reagent for detection of the compound ($R_f \sim 0.54$). The compound was identified as naringenin (mp 251-253 °C, $C_{15}H_{12}O_5$). The physical and spectral data of these isolated compounds were in agreement with the data of reference compounds reported in literature (Torres et al. 1996; Modak et al. 2012).

Pinocembrin (III) and tricetin (IV)

The other fractions (5–8) were also combined together (4.773 g). The TLC plates were developed using the following solvent system: Hexane-EtOAc-MeOH-H_2O; 3:7:6:4. The TLC plates were sprayed with the Naturstoff reagent and both compounds were detected. The first compound was identified as pinocembrin ($R_f \sim 0.43$, mp 218-220 °C, $C_{15}H_{12}O_4$) and the other was tricetin ($R_f \sim 0.53$, mp 232–234 °C, $C_{15}H_{10}O_7$). The physical and spectral data of isolated known flavonoids were identical with their respective standard compounds (Campos et al. 2002; Ching et al. 2007).

Eriodictyol (V)

The remaining two fractions (9 and 10) were combined together (2.894 g) and the compound was purified by PTLC (solvent used: $CHCl_3$: MeOH; 8.5–1.5; v/v). The TLC plates were sprayed with $AlCl_3$ reagent for the detection of compound ($R_f \sim 0.37$, mp 259–261 °C, $C_{15}H_{12}O_6$) and the compound was identified as eriodictyol. The identity of isolated compound was confirmed by comparison (ir, nmr and ms) with an authentic sample of eriodictyol (Backheet et al. 2003).

HPLC analysis of dichloromethane fraction

HPLC determination of isolated compounds was performed using the following conditions - HPLC model JASCO coupled to CD 2095 (Tokyo, Japan), column size (250x4.6 mm), valve model as Rheodyne (7725i). Two solvents were used as mobile phase: methanol (solvent A) and aqueous AcOH (solvent B). The column was eluted in isocratic [(0-12 min, solvent A (30%) and B (70%)] and linear gradient phases [(0-12 min, solvent A (98%) and B (2.0%)]. The flow rate was adjusted to 0.5 ml/min and the volume of the injection loop was 20 µl. The absorbance was recorded at 287-342 nm and the program was adjusted to 30 min. The isolated flavonoids were estimated on basis of peak areas in chromatograms taken at different absorbance values: galangin (287 nm), naringenin (312 nm), pinocembrin (321 nm), tricetin (330 nm), eriodictyol (342 nm). Different dilutions of standard solutions were prepared from stock solutions of galangin, naringenin, pinocembrin, tricetin, eriodictyol. All solutions of reference compounds and isolated compounds were stored at 4 °C. For the estimation of isolated compounds, the calibration lines were obtained by plotting peak areas against the concentrations of the reference compounds; these lines were used to calculate the quantities of isolated compounds. Each value of compounds was determined in triplicate readings (Modak et al. 2011a, b).

In vitro antioxidant activities

DPPH scavenging assay

DPPH scavenging assay of dichloromethane fraction, isolated flavonoids and standard compound was assessed by using a well established protocol (Yokozawa et al. 1998). 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.5 ml in MeOH, 150 µM), was mixed to the solutions of dichloromethane fraction, isolated flavonoids and standard compound with the different concentrations tested (10, 20, 30 µM). With continuous agitation, the reaction mixture was incubated at room temperature for 1 h. After 1 h incubation, the reaction mixtures were used for the calculation of inhibition. The readings were taken at 517 nm on a UV spectrophotometer (Schimadzu UV–Vis 1700) by using MeOH as blank. For this experimental study, (±)-α-tocopherol was used as positive control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The rate of inhibition (%) of DPPH assay was calculated as follows:

$$\text{Inhibition rate } (\%) = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}})/\text{Absorbance}_{\text{control}} \times 100$$

Reducing power

Different concentrations (10, 20, 30 μM) of dichloromethane fraction, isolated flavonoids and standard sample were assessed for reducing power activity. The experimental concentrations were added to the phosphate buffer (pH 6.8) and potassium ferrocyanide (2.5 ml of 1.0%). With continuous shaking, reaction mixtures were incubated at 50 °C for 20 min. After 20 min incubation, 2–3 drops of trichloroacetic acid were added to the reaction mixture and centrifuged at 1000×*g* for 15 min (Shimada et al. 1992). The supernatant layer of the mixture was collected and 2.5 ml of distilled water and $FeCl_3$ (0.5 ml) were added to this mixture. The solution of control was prepared in same manner excluding the test samples. After 15 min, the absorbance was measured at 700 nm on UV spectrophotometer (Schimadzu UV–Vis 1700). The inhibition rate of reducing power was calculated as follows:

$$\text{Inhibition rate } (\%) = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}})/\text{Absorbance}_{\text{control}} \times 100$$

Superoxide anion scavenging activity

The superoxide radicals were prepared in phenazine methosulfate-nicotinamide adenine dinucleotide systems by NAD oxidation. The superoxide anion scavenging assay was assessed by nitroblue tetrazolium reduction method (Robak and Gryglewski 1998). The dichloromethane fraction, isolated flavonoids and standard compound were added to the freshly prepared superoxide radicals. Phenazine methosulfate (1.0 ml; 100 μM) was added to the reaction mixtures of the test samples and incubated at room temperature for 10 min. The absorbance of the reaction mixtures was recorded at 560 nm against a blank (Schimadzu UV–Vis 1700). Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition rate (%) of superoxide anion scavenging activity was calculated as follows:

$$\text{Inhibition rate } (\%) = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}})/\text{Absorbance}_{\text{control}} \times 100$$

Metal chelating scavenging activity

The dichloromethane fraction, isolated flavonoids and standard compound (10, 20, 30 μM) were added to the 3.7 ml of MeOH and 0.1 ml of 2.0 μM ferrous chloride (Dinis et al. 1994). After proper mixing, 0.2 ml of 5.0 μM ferrozine was added to the reaction mixtures of the test samples and incubated for 10 min at room temperature. The absorbance of reaction mixtures was measured at 562 nm. The rate of inhibition (%) of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$\text{Inhibition rate } (\%) = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}})/\text{Absorbance}_{\text{control}} \times 100$$

In vivo antioxidant activities

Animals

For the assessment of in vivo antioxidant activity, in total 48 Wistar albino rats were used and divided into eight groups. Healthy laboratory diet was provided to the experimental animals which were housed in the Animal House of Pacific University Udaipur, India. Group 1 and 8 animals were used for negative (distilled water) and positive {(±)-α-tocopherol} control. To the animals of 2nd group, the dichloromethane fraction and to the animals of 3rd to 7th groups, the isolated flavonoids were administered orally. For the oral administration of test samples, 25, 50, 100 mg/kg body weight doses were used respectively. Two experimental animals were used for the testing of single dose of compounds (e.g. 25, 50, 100 mg/kg body; 2 × 3 = 6 animals per group).

For liver damage induction, on 7th day after test sample administration, the animals of groups 2–8 were intraperitoneally injected with carbon tetrachloride and olive oil (1:1) with a dose of 2.5 ml/kg body weight. The animals of the negative control group were injected with distilled water and olive oil (1:1).

After 24 h fasting, the experimental animals were sacrificed by cardiac puncture and the liver from each

rat was excised and washed with normal saline. The liver homogenate (10%) was prepared by mixing of 0.25 M sucrose in phosphate buffer and centrifuged at 8814×*g* for 5 min. After centrifugation, the supernatant was separated and used for assessment of catalase and lipid peroxidation activities.

Catalase assay

For the assessment of catalase activity, the protocol of Pari and Latha (2004) was used. The liver samples were homogenized in 0.01 M phosphate buffer (pH 7.0) and the reaction mixture centrifuged at 1530×*g* for 5 min. After centrifugation, 0.4 ml of hydrogen peroxide (0.2 M) was added to the reaction mixture and incubated for 2–3 min. Two ml dichromate acetic acid reagent (5% $K_2Cr_2O_7$ prepared in glacial acetic acid) was added to stop the reaction of reaction mixture. The absorbance of reaction mixture was measured at 620 nm and the inhibition percentage was calculated as follows:

$$\text{Catalase inhibition } (\%) = \text{Normal activity} - \text{inhibited activity}/\text{Normal activity} \times 100$$

Where:

Normal activity = hydrogen peroxide + phosphate buffer; inhibited activity = hydrogen peroxide + phosphate buffer + liver homogenate.

Lipid peroxidation assay

The method of Niehius and Samuelson (1968) was employed for the determination of lipid peroxidation activity. The activity of liver was calculated colorimetrically by recording the values of thiobarbituric acid reactive substances. Two ml of (1:1:1 ratio) TBA–TCA-HCl reagent (thiobarbituric acid 0.37%, 15% trichloroacetic acid and 0.25 N HCl) was added to the 0.1 ml of liver homogenate. The reaction mixtures were heated on a boiling water bath for 30 min and were cooled down. The absorbance of developed malondialdehyde (MDA) in each reaction mixtures was measured at 535 nm.

MDA concentration (C) was assessed as follows:

$$C = \frac{A}{E \times L}$$

where A = absorbance of the reaction mixtures, E = extinction coefficient of malondialdehyde (1.56×10^5 M^{-1}/cm) and L = length of the light path (1 cm).

Antineoplastic activity

Animals

Antineoplastic activity of dichloromethane fraction and of isolated flavonoids was assessed with ICR albino mice weighing 30–35 g each, 4-weeks old, reared in an air conditioned room and given a standard laboratory diet and tap water during the experimental work.

The packed cell volume (PCV) method was used for the determination of antineoplastic activity (Itokawa et al. 1979). Sarcoma 180 (1.0×10^{-6} cell/0.1 ml ascite fluid) was implanted (i.p.) in the experimental animals. The dichloromethane fraction and isolated flavonoids were dissolved in saline solution and suspended in carboxymethyl cellulose. The drug mixture was administered (i.p.) at 25, 50, 100 µg/kg/day dose of dichloromethane fraction while 5, 10, 20 µg/kg/day dose of isolated flavonoids were administered to the experimental animals for 5 days consecutively. The control group of animals was administered saline solution only for five days. The experimental animals were sacrificed individually on 7th day of ascite tumor implantation and the tumor cells were separated from ascite tumor by centrifugation (550.9×*g* for 7–8 min). The PCV of tumor cells and total volume (TV) of tumor cells was assessed individually in each case and body weight changes were also recorded on the 7th day of ascite tumor and implanted tumor. The inhibition rate (%) was calculated as follows:

$$\text{Inhibition rate } (\%) = \text{Average PCV of control} - \text{Average PCV of treated/Average PCV of control.}$$

Cytotoxic activity

The cytotoxic activity of dichloromethane fraction, isolated flavonoids was assessed on cloned Chinese hamster V79 cells (Itokawa et al. 1979). The cells (obtained from Dr. Tsukagoshi, JFCR, Japan) were

grown on RPMI 1640 culture medium with supplementation of 10% fetal calf serum and kanamycin (1000 µg/ml). Chinese hamster cells (3×10^5 cells/well) were cultured in 6 well plates and the each plate containing 2 ml growth culture medium, the cells were incubated at 30 °C in humid atmospheric condition (5% CO_2). Various concentrations (1.0–100 µg/ml) of dichloromethane fraction and isolated flavonoids were added to the cultures (10 µl) at day 1 after transplantation. Later, the colonies of cultured cells were fixed with HCHO solution (2.0 ml; 20 min). The developed cell colonies were stained with 0.05% crystal violet (0.75 ml) at day 5. The cytotoxic activity of dichloromethane fraction and isolated compounds was determined by recording the number of stained colonies of the test group/colonies of the control group x 100. Number of colonies in the medium which contained drug relative to colony growth (0.5%) at day 5 after the treatment with drug.

Statistical analysis

The data were expressed as mean ± SD and statistically assessed by analysis of variance (ANOVA). The inhibition percentage of antioxidant, antineoplastic and cytotoxic activities of dichloromethane fraction and isolated flavonoids are reported as mean ± standard deviation.

Results

Antioxidant activity of isolated compounds

The resinous exudate of *H. subulatum* was analyzed for the presence of phytochemicals. In total five flavonoids (I, 407 mg; II, 332 mg; III, 443 mg; IV, 567 mg; V, 303 mg; Fig. 1) were characterized from the dichloromethane fraction and investigated for their antioxidant, antineoplastic and cytotoxic activities. HPLC determination of dichloromethane fraction also revealed that tricetin as present at higher concentration (21.33 ± 0.21%) than the other isolated flavonoids (galangin-12.68 ± 0.37%, naringenin-10.21 ± 0.59%, pinocembrin-16.81 ± 0.39% and eriodictyol-09.64 ± 0.28%) in *H. subulatum*. Flavonoids are polyphenolic compounds isolated from various species of higher plants and these flavonoids protect the plants from abiotic and biotic stresses. The potentialities of the isolated

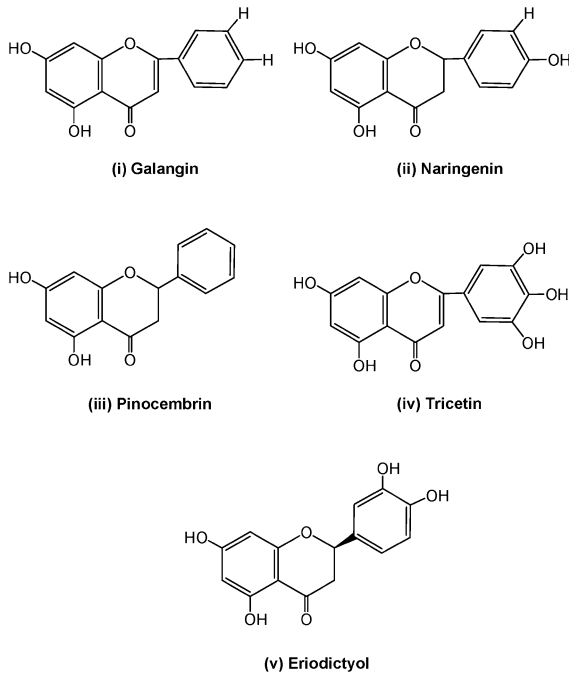

Fig. 1 Structures of isolated flavonoids from resinous exudates of *H. subulatum*

flavonoids to scavenge free radicals were assessed via a chemical reaction with DPPH. Results obtained in this study revealed that all tested flavonoids showed different DPPH radical scavenging inhibition rates at the different concentrations tested (10, 20, 30 µM). Among the tested flavonoids, tricetin demonstrated maximum radical scavenging activity (220.18 ± 0.31%) while the dichloromethane fraction exhibited minimum scavenging activity (118.63 ± 0.54%) at 30 µM concentration (Fig. 2). The variations in antioxidant potential between tricetin and other flavonoids were observed at each tested concentration. The obtained results of inhibition of tested compounds were compared with (±)-α-tocopherol (standard compound; 225.76 ± 0.74%).

The reducing power activity was assessed by Fe^{3+}–Fe^{2+} transformation system in case of dichloromethane fraction and isolated flavonoids and this system is considered as important indicator for antioxidant activity. Different mechanisms have been suggested—such as interruption of chain initiation, degradation of peroxidases, and the potentiality to reduce the complex formation. Highest reducing power activity was exhibited by tricetin (489.31 ± 0.52%) but which was lower than the standard compound (522.24 ± 0.78%) at 30 µM

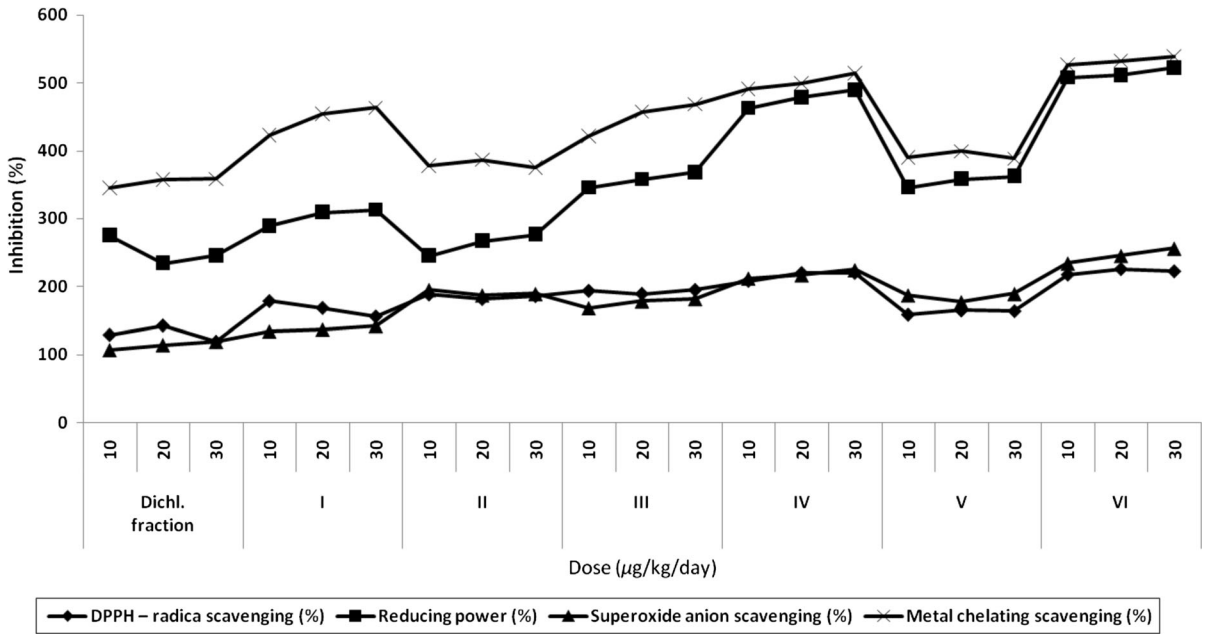

Fig. 2 In vitro antioxidant activity of the active fraction and the isolated flavonoids from *H. subulatum*

concentration (Fig. 2). All the isolated flavonoids showed moderate activity and it was found that the reducing power of isolated compounds and standard compound increased relative to their increase in concentration.

Superoxide anion scavenging is a well known reactive oxygen species among other free radicals and accepted worldwide by researchers. The combination of phenazine methosulfate-nicotinamide adenine dinucleotide generates superoxide radicals that can be determined by their capability to reduce nitroblue tetrazolium. The decrease in absorbance with antioxidative agents indicates the utilization of superoxide anion radicals in the reaction mixture. The maximum superoxide anion activity (224.61 ± 0.28%) was exhibited by tricetin and weaker activity was shown by the dichloromethane fraction (119.58 ± 0.69%) at 30 µM concentration (Fig. 2). The moderate superoxide anion scavenging activity was exhibited by the other isolated flavonoids.

The chelating activity of isolated flavonoids was compared with (±)-α-tocopherol. Plant secondary metabolites have the potential to chelate transition metals, which have the ability to catalyze fenton type reactions. These chelating agents act as secondary antioxidative agents because they have ability to reduce redox potential. Tricetin demonstrated highest chelating ability (514.21 ± 0.79%) while dichloromethane fraction exhibited minimum potential to scavenge chelate transition metals at 30 µM concentration (Fig. 2). The other isolated flavonoids such as galangin (463.55 ± 0.38%) and pinocembrin (468.76 ± 0.64%) also exhibited moderate chelating activity at 30 µM concentration (Fig. 2). The change of color of the test solution from yellow to green and blue indicate the strong chelating activity of dichloromethane and isolated flavonoids from *H. subulatum*.

For the assessment of in vivo antioxidants activity, the test samples were administered to the seven groups (2–8) of animals and the 1st group of animals was administered distilled water only. The maximum catalase activity was shown by tricetin (74.22 ± 0.62%) at a dose of 100 mg/kg body weight as compared with (±)-α-tocopherol (reference drug) at 50 mg/kg body weight dose (76.24 ± 0.74%; Fig. 3). As per the observed results, the catalase activity of tricetin was closer to that of the reference compound. The dichloromethane fraction exhibited the lowest activity (34.21 ± 0.76%) at 25 mg/kg body weight dose amongst all tested samples. The concentration of formed MDA was used for the determination of the degree of lipidic peroxidation. The lowest MDA level was (4.05 ± 0.46 µM) recorded of the reference compound while in case of tricetin it was higher

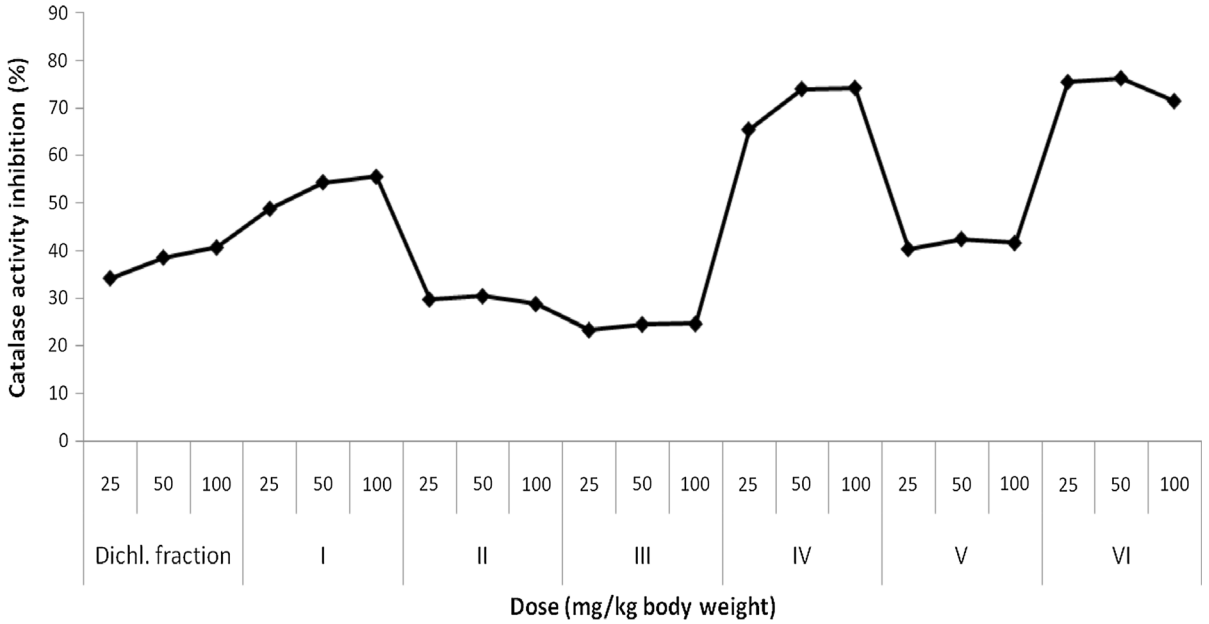

Fig. 3 Catalase activity of the active fraction and the isolated flavonoids from *H. subulatum* assessed in the mouse model

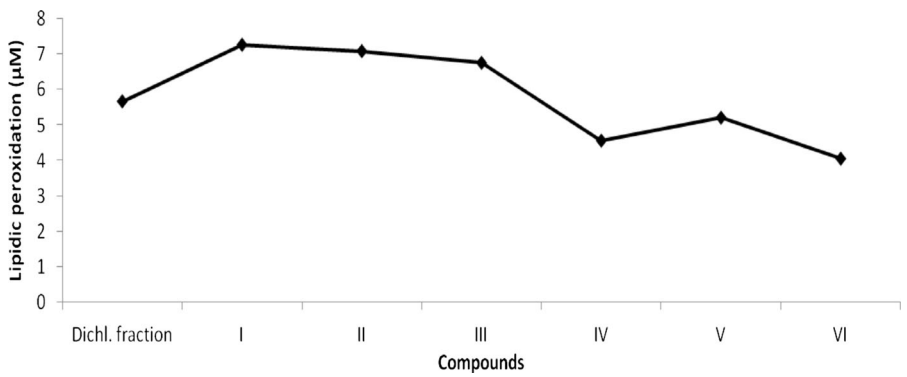

Fig. 4 Lipid peroxide activity of the active fraction and the isolated flavonoids from *H. subulatum* assessed in the mouse model

(4.56 ± 0.72 µM) than for the standard compound (Fig. 4).

Antineoplastic and cytotoxic effects of isolated compounds

Sarcoma 180 and Chinese hamster V79 cells were used for the determination of antineoplastic and cytotoxic activities. The antineoplastic activity was assessed by analyzing the PCV and body weight change in the Sarcoma 180 cells injected animals (tumor and ascites tumor) before and after treatment. The tested samples (dichloromethane fraction and the isolated flavonoids) were administered to the experimental animals up to 5 days continuously and at day 7, the experimental animals were sacrificed. The PCV of ascites tumor and tumor cells were measured. Similarly, the body weight change of ascites tumor and tumor cells injected animals was also recorded before and after the treatment. The observed results revealed that galangin showed maximum antineoplastic effects (78.35%) against Sarcoma 180 at 10 µg/kg/day dose (Fig. 5). Galangin also reduced the PCV and body weight of the animals treated with Sarcoma 180 ascites tumor. In an other experiment, the cytotoxic effects of the dichloromethane fraction and the isolated flavonoids were assessed in Chinese hamster V79 cells. For this study, various

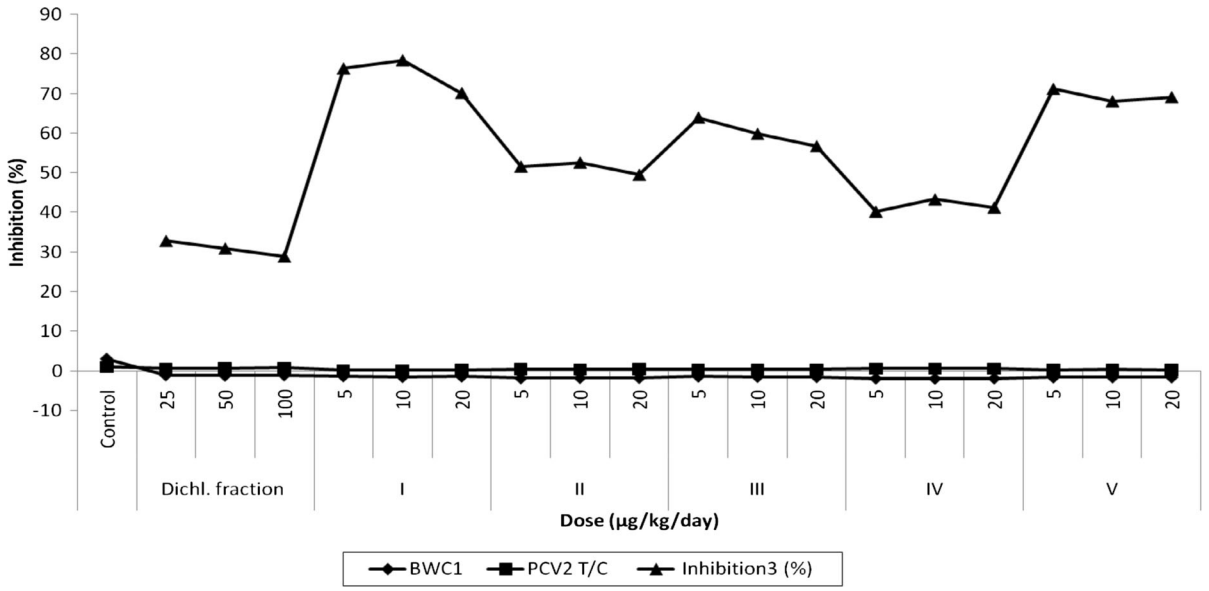

Fig. 5 Antineoplastic activity of the active fraction and the isolated flavonoids from *H. subulatum* in Sarcoma 180

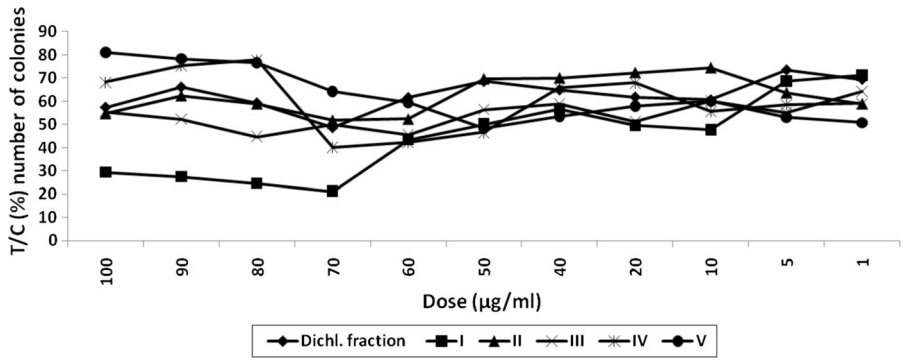

Fig. 6 Cytotoxic activity of the active fraction and the isolated flavonoids from *H. subulatum* in Chinese hamster V79 cells

concentrations (1.0–100 µg/ml) of both fraction and isolated compounds were evaluated and maximum cytotoxic effect (21.1 ± 0.15%) was demonstrated by galangin at 70 µg/ml concentration (Fig. 6).

Discussion

Recently, many researchers have shown their interest in the investigation of bioactivities of flavonoids from higher plants because they have no side effects and can be taken as major dietary constituents, (Rice-Evans et al. 1996). During physiological processes of cells in human beings, oxygen free radicals and their derivatives are generated and in excess they cause degradation of proteins, lipids and DNAs (Tiwari 2001; Majewska et al. 2011). Several scientists have suggested that natural flavonoids demonstrate antioxidant activity because they have the ability to scavenge free radicals (Pietta 2000). The DPPH system of screening for antioxidant activity is well known and accepted worldwide. The effectiveness of flavonoids depends on the structure of the molecules and their hydrophobicity. It has been published that the ability of flavonoids to disconnect the chain reaction depend on the presence of at least two hydroxyl groups in the B-ring of flavonoids (Torres et al. 1994). In our study, we observed that tricetin demonstrated maximum

DPPH scavenging activity because it has three hydroxyl groups in the B-ring of its structure. All the isolated flavonoids showed antioxidant activity because they have one to three hydroxyl groups in the B-ring of their structure (Urzúa et al. 2001). It has also been proven that a higher number of hydroxyl groups exhibited stronger antioxidant activity but lack of them reduced the ability to scavenge free radicals (Moreira et al. 2007). The DPPH scavenging activity of galangin was in agreement with the other reported results of an earlier study (Laskar et al. 2010).

The reducing power activity of flavonoids suggests that flavonoids have the potential to donate electrons to free reactive radicals and change them to non-reactive compounds by degrading the chain reactions of free radicals (Zha et al. 2009). In this assay, the flavonoids with reducing potential react with potassium ferrocyanide and $FeCl_3$ to produce Fe^{3+}–Fe^{2+} complex (Jothy et al. 2012). It has been suggested that the hydroxyl groups at C-3′ and C-4′ positions of the B-ring possesed as stronger reducing potency (Moran et al. 1997). In our study, we observed that tricetin (having hydroxyl groups at the C-3′ and C-4′ positions in the B-ring) showed maximum reducing activity and results were similar with an earlier study (Moran et al. 1997).

Superoxide anion scavenging assay is known as most important for determination of the antioxidant activity among other free radical assays. This assay involves the synthesis of superoxides from phenazone methosulphate-nicotinamide adenine dinucleotide system. The antioxidant agents inhibit the synthesis of nitroblue tetrazolium. The capability of isolated compounds to quench the superoxide radicals from the reaction mixture is reflected in the decrease of absorbance at 560 nm (Farhan et al. 2012). Our results revealed that tricetin was most active to superoxide anion radicals. Other studies have also confirmed the antioxidative potency of tricetin against superoxide anion radicals (Halliwell 1991). As per available report, the galangin having 2, 3 double bond with 4-oxo function which play major role to scavenge the superoxide radicals (Dorta et al. 2008).

The metal chelating scavenging activity is dependent on the chemical synthesis of ferrozine in the chemical reaction mixture. The intensity of red color reduction gives the chelating effects of tested compounds By measuring the intensity of the color reduction via spectrophotometry, the metal chelating activity of flavonoids was estimated. The ability of transition metal ion (Fe^{2+}) to transfer single electron can allow the formation of many free radicals. Out of all isolated flavonoids, tricetin exhibited maximum metal chelating activity compared with the reference compound. Some chelating compounds stop the synthesis of radicals by providing stability to transition metals while some other phenolic compounds demonstrate antioxidant activity by chelating metal ions (Zhao et al. 2008; Olennikov et al. 2014).

Both in vitro and in vivo antioxidative assays are important and to be conducted simultaneously to accede therapeutic potential of secondary metabolites of plants. In case of in vivo studies, the oxidative stress was generated in experimental animals by injecting carbon tetrachloride for damaging their liver. Liver damage caused by the formation of chain of free radicals (Liu et al. 1993). Catalase is an important enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. This enzyme protects the cells of organisms from oxidative damage by ROS (Sharma et al. 2012). The depletion of this enzyme activity in organisms may cause toxic effects due to the accumulation of hydrogen peroxide in animal cells (Ferraris et al. 1996). In the present study, the maximum inhibition was exhibited by tricetin while lower to moderate activity was exhibited by the other isolated flavonoids. As per available report, flavonoids have the ability to neutralize the toxicity of free radicals in organisms (Scalbert et al. 2005). In lipid peroxidation, molecular oxygen accepts electrons from foreign compounds which enter into cells and start to damage unsaturated lipids, resulting in formation of a continuous chain of free radicals. Continuity in the chain formation is terminated by the MDA or lipid alcohols (Cotelle 2001). MDA is being used as biomarker that helps in determination of oxidative stress as well as it is very relevant in biomedical research. In this present study, the rats treated with tricetin, the MDA level was significantly lowered in the 6th group of animals which indicates that tricetin was stopping the process of liver damage.

Flavonoids are secondary plant metabolites occurring in flowers, fruits, and vegetables. During the last few years, an enormous interest has been developed in research on the use of dietary supplements (Amic et al. 2007). By surveying human clinical laboratories, it was found that flavonoids have significant chemotherapeutic effects on cancers. Several mechanisms of action have been proposed by the scientists to

understand the different types of interactions between cancerous cell and genes (Chahar et al. 2011). As per available report, it is suggested that galangin suppresses the β-catenin regulated transcription in case of liver cancers (JungSug et al. 2011). Galangin disrupts cell division of cancer cells at the G_0–G_1 phase of cell cycle and it is also a strong inhibitor of Hs578T cell proliferation (Tolomeo et al. 2008). Our results revealed that galangin demonstrated maximum antineoplastic effects against Sarcoma 180 and are in agreement with earlier reported results (Murray et al. 2006). The potent cytotoxic activity was exhibited by galangin against Chinese hamster V79 cells. Galangin even creates apoptosis like conditions for PANC-1 cells (Li et al. 2010). On the basis of galangin activity against Sarcoma 180 and Chinese hamster V79 cells, it is suggested that the isolated flavonoids may regulate the expression of a regulatory protein, which stops G_0–G_1 cell cycle progression of Sarcoma 180 and Chinese hamster V79 cells.

Conclusions

The dichloromethane fraction and isolated flavonoids displayed significant antioxidant, antineoplastic and cytotoxic activities. Our findings suggest that tricetin showed promising antioxidant activity in both in vitro and in vivo models while galangin demonstrated excellent antineoplastic and cytotoxicity activities. Moreover, the investigation of mechanisms of action of these isolated compounds against antioxidant and antineoplastic models is needed in further course of study.

Authors' contribution BS and PMS designed and perform the experimental work and analyzed the data. RAS reviewed the manuscript and facilitated the help in equipments and chemicals. We are grateful to Professor P. Singh, Department of Chemistry, University of Rajasthan, Jaipur for providing the standard compounds and Professor Dr. H. Itokawa, College of Pharmacy, Tokyo, for his kind help in the screening process of antineoplastic and cytotoxic activities as well as Dr. H. Singh, Pacific University for helping in study of in vivo antioxidant activities.

Compliance with ethical standards

Conflict of interest The authors hereby declare that no financial support associated to this research work.

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