

Original article

Bioactive compounds, antioxidant and antineoplastic activities of Asian herbs

Tahira Yasmin, Muhammad Azam, Ummaimah, Muhammad Asim Raza Basra*

Institute of Chemistry, University of the Punjab, New Campus, Lahore 54590, Pakistan

Background: Herbage are the most sophisticated medicinal factories on earth which produce phytochemicals that possess biological activities and are used for the cure of various diseases.

Objectives: The aim of this study was to explore anti-neoplastic activities and antioxidant potential of selected herbs along with their total phenolic content and phytochemical screening.

Methods: 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺) methods were used to determine the antioxidant capacity. *In vivo* anti-neoplastic activities were determined by chick chorioallantoic membrane (CAM) and crown gall tumor (potato disc) assay, respectively.

Results: The highest antioxidant activity in aqueous and methanol extracts was found in *Centella asiatica* and *Spheranthus indicus*, respectively. Results of anti-neoplastic activities were in accordance to their antioxidant activity. Phytochemical analysis showed that all herbs contained tannins, saponins, glycosides, terpenoids, flavonoids and reducing sugars.

Conclusion: These findings suggest that these herbs are potential natural sources of bioactive compounds and natural antioxidants hence these herbs might be used in prophylaxis and treatment of neoplasms.

Keywords: Medicinal plants, antioxidants, anti-neoplastic, anti-angiogenic, anti-tumor, phytochemicals.

Medicinal herbs manifest sophisticated traditional medicine systems as a promising source of natural medicines. The natural components of these herbs are plant secondary metabolites, produced for their defense mechanisms and classified as terpenoids, alkaloids, flavonoids, tannins, saponins and polyphenolic compounds.⁽¹⁾ The crude extract of various herbs has been used as traditional medicine for the treatment of different diseases or to ameliorate the health. More than 80% of the world's population relies on traditional medicine for their primary health care.⁽²⁾ Medicinal plants contain mixtures of different chemical compounds and nutrients that may act individually, additively or in synergy to improve health. Polyphenolic compounds have been found to have therapeutic applications against various diseases caused by oxidative stress.⁽³⁾ Different phenolic

compounds act as antioxidants; tannins act as natural antibiotics; diuretic substances enhance the elimination of toxins and alkaloids enhance mood and give a sense of well-being.⁽⁴⁾ Various physiological and biochemical processes occur inside the body and free radicals have been produced as by products.⁽⁵⁾ They are valuable in sustaining the healthy life as they take part in the process of biological evolution and origin of life.⁽⁶⁾ Formation and utilization of the free radicals is imbalanced, resulting in overproduction of the radicals in various conditions including diabetes mellitus, inflammation, neurodegenerative disorders, cancer, coronary heart disease, aging, AIDS, and gastric problems.⁽⁷⁾ These free radicals interact with biomolecules such as proteins, DNA and lipids and affect their biological functions which lead to oxidative stress and ultimately cause lethal diseases.^(6, 8) Oxidative stress jeopardizes the normal process of cell division resulting in rapid, uncontrolled and undifferentiated cells formation and abnormal growth of tissues leading to formation of neoplasms.⁽⁹⁾ Cancerous cells and developing tumors require extra supply of blood to fulfill their food and energy requirements, hence these cells start producing highly

*Correspondence to: **Muhammad Asim Raza Basra**, Institute of Chemistry, University of The Punjab, New Campus, Lahore 54590, Pakistan.

E-mail: asimarbasra.chem@pu.edu.pk, asimbasra@gmail.com

Received: September 16, 2019

Revised: November 21, 2019

Accepted: December 6, 2019

dividing blood vessels by angiogenesis. This multistep, progressive physiological process, which is major contributor in tumor and cancer development, could be inhibited by anti-neoplastic agents. Anti-angiogenesis is a way to treat primary tumors by reducing their metastases.⁽¹⁰⁻¹²⁾ Nowadays, medicinal plants attain central position in the discovery of variety of medicines because they mostly possess antioxidant, antibacterial, anti-angiogenic and antifungal properties and also due to the unavailability of synthetic drugs for the treatment of certain lethal diseases. As it has become very difficult to fulfill the demand of drugs for the maintenance of human health, therefore most of the researches have been diverted towards herbal medicine for health remedies.⁽¹³⁻¹⁵⁾

The present study was designed to evaluate and compare the antioxidant and anti-neoplastic activities including anti-angiogenic and anti-tumor activities along with their phytochemical constituents and phenolic contents of methanol and aqueous extract of five different Asian herbs to aid the determination of new potential sources of natural antioxidants.

Materials and methods

Herbs collection and processing

Herbs including *Cassia angustifolia*, *Sphaeranthus indicus*, *Centella asiatica*, *Echinop chainatus*, and *Fagonia cretica*, were purchased from local herbal pharmacies in Lahore, Pakistan. The identity of herbs was confirmed by a taxonomist at the Department of Botany, University of Punjab, Lahore. The dried plant materials were cleaned and crushed into fine powder. Powdered samples were stored in the dark to protect them from light until further use.

Preparation of extracts

Methanol (commercial) and water were used as solvents for extract preparation of the selected herbs.⁽¹⁶⁾ In brief, 3 g of herbs powder was mixed individually in 120 ml of water or methanol and placed in an electrical shaking water bath. As for methanolic extraction, the contents were placed in shaking water bath for 3 h at 37°C whereas the aqueous extraction shaking time was one hour at 80°C. Then contents were cooled down to room temperature under running tap water and centrifuged at 3,500 rpm for 15 min; the clear supernatants were stored in dark bottles at 4°C for further analysis.

Scavenging of DPPH free radicals

Antioxidant activity of herb extracts was determined through 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) free radical scavenging assay.⁽¹⁷⁾ To the 0.5 ml of herb extracts of concentrations ranging from (0.01 - 20 mg/ml), 2.5 ml of 5×10^{-5} M DPPH solution was added and mixed. Control was prepared using methanol instead of herb extract by the same procedure. The reaction mixture was allowed to stand in the dark in the incubator (memmert) at 37°C for 30 min. Absorbance was measured at 517 nm against blank using a UV-VIS spectrophotometer (S-200D R and M England). Percentage scavenging activity was determined by taking methanol treated group as control. The percentage scavenging was calculated by using the following formula:

$$\text{Percentage scavenging} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

ABTS⁺ Free radical scavenging assay

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺) free radical scavenging method was used to determine the antioxidant potential of selected herbs.⁽¹⁸⁾ To 0.5 ml of herb extracts of different concentration ranging from (0.01- 20 mg/ml), 2.5 ml of diluted ABTS⁺ solution was added and mixed. Control was prepared by adding ethanol instead of sample and ABTS⁺ solution. The reaction mixture was incubated for 5 minutes at 37°C and absorbance was measured immediately at 734 nm using a UV-VIS spectrophotometer (S-200D R and M England). The percentage scavenging was calculated by using the following formula:

$$\text{Percentage scavenging} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Determination of total phenolics

Total phenolic contents (TPC) of herb extracts were estimated by previously described method by Singleton and Rossi (1965).⁽¹⁹⁾

Phytochemical screening

Different phytochemical constituents present in the extract of all herbs were estimated by using different standard methods.^(20, 21)

Detection of terpenoids (Salkowski test)

To 2 ml chloroform (CHCl_3), 500 μl of herb extract was added, and then 3 ml of concentrated H_2SO_4 was added carefully along the side of the test tube wall so that a layer can be formed. The formation of a greyish brown coloration or ring at the interface is a sign of terpenoids presence.

Detection of tannins

To 0.5 ml of herb extract, 5 ml of distilled water was added. It was boiled in water bath. The extract was cooled to room temperature and then 500 μl of 0.1% FeCl_3 solution was added. Brownish green or blue-black precipitates were formed and gave the indication of tannins presence in the herb extract.

Detection of saponins

Saponins were detected by adding 0.5 ml of the herb extract to 5 ml of distilled water and the solution was vigorously shaken to form a stable unrelenting froth. A few drops of olive oil were added to the mixture and again shaken vigorously; the formation of an emulsion indicated the presence of saponins.

Detection of flavonoids

For the detection of flavonoids, 5 ml of diluted ammonia was added to 0.5 ml of aqueous and methanolic herb extracts separately then added 1 ml of concentrated H_2SO_4 to this solution. With the passage of time yellow color disappears gave the indication of flavonoids in the herb extracts.

Detection of anthraquinones

To 3 ml of the herb extract 3 ml of benzene was added and mixed thoroughly, then 5 ml of 10% ammonia solution was added and again mixed the solution. Production of red, violet or pink color in the ammonical layer gave the indication of free anthraquinones presence in extract.

Detection of reducing sugars (Benedict's test)

2 ml of freshly prepared Benedict's solution was taken in a test tube and heated for few minutes, then added 2 ml of the herb extract to the boiling solution. The formation of red colored precipitates gave the indication for the presence of reducing sugar in extract of herbs.

Determination of carbohydrates (Molisch test)

The carbohydrates were determined by Molisch test. To 3 ml of herb extracts, 3 - 4 drops of α -naphthol

was added and mixed the solution by shaking gently then added 3 - 4 ml of concentrated H_2SO_4 slowly along the side of test tube. Formation of violet ring at the junction indicates the presence of carbohydrate.

Detection of steroids

For the detection of steroids, 2 ml of each extract was added to 2 ml of chloroform and mixed the solution gently then added 2 ml of concentrated sulphuric acid and shaken the solution gently. Production of red color in chloroform layer gave the indication of steroids presence.

Detection of cardiac glycosides (Salkowski's test)

2 ml of chloroform was added to the 2 ml of each extracts and mixed the solution gently, then added 2 ml of concentrated sulphuric acid slowly along the side of test tube. A reddish brown ring at junction of two solvents indicates the presence of deoxy sugars which is distinguishing feature of cardenolides.

Detection of phlobatannins

Red colored precipitates were produced when each extract (2 ml) were added to 2 ml of 1% HCl and boiled the solutions. Formation of red precipitates indicates the presence of phlobatannins.

In vivo chorioallantoic membrane (CAM) assay

Anti-angiogenic activity was performed by using chick chorioallantoic membrane (CAM) assay. Eggs were sterilized and incubated at 37°C for three days, and the small window was made by carefully removing egg shell along with internal membrane. The window was sealed with surgical cotton tape and eggs were again incubated. On the 8th day of incubation, 20 μl of each sample (25 mg/ml) of each herbal extract was loaded on the disc and placed this disc on the egg yolk at the point of emergence of new blood vessels. Phosphate buffer saline of pH 7.30 was used as control. On the 12th day of incubation, chorioallantoic membrane along with developed chick embryo was removed carefully such that blood vessels are not damaged. Formalin was used as fixative agent to stop the development of embryo and recorded the results.⁽²²⁾ The outcomes were quantified and evaluated by semi-quantitative scoring system represented in Table 4.⁽²³⁾

Potato disc (crown gall tumor) assay

Antitumor activity was measured through previously established potato disc assay.⁽²⁴⁾ 20 ml of

autoclaved agar (1.5%) was added to each Petri dish under aseptic conditions and allowed to cool. Red skinned potatoes were washed and soaked in sodium hypochlorite solution (10%) for 15 minutes. Rinsed thoroughly with water, peeled off skin and cut potato disc of (8 mm × 15 mm) diameter. Three discs were placed in each petri dish by gently pressing them into the agar and added one drop of prepared inoculum in the center of disc. The inoculum was prepared by adding 1.5 ml distilled water in 2 ml of 48 hours old bacterial culture followed by addition of 0.5 ml sample dissolved as 4 mg/ml in DMSO. The control was prepared by adding 0.5 ml of DMSO in place of sample in the inoculum. Petri dishes (n = 3) were prepared for each sample including control and edges were sealed with parafilm strips to maintain moisture content inside the dish which was covered with aluminum foil and placed in the dark at 27°C. After 12 - 18 days, the results were carefully recorded. The percentage inhibition was found by following formula: ⁽²⁵⁾

$$\% \text{ Inhibition} = \left(1 - \frac{\text{No. of tumors on sample disc}}{\text{No. of tumors on control disc}} \right) \times 100$$

Statistical analysis

The data were analyzed using IBM-SPSS version 22. Pearson's correlation and linear regression relation was applied to check the relationship between means of antioxidant capacities and TPC with the CAM antiangiogenic assay of herbs.

Results

Antioxidant activity analysis

The antioxidant activity of methanolic and aqueous extracts of herbs was investigated by DPPH and ABTS scavenging methods. Among the herbs, aqueous extract of *S. indicus* was found to be the most potent scavenger of DPPH radicals at a concentration of 0.455 mg/ml (IC₅₀) followed by *C. asiatica*, *C. angustifolia*, *E. echinatus Roxb.* and *F. cretica* (Table 1). Therefore, the order of antioxidant activity by DPPH method observed in aqueous extracts was: *S. indicus* > *C. Asiatica* > *C. angutifolia* > *E. echinatus Roxb.* > *F. cretica*. IC₅₀ values of medicinal herbs obtained from ABTS are shown in Table 1. The following order of antioxidant activity was observed, *C. angutifolia* > *S. indicus* > *C. asiatica* > *F. cretica* > *E.*

echinatus Roxb. The respective IC₅₀ values of herbs were calculated from ABTS method were 0.422, 0.436, 0.459, 0.612 and 0.725 mg/ml, respectively.

Similarly, DPPH free radicals assay was performed for each methanolic extracts of herbs and found the antioxidant activity in the order such as *C. asiatica* > *C. angustifolia* > *S. indicus* > *E. echinatus Roxb.* > *F. cretica*. Their IC₅₀ values were depicted as 1.172, 1.275, 1.779, 2.378 and 18.291 mg/ml, respectively. Whereas, the ability of methanolic extracts to sequester the ABTS radicals was observed in descending order (*C. angutifolia* > *C. asiatica* > *E. echinatus Roxb.* > *S. indicus* > *F. cretica*) (Table 1). Aqueous extracts of all herbs showed higher potential to scavenge the free radicals in comparison with methanolic extracts.

Total phenolic content

The antioxidant activity of medicinal plants has been reported to be positively correlated with their total phenolic contents due to their ability to scavenge the free radicals.⁽³³⁾ The TPC ranged from 64 to 400 mg GAE/100 g DW. Aqueous extract of *C. angustifolia* contained highest phenolics (400 mg GAE/100 g DW), followed by *F. cretica* having phenolics value 376 mg GAE/100 g DW. The following order of TPC was observed in aqueous extracts of all herbs is *C. angustifolia* > *F. cretica* > *C. asiatica* > *E. echinatus Roxb.* > *S. indicus*. Almost the same pattern was observed for TPC in methanolic extracts of herbs. *C. angustifolia* showed highest phenolic contents while *F. cretica* showed lowest content (360 and 64 mg GAE/100 g DW, respectively) compared with other herbs (Table 2).

Phytochemical constituents

Phytochemical constituents present in both aqueous and methanolic extracts of herbs were determined and results are shown in Table 3. Aqueous and methanolic extracts of all herbs contained tannins, saponins, glycosides, terpenoids, flavonoids and reducing sugars. However, anthraquinones were present only in aqueous extract of *C. angustifolia* whereas phlobatannins were absent in all extracts of herbs. Similarly, steroids were absent in the aqueous extracts of *S. indicus*, *C. asiatica* and *F. cretica*. Along with phytochemical constituents, carbohydrates presence was also confirmed.

Table 1. Antioxidant activity of herbs.

| Herbs | Antioxidant activities (IC ₅₀ mg/ml) | | | |
|----------------------------|-------------------------------------------------|----------|------------|---------|
| | DPPH assay | | ABTS assay | |
| | AE | ME | AE | ME |
| <i>Cassia angustifolia</i> | 1.5±0.9 | 1.3±1.4 | 0.4±0.6 | 0.4±0.5 |
| <i>Spheranthus indicus</i> | 0.5±0.7 | 1.8±1.2 | 0.4±0.7 | 0.9±0.6 |
| <i>Centella asiatica</i> | 0.8±0.7 | 1.2±1.1 | 0.5±0.2 | 0.8±0.5 |
| <i>Echinops echinatus</i> | 2.1±1.4 | 2.4±1.5 | 0.7±0.8 | 0.8±0.7 |
| <i>Fagonia cretica</i> | 10.6±2.3 | 18.3±1.7 | 0.6±0.3 | 3.5±0.3 |

AE = aqueous extract, ME = methanolic extract.

Antioxidant activities of herbal extracts were determined through DPPH and ABTS assays and data was represented as Mean ± SD of three determinations.

Table 2. Total phenolic contents in herbal extracts.

Total phenolic contents of extracts were determined through Folin-Ciocalteu reagent. The standard calibration curve for total phenolics is made by using Gallic acid standard solution (1 - 10 mg/100 ml) and phenolic contents in extract are expressed as milligrams of Gallic acid equivalents per grams of the dry herb.

| Herbs | Total phenolic contents in mg/100 g of dry weight | |
|----------------------------|---------------------------------------------------|-------------------------|
| | Aqueous Extract (AE) | Methanolic Extract (ME) |
| <i>Cassia angustifolia</i> | 400.0 | 360.0 |
| <i>Spheranthus indicus</i> | 332.0 | 280.0 |
| <i>Centella asiatica</i> | 368.0 | 248.0 |
| <i>Echinops echinatus</i> | 348.0 | 208.0 |
| <i>Fagonia cretica</i> | 376.0 | 64.00 |

Table 3. Screening of phytochemical compounds.

| Test | <i>C. angustifolia</i> | | <i>S. indicus</i> | | <i>C. asiatica</i> | | <i>E. echinatus Roxb.</i> | | <i>F. cretica</i> | |
|-------------------|------------------------|----|-------------------|----|--------------------|----|---------------------------|----|-------------------|----|
| | AE | ME | AE | ME | AE | ME | AE | ME | AE | ME |
| Reducing sugar | + | + | + | + | + | - | + | + | + | - |
| Anthraquinones | + | - | - | - | - | - | - | - | - | - |
| Carbohydrates | + | + | + | + | + | + | + | + | + | + |
| Flavonoids | + | + | + | + | + | + | + | + | + | + |
| Terpenoids | + | + | + | + | + | + | + | + | + | + |
| Tannins | + | + | + | + | + | + | + | + | + | + |
| Saponins | + | + | + | + | + | + | + | + | + | + |
| Steroids | + | + | - | + | - | + | + | + | - | - |
| Cardiac glycoside | + | + | + | + | + | + | + | + | + | + |
| Phlobatannins | - | - | - | - | - | - | - | - | - | - |

+ = Present, - = Absent, AE = aqueous extract, ME = methanolic extract.

Anti-angiogenic activity (CAM assay)

The process of formation of new blood vessels from pre-existing blood vasculature is known as angiogenesis.⁽¹⁰⁾ Anti-angiogenic effects of these herbs were studied by using CAM assay. The result obtained clearly indicates that aqueous and methanol extracts of herbs greatly inhibited the development

of new blood vessels on the CAM as compared to control (Figures 1 - 2). The scores calculated by semi-quantitative scoring system revealed the digital values of the CAM outcomes of the herbal extracts (Table 4 and 5). The results showed that aqueous extracts of all herbs were more anti-angiogenic as compared to methanol extracts.

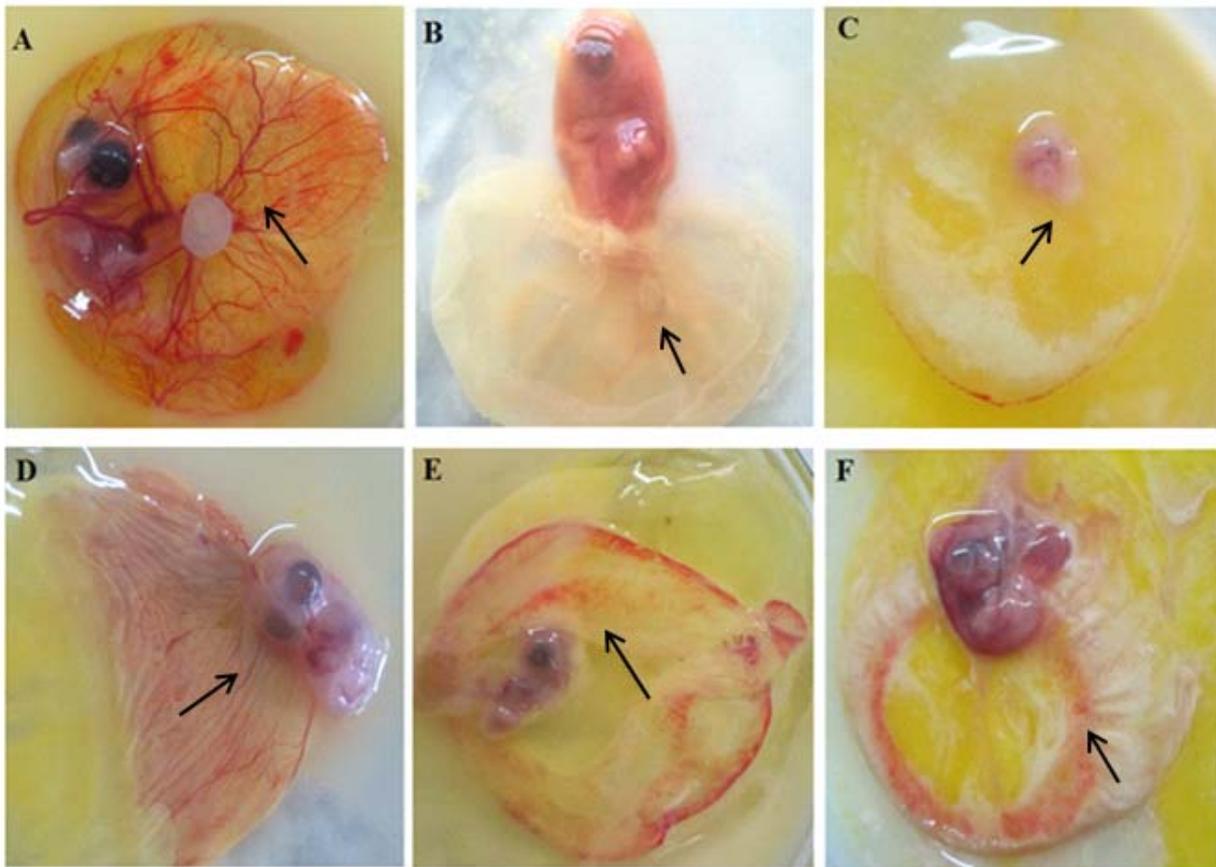


Figure 1. Anti-angiogenic effect of aqueous extract of herbs on chick chorioallantoic membrane (CAM). Zero days fertilized chick eggs were incubated at 37°C and loaded with 20 μ l (25 mg/ml of each aqueous extract) sample on 8th day of incubation. Results were recorded on 12th day. (A) control group, (B) *Cassia angustifolia*, (C) *Spheranthus indicus*, (D) *Centella asiatica*, (E) *Echinops echinatus*, and (F) *Fagonia cretica*. Arrow shows the reduced blood vessels growth (anti-angiogenic effect) in treated chick CAM compared with control. The experiments were performed in triplicate (n = 3).

Table 4. Scale for semi-quantitative scoring system for anti-angiogenic estimation.

| Scale | Level of anti-angiogenesis in CAM assay | Attributed anti-angiogenic effect |
|-------|---------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|
| 0 | No damage to blood Vessels | No effect |
| 0-0.5 | Slightly damaged blood Vessels | Slight effect |
| 0.5-1 | Small capillary free area in vicinity of treatment; a few micro vessels congregate, growth of blood vessels marginally reduced. | Moderate effect |
| 1-1.5 | Moderate capillary free area; Minute number of micro vessels visible | Good effect |
| 1.5-2 | Capillary free area below the area of treatment; micro vessels no longer visible and large vessel merging. | Strong effect |

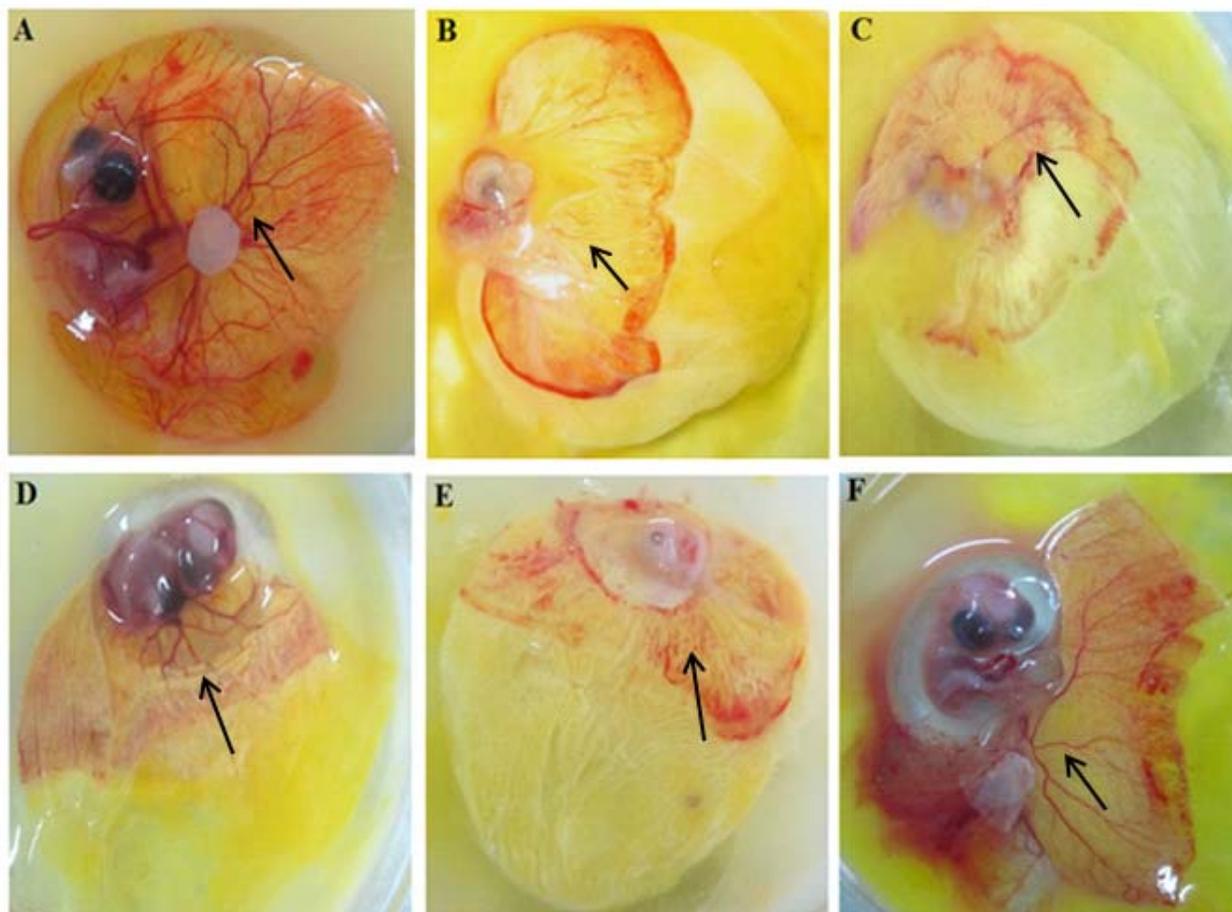


Figure 2. Anti-angiogenic effect of methanolic extract of herbs on chick chorioallantoic membrane (CAM). Zero days fertilized chick eggs were incubated at 37°C and loaded with 20 µl (25 mg/ml of each methanolic extract) sample on 8th day of incubation. Results were recorded on 12th day. (A) control group, (B) *Cassia angustifolia*, (C) *Spheranthus indicus*, (D) *Centella asiatica*, (E) *Echinops echinatus*, and (F) *Fagonia cretica*. Arrow shows the reduced blood vessels growth (anti-angiogenic effect) in treated chick CAM compared with control. The experiments were performed in triplicate (n = 3).

Table 5. Quantitative anti-angiogenic effect of herb extracts.

| Extracts | | Score | Anti-angiogenic effect |
|---------------------------|---------------------------|--------------------|------------------------|
| Methanolic Extract | <i>C. asiatica</i> | 1 | Weak |
| | <i>C. angustifolia</i> | 1.5 | Good |
| | <i>S. indicus</i> | 1.5 | Good |
| | <i>E. echinatus Roxb.</i> | 1.5 | Good |
| | <i>F. cretica</i> | 0.5 | Slight |
| | Aqueous Extract | <i>C. asiatica</i> | 0.5 |
| <i>C. angustifolia</i> | | 1.5 | Good |
| <i>S. indicus</i> | | 2 | Strong |
| <i>E. echinatus Roxb.</i> | | 1 | Weak |
| <i>F. cretica</i> | | 1 | Weak |

Anti-tumor activity (Crown Gall assay)

Results showed that methanolic extracts of selected herbs inhibit the tumor growth more effectively (Figure 3). The percentage inhibition of

tumor for methanolic extracts of *C. asiatica*, *E. echinatus* Roxb. and *F. cretica* and that of aqueous extract of *S. indicus* was 46.55, 57.1, 68.97 and 70.69%, respectively (Table 6).

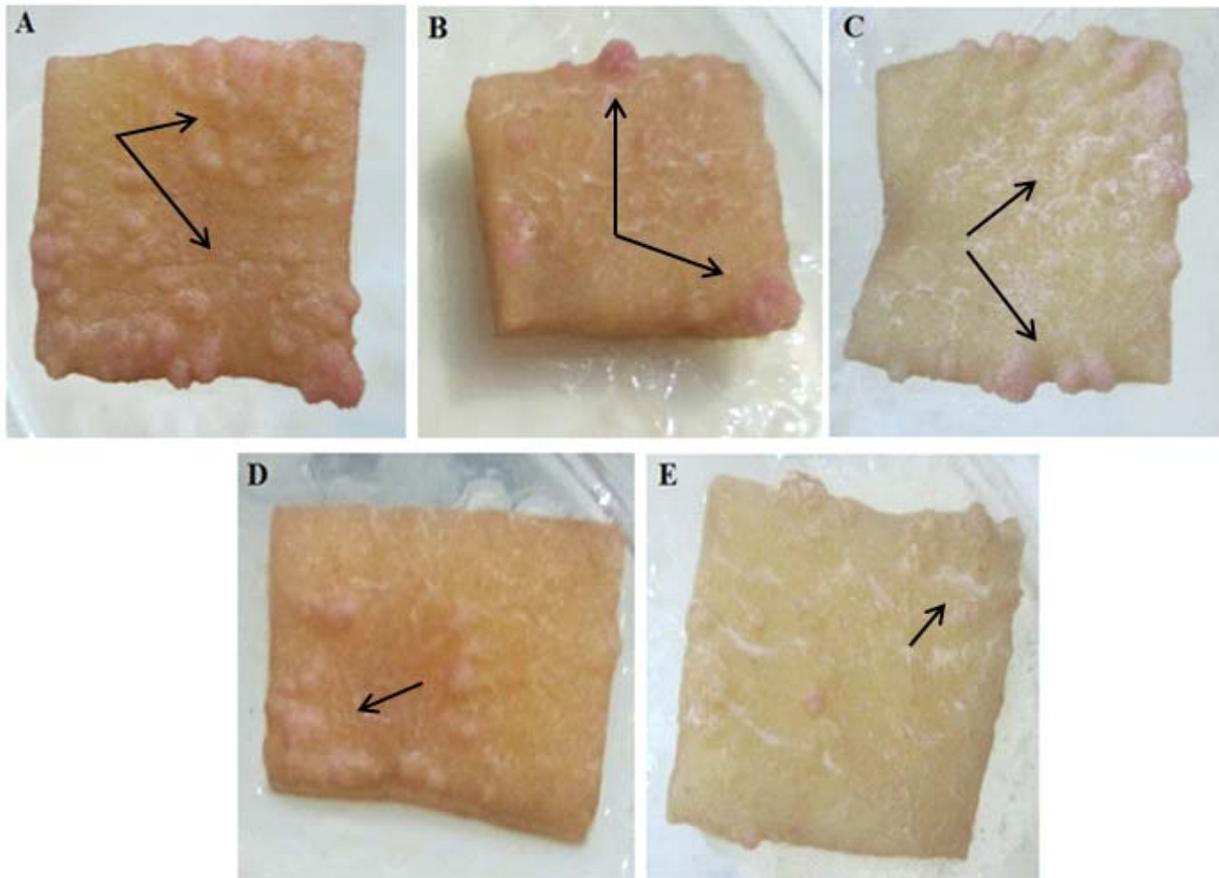


Figure 3. Anti-tumor activity of herb extract on potato discs. Potato disc of (8 mm × 15 mm) diameter were placed in agar containing petri dish and added one drop of prepared bacterial inoculum of 0.5 ml of methanolic and aqueous extract (4 mg/ml in DMSO) on the center of the disc and the edges were sealed with parafilm strips. Then it was covered with aluminum foil and placed in the dark at 27°C. After 12 - 18 days, the results were recorded. (A) control group (B) methanolic extract of *Fagonia cretica*, (C) methanolic extract of *Centella asiatica*, (D) methanolic extract of *Echinops echinatus*, and (E) aqueous extract of *Spheranthus indicus*. Arrow shows the reduced crown gall (tumor) development on potato disc treated with herb extract compared to control. The experiments were performed triplicate (n = 3).

Table 6. Percentage inhibition of crown gall tumor by herbal extracts.

| Extracts | | No. of tumor outgrowths | Percent inhibition of tumors |
|--------------------|---------------------------|-------------------------|------------------------------|
| Methanolic Extract | <i>C. asiatica</i> | 31 | 46.6 |
| | <i>E. echinatus</i> Roxb. | 26 | 55.2 |
| | <i>F. cretica</i> | 18 | 69.0 |
| Aqueous Extract | <i>S. indicus</i> | 17 | 70.7 |

* No. of tumor outgrowths on control potato disc = 58

Correlation between antioxidant capacities, total phenolic contents and CAM assay

The scores of antiangiogenic CAM assay of methanolic extracts of herbs (MECAM) depicted inverse relation with IC_{50} values obtained from DPPH and ABTS activities of methanolic extracts of herbs (MEDPPH and MEABTS). The calculated values of Pearson correlation coefficient (r) and coefficient of determination (r^2) for MEDPPH and MECAM were 0.855 and 0.731, for MEABTS and MECAM the values were 0.873 and 0.763 while for METPC and MECAM the values were 0.819 and 0.670, respectively (Figure 4).

Discussion

Medicinal plants are rich sources of phytochemicals that show biological activities. These compounds help plants in defense against microbial, bacterial and pathogenic infections. The comparison of antioxidant activities determined by DPPH and ABTS in both aqueous and methanolic extract of herbs showed the most potent extract to be those of *C. asiatica*, *S. indicus* and *C. angutifolia*. Presently, these exhibited higher antioxidant ability. Antioxidant capacity of different herbs estimated previously can be comparable with the present study. ^(26, 27) Previously antioxidant activity was determined and compared in the different parts of *C. asiatica*

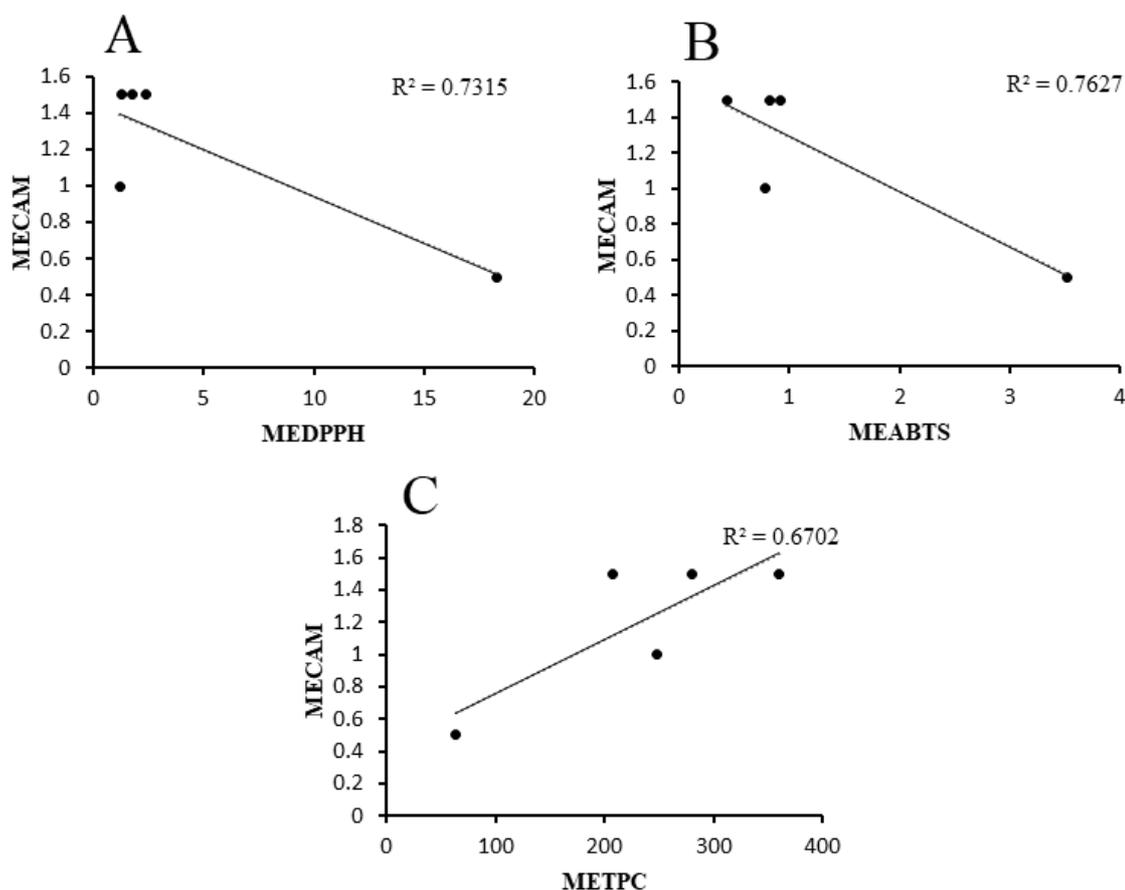


Figure 4. Correlation between antioxidant activity, total phenolic content (TPC) and CAM assay of methanolic extract herbs (MECAM). (A) Correlation between IC_{50} values of DPPH activity (MEDPPH) and MECAM. (B) Correlation between IC_{50} values of ABTS activity (MEABTS) and MECAM. (C) Correlation between METPC and MECAM.

separately⁽²⁸⁾ whereas antioxidant activity of whole plant of *C. asiatica* studied presently showed that it exhibits high antioxidant activity. *F. cretica* is also an important herb frequently used in folk medicine in Pakistan. Antioxidant ability of *F. cretica* to scavenge the free radicals gives the scientific reason for its use as herbal medicine to cure the illness. Previous studies reported that methanol and aqueous extracts of roots of *F. cretica* showed high antioxidant activity as compared to other solvents.⁽²⁹⁾ The antioxidant activity results obtained by DPPH and ABTS assays for alcoholic extract of *S. indicus* can be comparable to those reported in previous studies in Indian species.⁽³⁰⁾ These differences may be due to different factors such as location, agricultural practices, growing conditions, climatic factors, storage conditions.⁽³¹⁾ So, the estimation of antioxidant activity is not identical. Moreover, the similar observations have reported during the optimization of antioxidant activity of herbs using different solvents which suggests that aqueous extracts showed more antioxidant potential.⁽³²⁾

Total phenolic contents

Aqueous extract of each herb contains more phenolic contents as compared to methanolic extracts. This complies with the TPC observations which reported in Jordanian plants that showed aqueous extracts give higher value of TPC as compared to the extracts in other solvents.⁽¹⁶⁾ The TPC content in methanol extract of *S. amaranthiodes* was reported to be 2.15 mg which is lower than that reported in the thesis for *S. indicus* (280 mg).⁽³³⁾ The difference in polyphenolic contents may be due to different plant species. Thus, results of TPC support that high antioxidant activity of aqueous extracts that compared methanolic extracts to scavenge of free radicals could be explained on the basis of their TPC.

Phytochemical analysis

Different phytochemicals are present in herbs that interact with ROS in human to protect from damage. Previous studies indicate the presence of active phytochemical constituents such as saponins, triterpene glycosides, madecassoside and asiaticoside in different medicinal plant extracts⁽³⁴⁾ which are comparable to our findings of phenolic compounds. Likewise, presences of flavonoids, carbohydrates, alkaloids and essential oils were confirmed in the flowers and roots of *S. indicus* and *E. echinatus*.^(35, 36) These analyses indicate that

the plant extracts have potent antioxidant properties; thus, providing evidence in support for their uses as significant source of natural antioxidant which might be helpful in preventing the progress of various oxidative stress related diseases. It has been reported that the antioxidant, anti-angiogenic and anti-tumor and anti-fungal activities of edible plants are related to the free radical scavenging property of phytochemical components and flavonoids.⁽³⁷⁾

Anti-angiogenic CAM assay

The results indicate aqueous extract of all herbs are more anti-angiogenic as compared to methanol extracts. These results are in accordance with the antioxidant ability of herbs measured by DPPH assay (Table 1). However, the methanolic extracts of herbs showed significantly correlated anti-angiogenic effect with respect to their DPPH, ABTS as well as TPC values.

Potato gall anti-tumor assay

Crown Gall is a neoplastic disease of plants induced by specific strains of the gram-negative bacterium *Agrobacterium tumefaciens*. Different phytochemical components present in all herbs might be involved to resist and inhibit the tumor development. Anti-tumor activity of herbs was studied by using the potato disc assay. The results showed that methanolic extracts of selected herbs inhibited the tumor growth more effectively.

The results obtained from statistical analysis suggested that the greater amounts of total phenolic contents might be involved in enhancing the antioxidant activities and lower IC₅₀ values of the samples. Hence the relation between antioxidant assays and antiangiogenic CAM assay was inverse while that of TPC and CAM was direct.

Acknowledgements

We are thankful to the Institute of Chemistry, University of the Punjab for providing research funding and Dr. Asmat Ullah, the Department of Zoology for the use of his laboratory facilities and his cooperative behavior was extended to carry our anti-angiogenic studies.

Conflict of interest

The authors, hereby, declare no conflicts of interest.

References

1. Znati M, Ben Jannet H, Cazaux S, Souchard J, Harzallah Skhiri F, Bouajila J. Antioxidant, 5-lipoxygenase inhibitory and cytotoxic activities of compounds isolated from the *Ferula lutea* flowers. *Molecules*. 2014;19(10):16959-75.
2. Kumari A, Sharma R. A review on *Millingtonia hortensis* Linn. *Int J Pharm Sci Rev Res*. 2013;19(2):85-92.
3. Sen S, Chakraborty R. The role of antioxidants in human health. *Oxidative stress: diagnostics, prevention, and therapy*. 2011;1083:1-37.
4. Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine*. 2006;27(1):1-93.
5. Choi CW, Kim SC, Hwang SS, Choi BK, Ahn HJ, Lee MY, et al. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant science*. 2002;163(6):1161-8.
6. Fang Y-Z, Yang S, Wu G. Free radicals, antioxidants, and nutrition. *Nutrition*. 2002;18(10):872-9.
7. Tupec M, Hýsková V, Bilonožníková K, Hraníček J, Červený V, Ryšlavá H. Characterization of some potential medicinal plants from Central Europe by their antioxidant capacity and the presence of metal elements. *Food Bioscience*. 2017;20:43-50.
8. Nanda R, Agrawal V. Elucidation of zinc and copper induced oxidative stress, DNA damage and activation of defence system during seed germination in *Cassia angustifolia* Vahl. *Environmental and experimental botany*. 2016;125:31-41.
9. Finsterer J, Frank M. Prevalence of neoplasms in definite and probable mitochondrial disorders. *Mitochondrion*. 2016;29:31-4.
10. Deryugina EI, Quigley JP. Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules. *Methods in enzymology*. 2008;444:21-41.
11. Davies KJ. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB life*. 2000;50(4 5):279-89.
12. Fam SS, Morrow JD. The isoprostanes: unique products of arachidonic acid oxidation-a review. *Current medicinal chemistry*. 2003;10(17):1723-40.
13. Kamble SS, Gacche RN. Evaluation of anti-breast cancer, anti-angiogenic and antioxidant properties of selected medicinal plants. *European Journal of Integrative Medicine*. 2019;25:13-9.
14. van den Bogaard AE, Stobberingh EE. Epidemiology of resistance to antibiotics: links between animals and humans. *International journal of antimicrobial agents*. 2000;14(4):327-35.
15. Levy SB. The challenge of antibiotic resistance. *Scientific American*. 1998;278(3):32-9.
16. Tawaha K, Alali FQ, Gharaibeh M, Mohammad M, El-Elimat T. Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chemistry*. 2007;104(4):1372-8.
17. Chen Y, Wang M, Rosen RT, Ho C-T. 2, 2-Diphenyl-1-picrylhydrazyl radical-scavenging active components from *Polygonum multiflorum* Thunb. *Journal of agricultural and food chemistry*. 1999;47(6):2226-8.
18. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*. 1999;26(9):1231-7.
19. Singleton V, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*. 1965;16(3):144-58.
20. Ayoola G, Coker H, Adesegun S, Adepoju-Bello A, Obaweya K, Ezennia E, et al. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research*. 2008;7(3):1019-24.
21. Njoku OV, Obi C. Phytochemical constituents of some selected medicinal plants. *African Journal of Pure and Applied Chemistry*. 2009;3(11):228-33.
22. Lokman NA, Elder AS, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *International journal of molecular sciences*. 2012;13(8):9959-70.
23. Dhara M, Adhikari L, Majumder R. Chorioallantoic Membrane (CAM) Assay of Different Extracts of Rhizome and Inflorescence of *Heliconia rostrata*. *Indian Journal Of Pharmaceutical Education And Research*. 2018;52(4):S246-S51.
24. McLaughlin JL, Rogers LL, Anderson JE. The use of biological assays to evaluate botanicals. *Drug information journal*. 1998;32(2):513-24.
25. Ashraf Z, Mahmood T, Hassan M, Afzal S, Rafique H, Afzal K, et al. Dexibuprofen amide derivatives as potential anticancer agents: synthesis, in silico docking, bioevaluation, and molecular dynamic simulation. *Drug Des Devel Ther*. 2019;13:1643-57.
26. Rahman MM, Sayeed MSB, Haque MA, Hassan MM,

- Islam SA. Phytochemical screening, antioxidant, anti-Alzheimer and anti-diabetic activities of *Centella asiatica*. *J Nat Prod Plant Resour*. 2012;2(4):504-11.
27. Pittella F, Dutra RC, Junior DD, Lopes MT, Barbosa NR. Antioxidant and cytotoxic activities of *Centella asiatica* (L) Urb. *International journal of molecular sciences*. 2009;10(9):3713-21.
28. Singh M, Roy B, Tandon V, Chaturvedi R. Extracts of dedifferentiated cultures of *Spilanthes acmella* Murr. possess antioxidant and anthelmintic properties and hold promise as an alternative source of herbal medicine. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*. 2014;148(2): 259-67.
29. Iqbal P, Ahmed D, Asghar MN. A comparative in vitro antioxidant potential profile of extracts from different parts of *Fagonia cretica*. *Asian Pacific journal of tropical medicine*. 2014;7:S473-S80.
30. Shirwaikar A, Prabhu KS, Punitha I. In vitro antioxidant studies of *Sphaeranthus indicus* (Linn). 2006.
31. Singh M, Jha A, Kumar A, Hettiarachchy N, Rai AK, Sharma D. Influence of the solvents on the extraction of major phenolic compounds (punicalagin, ellagic acid and gallic acid) and their antioxidant activities in pomegranate aril. *Journal of food science and technology*. 2014;51(9):2070-7.
32. Singh M, Pandey N, Agnihotri V, Singh K, Pandey A. Antioxidant, antimicrobial activity and bioactive compounds of *Bergenia ciliata* Sternb.: A valuable medicinal herb of Sikkim Himalaya. *Journal of traditional and complementary medicine*. 2017;7(2): 152-7.
33. Geethalakshmi R, Sakravarthi C, Kritika T, Arul Kirubakaran M, Sarada D. Evaluation of antioxidant and wound healing potentials of *Sphaeranthus amaranthoides* Burm. f. *BioMed research international*. 2013;2013.
34. Gill T, Kumar M, Kumar S, Kaur S. DNA-protective effect of extract/fractions from *Centella asiatica* (L.) Urban in single cell gel electrophoresis assay. *Spatula DD-Tamamlayici Tip ve İlaç Geliştirme Alanında Hakemli Dergi*. 2011;1(4):207-12.
35. Ramachandran S, Asokkumar K, Uma Maheswari M, Ravi T, Sivashanmugam A, Saravanan S, et al. Investigation of antidiabetic, antihyperlipidemic, and in vivo antioxidant properties of *Sphaeranthus indicus* Linn. in type 1 diabetic rats: an identification of possible biomarkers. *Evidence-Based Complementary and Alternative Medicine*. 2011;2011.
36. Singh S, Upadhyay R, Pandey M, Singh J, Pandey V. Flavonoids from *Echinops echinatus*: note. *Journal of Asian natural products research*. 2006;8(3):197-200.
37. Zida A, Bamba S, Yacouba A, Ouedraogo-Traore R, Guiguemdé R. Anti-*Candida albicans* natural products, sources of new antifungal drugs: A review. *Journal de Mycologie Médicale/Journal of Medical Mycology*. 2017;27(1):1-19.