

Full Length Research Paper

***In vivo* cytotoxic effects of methanol extract of *Convolvulus arvensis* on 7-12-dimethyl benz(a)anthracene (DMBA) induced skin carcinogenesis**

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A current study was conducted to evaluate the possible cytotoxic effect of Asian herb *Convolvulus arvensis* (methanolic extract), followed by 2 stage skin carcinogenesis protocol by tumor initiator, 7-12-dimethyl benz(a)anthracene (DMBA) and tumor promoter, Croton oil in Swiss albino mice. They induced 100% skin ulceration in carcinogen control and cumulative number of papilloma (CP), tumor yield (TY) and tumor burden (TB) were calculated as 18.20 ± 1.643 , 3.640 ± 0.3286 and 3.640 ± 0.3286 , respectively. Local application of the extract at 300 mg/kg/day inhibited the tumor incidence up to 20% in 16 weeks and showed a significant decline in continuous group in CP 4.800 ± 6.611 and TY 0.9600 ± 1.322 compared to carcinogen group. For assistance of morphological alteration, biochemical investigations were performed. Extract increased the reduced glutathione (GSH) from 3.286 ± 0.207 to 7.1260 ± 0.4953 $\mu\text{mol/g}$, superoxide dismutase (SOD) 1.722 ± 0.1262 to 6.5160 ± 0.3710 $\mu\text{mol/g}$ and catalase (CAT) 13.624 ± 0.813 to 18.792 ± 0.714 of H_2O_2 reduction/mg protein/min and decreased lipid peroxidation (LPO) 7.652 ± 0.1863 to 4.2340 ± 0.5928 nmol/mg levels compared to carcinogen group. Histopathological changes showed papillomatosis and ulceration in carcinogen while acanthosis with normal psychological features in the continuous group. In conclusion, cytotoxic potential of *Convolvulus arvensis* methanol extract is due to the presence of quercetin, that is, flavonoid that have the ability to capture the reactive oxygen species, over expressions of tumor causing genes and topoisomerase II; its presence was confirmed by high performance of liquid chromatography (HPLC) chromatogram. These investigations may have introduced new phytochemicals that are easily available and effective for cancer prevention.

Key words: *Convolvulus arvensis*, skin carcinogenesis, tumor, 7-12-dimethyl Benz (a) anthracene (DMBA), croton oil, quercetin.

INTRODUCTION

Skin cancer is one of the most widespread diseases, characterized by malignancies on the outermost layer of

skin and related to the race, gender, age, geography (Marks, 2007). Sun rays, artificial light sources, chemicals and polycyclic aromatic hydrocarbons become the cause of carcinogenesis (Narayanan et al., 2010) which initiates by promotion and progression, and after metabolism, radicals of reactive oxygen species (ROS) are produced which mutate the properties, functions and sequence of amino acids (Dixon and Koprass, 2008; Park, 2005). In the present study an attempt has been made to study the cytotoxic activity of bindweed (*Convolvulus arvensis*) on the skin carcinogenesis induced by 7-12-dimethyl Benz (a) anthracene (DMBA) and croton oil in Swiss albino mice.

Convolvulus arvensis from family Convolvulaceae is commonly known as Morning-glory or bindweed widely found in Asia. Its aerial parts have flavonoids, tannins, polyphenols, caffeic acid, saponins, δ -amino levulinic acid, lipids and tropane alkaloids (Kaur and Kalia, 2012; Montes-Holguin et al., 2006) including cuscohygrine and calystegines (Atta and Mounier, 2004) and its leaves also contain various flavanoids and glycosides like Quercetin, Kaempferol-3-mono glycosides, etc. (Yusuf et al., 2002). Traditionally, the plant has been used for the treatment of fever, inflammation, hemorrhage, abdominal pain and worms in children (Atta and Mounier, 2004; Al-Bowait et al., 2010), skin ulcers and wound healing (Meng et al., 2002) flowers have laxative activity and useful for muscle weakness and leaves are immunostimulant and effective in asthma and jaundice (Al-Bowait et al., 2010). It has anti-angiogenesis and antioxidant activity at high doses due to the presence of proteoglycans and glycoproteins (Meng et al., 2002; Calvino, 2002). Aerial parts have shown antioxidant and cytotoxic activity against Hela cells due to flavonoids (quercetin) and tannins (Sadeghi-aliabadi et al., 2008, 2010). As we know that conventional medicines are in easy access, but due to their solemn side effects and high cost, phytomedicine are grabbing high interest (Surh, 2003). In ages proved antioxidant and free radical scavenging potential due to polyphenols, flavonoids, lignins, carotene, polysaccharides and xanthines like other anticancer agents vincristine, vinblastine, vincarosa, we had selected whole plant of *Convolvulus arvensis* for determining its *in vivo* cytotoxic potential.

MATERIALS AND METHODS

Collection of plant

Whole plants of *Convolvulus arvensis* were collected during the months of April and May in 35 to 40°C from the backyard of Govt. College, University Faisalabad, Pakistan and identified by a plant

taxonomist Dr. Mansoor Hameed Head of Botany department, Agriculture University Faisalabad.

Preparation of plant extract

The whole plants were washed, chopped and dried under shade at room temperature for many days until fully dried, ground by electric grinder, powdered and sieved. This material was macerated in methanol for 7 days with frequent shaking every day, filtered out by using Whatman filter paper, separating out solvent from solid material by using a rotary evaporator at 40 to 50°C and residues obtained were stored in small amber jars at 4°C.

Drug or Chemical

Carcinogen 7-12-dimethyl benz(a)anthracene (DMBA) and Croton oil were obtained from Sigma Aldrich Chemical Company USA. Acetone was used as a vehicle for all topically applied carcinogens and dilution of plant extract.

Experimental animals

6 to 8 weeks old male mice weighing 20 to 30 g were obtained from National Institute of Health Islamabad and kept in animal house of the Department of Pharmacology, Government College, University Faisalabad under controlled conditions of Temperature ($25 \pm 1^\circ\text{C}$) and humidity ($50 \pm 5\%$). They were given standard diet and water *ad libitum*. Mice were acclimatized to environment for one week prior to commencement of the experiment (Roslida et al., 2011).

Experimental plan

Dorsal skin of albino mice was shaved with an electric clipper for approximately 2×2 cm area and marked with permanent marker (Arya and Kumar, 2011). 50 animals were divided into 5 subgroups: (1) Carcinogen Control group: 10 mice were applied topically with a single dose of DMBA as a tumor initiator on the shaved area of the skin of mice and two weeks later Croton oil was applied as tumor promoter thrice a week till the end of 16th week. (2) Pre group: 10 mice received 300 mg/kg methanol (CA) extract topically for consecutive 7 days. DMBA single topical dose was applied at 8th day and two weeks later Croton oil was applied trihrice a week till the end of 16th week. (3) Peri group: 10 mice received DMBA single topical dose, then 300 mg/kg CA extract topically for consecutive 15 days. After that, Croton oil was applied thrice a week till the end of 16th week. (4) Post group: 10 mice were received DMBA single topical dose. After 2 weeks, 300 mg/kg CA extract applied topically daily with the half hour delay application of Croton oil thrice a week till the end of 16th week. (5) Continuous group: 10 mice were received 300 mg/kg C.A (methanol) extract topically throughout the experimental period daily and at 7th day DMBA single topical dose was applied and two weeks later Croton oil was applied thrice a week till the end of 16th week.

Preparation of stock solution

1 molar 100 μg DMBA was dissolved in 100 μl acetone and Croton

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oil at 1 µg/100 µl to make 1% (v/v) dilution prepared just before its use and kept in amber glass bottle at about 20°C. A stock solution of extract was prepared by dissolving 10 mg extract in 1.0 ml acetone. Serial dilution of 300 mg/kg was made (Roslida et al., 2011).

Preliminary phytochemistry

Determining the chemical constituents in CA extract was done by following protocols.

Screening for reducing sugars

In determining the presence of reducing sugars, weighed 0.5 g of ethanolic extract was used to form aqueous ethanolic extract in 5 ml distilled water in a test tube and few drops of both Fehling's solution A and B added. The change in color was observed. Color alterations showed the presence of reducing sugars (Khan et al., 2011).

Screening for alkaloids

For determining the alkaloids, to weighed 0.2 g of alcoholic extract was added 5 ml hydrochloric acid 2 N solution which was warmed in a boiling water bath, cooled and filtered properly. The obtained filtered solution was put in equal portions in two test tubes to which was added 2 to 3 drops of Mayer's reagent in one tube and Dragendoeff's in the other. Suspending white creamy solid in Mayer's reagent and reddish brown in Dragendoeff's showed the confirmation for the existence of alkaloids (Khan et al., 2011).

Screening for tannins

In determining the tannins presence, the weighed 0.5 g plant extract was dissolved in 10 ml distilled water, warmed till boiling then filtered properly. 0.1% solution of ferric chloride was added to filtrate solution. Changed color from dark brown green or blue green showed the existence of tannins (Egwaikhidi et al., 2007).

Screening for saponins

In determining the saponins presence, a weighed 0.5 g extract was put in the test tubes and to it was added 5 ml distilled water. This mixture was shaken and waited for visible foam to appear. Then a few drops of olive oil was added in this stable foam and an emulsion was formed which showed the presence of saponins (Khan et al., 2011).

Screening for terpenoids

In determining the presence of terpenoids, a weighed 0.2 g extract was mixed with 2 ml of chloroform and then sulfuric acid was added in small parts within this solution. A fine thin layer was formed and inner solution showing reddish brown color indicated the presence of terpenoids (Mojab et al., 2003).

Screening for flavonoids

In determining the presence of flavonoids, to a weighed 0.2 g extract, 5 ml of diluted sodium hydroxide and 5 ml of 1 M

hydrochloric acid was added in plant extract. Yellowish red solution was formed that turned into a transparent white solution which indicated the existence of flavonoids (Mojab et al., 2003).

Screening for anthraquinones

In determining the presence of anthraquinones, a weighed 0.5 g plant extract was warmed for 3, 4 min with 10% hydrochloric acid in a boiling water bath then filtered properly and cooled for a few minutes. Methyl chloride in equal volume of filtrate was added in this solution. 10% ammonia was mixed with this solution before heating. A fine pink or dark pink color indicated the existence of anthraquinones (Egwaikhidi et al., 2007).

Screening for glycosides

In determining the presence of glycosides, a weighed 1.2 g of plant extract was mixed in 10 ml of 1% hydro alcoholic acid and 10% sodium hydroxide and then to it was added 3 to 4 drops of both Fehling's solutions A and B. Reddish precipitates indicated the existence of glycosides (Mojab et al., 2003).

Determining the pharmacological activity

Screening was done by the following studies.

Morphological studies

Skin of each mice was weekly observed for loss of hair, redness, ulceration and outgrowths. These were counted and measured by digital vernier caliper till the end of 16th weeks. Recorded morphological parameters were

1. Tumor incidence: Number of tumor bearing mice,
2. Cumulative number of papilloma, tumor yield: Average number of tumors per mouse and,
3. Tumor Burden: Number of tumors per tumor bearing mice (Roslida et al., 2011).

Biochemical studies

Mice ulcerated skin was shaved, washed with cool and normal saline solution. Excised skin was used to prepare 10% tissue homogenate in 0.15 molar Tris potassium chloride having a pH of 7.4 and centrifuged for 10 min at 2,000 rpm. Reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) level were determined by protocols followed by earlier researchers.

Reduced glutathione level: For determining the GSH level, 10% tissue homogenate was mixed with 100 µl trichloroacetic acid (25%), centrifuged and the precipitates collected. Then, 200 µl of coloring reagent, 0.6 mM 5, 5' dithio-bis-2-nitrobenzoic acid was added in 0.2 molar phosphate buffer solution having a pH of 8.0 upto 100 µl. The absorbance of nonprotein sulphahdryl (SH) group was calculated by ultraviolet-VIS Systronics spectrophotometer at 412 nm wavelength. Reduced glutathione level was determined in µmol/g of tissue (Moron et al., 1979).

Catalase level: For determining the catalase (CAT) level, 10%

Table 1. Preliminary phytochemistry of whole plant methanol *Convolvulus arvensis* extract.

Phytochemicals	Methanol extract
Reducing sugars	+
Alkaloids	+
Tannins	+
Saponins	+
Terpenoids	+
Flavanoids	+
Antra-quinones	+
Glycosides	+

tissue homogenate was mixed with 50 mM phosphate buffer solution having a pH 7. After 10 min centrifugation, 30 mM hydrogen superoxide solution was added in obtaining 100 μ l solution. The absorbance was recorded by spectrophotometer at 240 nm. Catalase level was shown in μ mol of H₂O₂ reduction/mg protein/min (Aebi, 1984).

Superoxide dismutase level: For determining the superoxide dismutase (SOD) level, pyrogallol was added in 50 mM Tris-hydrochloride buffer solution having a pH 7.5. After auto oxidation, change in absorbance was calculated by spectrophotometer at 420 nm. Superoxide dismutase level was determined in μ mol/g of protein (Marklund and Marklund, 1974).

Lipid peroxidation level: Lipid peroxidation (LPO) level was calculated by 0.6% active substances of thiobarbituric acid, 0.1% sodium dodecyl sulphate and 20% trichloroacetic acid. Then 200 μ l 10% tissue homogenate were dissolved in these reagents and warmed this mixture for one hour. After cooling it, extraction was made with N butanol- pyridine in a ratio of 15:1 and the optical density values noted by spectrophotometer at 532 nm. All the results were expressed in nmol/mg of tissue (Ohkawa et al., 1979).

Histopathological study

Specimens of mice ulcerated skin was excised, washed with normal saline and fixed in 10% formalin for a day. Again it was fixed with paraffin wax, 5 micrometer portions of each specimen was cut and the histopathology observed (Parmar et al., 2011).

Chromatogram by HPLC for identification of active constituent:

High performance liquid chromatography (HPLC) was performed to confirm the presence of quercetin in *C. arvensis* methanol extract (Ali et al., 2013). The sample was dissolved in 5 ml distilled water and 12 ml methanol, kept for 5 min, again to it was added 6 ml distilled water, allowed to stay for 5 min and again was added 10 ml 5 M HCl in this solution. It was placed in oven for 2 h and the solution filtered by syringe filter. Acetonitrile, methanol and acetic acid were used as the mobile phase with the flow rate of 1 ml/min. The column was ODS 250 mm \times 4.6 mm and UV detector was used to obtain chromatogram at 280 nm at room temperature (Saleem et al., 2014).

RESULTS

All the obtained results were statistically analyzed by one way analysis of variance (ANOVA). Minitab 16.0 software was used for calculation and all values were represented as mean \pm standard deviation (SD). Values were taken as $p < 0.05$ (significant). According to this screening, the methanol extract of *C. arvensis* had shown the presence of phytochemicals including: reducing sugars, alkaloids, tannins, saponins, terpenoids, flavonoids, anthraquinones and glycosides (Table 1).

Morphological parameters

Results obtained from present study had shown that single topical application of carcinogen DMBA followed by a thrice/week repeated application of 1% Croton oil till 16th week produced 100% skin ulceration in the carcinogen control group. 18.20 ± 1.643 , 3.640 ± 0.3286 and 3.640 ± 0.3286 were calculated as the cumulative number of papilloma, tumor yield and tumor burden, respectively (Table 1 and Figure 1a and b). Ulcerated skin specimens were observed under microscope after staining with hematoxylin, a basic dye and eosin, an acidic dye, as shown in Figure 2.

Convolvulus arvensis (methanol) extract in 300 mg/kg was applied locally to pre, peri, post and continuous groups. Significant decline in cumulative number of papillomas from pre to continuous group 15.800 ± 2.387 to 4.800 ± 6.611 , tumor yield 3.160 ± 0.4775 to 0.9600 ± 1.322 and tumor burden 3.640 ± 0.329 to 1.200 ± 1.653 were calculated (Table 2). The tumor incidence decreased from 5 to 4 that is, 20% in continuous group compared with carcinogen group. When the results of the experimental group vs. carcinogen group was statistically analyzed, they showed a significant difference between groups ($p < 0.05$).

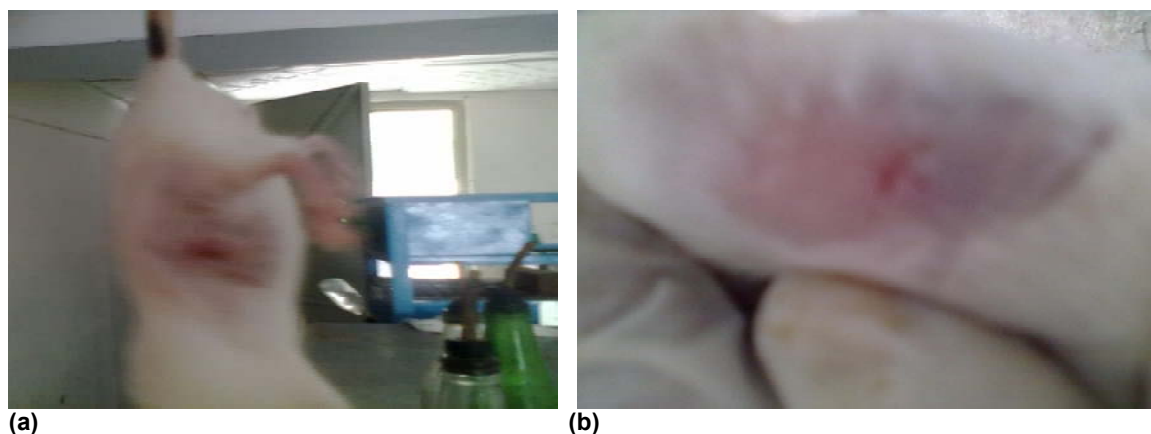


Figure 1. Morphologically representing papillomas on mice skin

Table 2. Inhibition of morphological parameters of DMBA/Croton oil induced skin tumors by *Convolvulus arvensis* (methanol) extract in 300 mg/kg topically applied dose.

Parameter	Control	Pre	Peri	Post	Continuous
Tumor incidence	5.000 ± 0.000	5.000 ± 0.000	4.000 ± 2.236	4.000 ± 2.236	4.000 ± 2.236
Cumulative no. of papillomas	18.20 ± 1.643	15.800 ± 2.387	10.600 ± 6.229	8.600 ± 5.030	4.800 ± 6.611
Tumor yeild	3.640 ± 0.3286	3.160 ± 0.4775	2.120 ± 1.2458	1.7200 ± 1.0060	0.9600 ± 1.3221
Tumor burden	3.640 ± 0.329	3.160 ± 0.477	2.650 ± 1.557	2.150 ± 1.257	1.200 ± 1.653

Each value was the mean of 3 readings and expressed as mean ± SD; Significance level between carcinogen control vs experimental groups ($p < 0.05$).

Table 3. Induction of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and inhibition of lipid peroxides (LPO) level by *C. arvensis* (methanol) extract in 300 mg/kg topically applied dose.

Parameter	Control	Pre	Peri	Post	Continuous
GSH	3.286 ± 0.207	4.3020 ± 0.449	5.5100 ± 0.1283	7.1260 ± 0.4953	7.1260 ± 0.4953
SOD	1.722 ± 0.1262	2.562 ± 0.5731	4.3300 ± 0.5665	5.3600 ± 0.2835	6.5160 ± 0.3710
CAT	13.624 ± 0.813	13.624 ± 0.813	14.240 ± 2.073	15.900 ± 0.900	18.792 ± 0.714
LPO	7.652 ± 0.1863	6.188 ± 0.2417	4.4760 ± 0.2370	4.5620 ± 0.2782	4.2340 ± 0.5928

Each value was the mean of 3 readings and expressed as mean ± SD; Significance level between carcinogen control vs experimental groups ($p < 0.05$).

Biochemical parameters

Results obtained from present study had shown that topical application of DMBA and Croton oil produced 100% skin ulceration in carcinogen control group and decreased the GSH, SOD and CAT level to $3.286 \pm 0.207 \mu\text{mol/g}$, $1.722 \pm 0.1262 \mu\text{mol/g}$ and $13.624 \pm 0.813 \mu\text{mol}$ of H_2O_2 reduction/mg protein/min levels, respectively and increased the LPO level as $7.652 \pm 0.1863 \text{ nmol/mg}$ (Table 3). *C. arvensis* (methanol) extract in 300 mg/kg caused a 20% decline in the tumor incidence in the continuous group. GSH, SOD and CAT increased up to $7.1260 \pm 0.4953 \mu\text{mol/g}$, 6.5160 ± 0.3710

$\mu\text{mol/g}$ and $18.792 \pm 0.714 \mu\text{mol}$ of H_2O_2 reduction/mg protein/min level while LPO level decreased up to $4.2340 \pm 0.5928 \text{ nmol/mg}$ as compared with a carcinogen (Table 3). When the results of experimental vs. carcinogen group was statistically analyzed and compared they showed a significant difference between groups ($p < 0.05$). The results were supported by HPLC chromatogram as shown in Figure 3.

DISCUSSION

Cancer chemoprevention by conventional means or

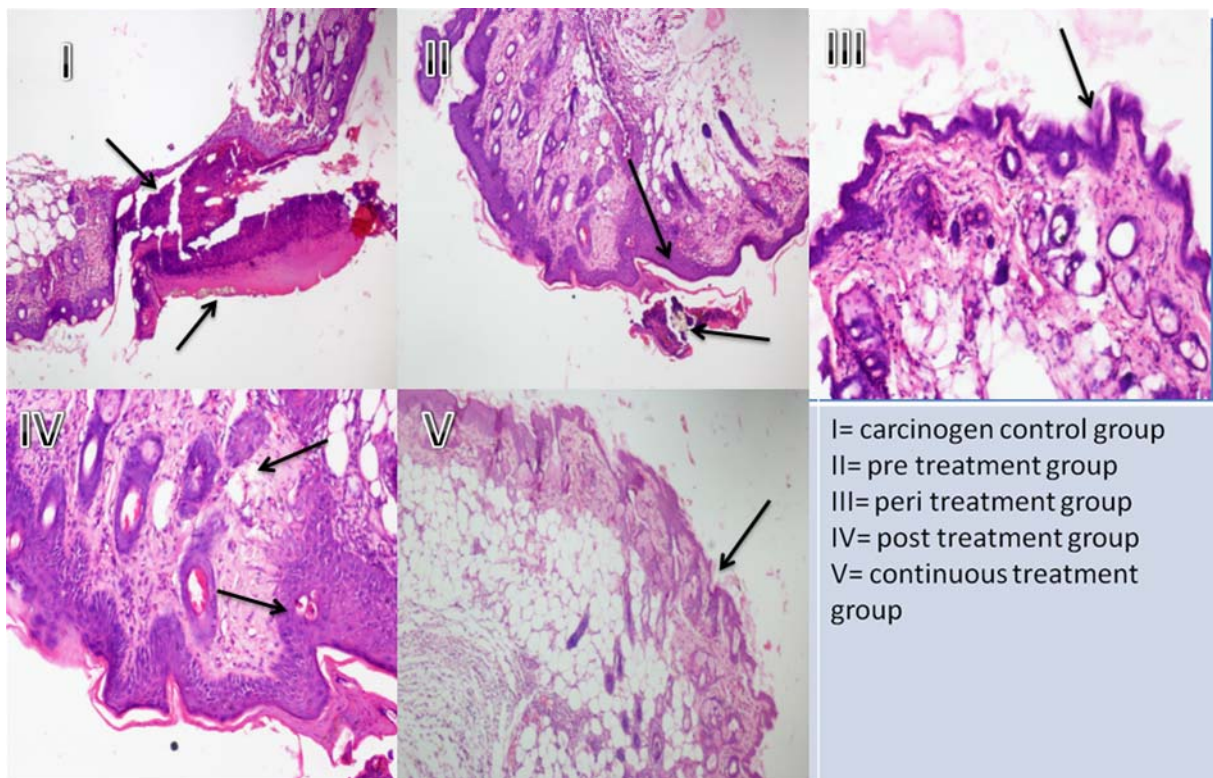


Figure 2. Histopathology of *C. arvensis* methanol extract. (I) Carcinogen control: epidermis show ulceration and inflammatory slough; (II) Pre group: epidermis show mild acanthosis, hyperkeratosis and mild papillomatosis with normal cytological features; (III) Peri group: epidermis show mild papillomatous changes; (IV) Post group: epidermis show mild acanthosis with normal cytological features and (V) Continuous group: epidermis show very mild degree of acanthosis with normal cytological features.

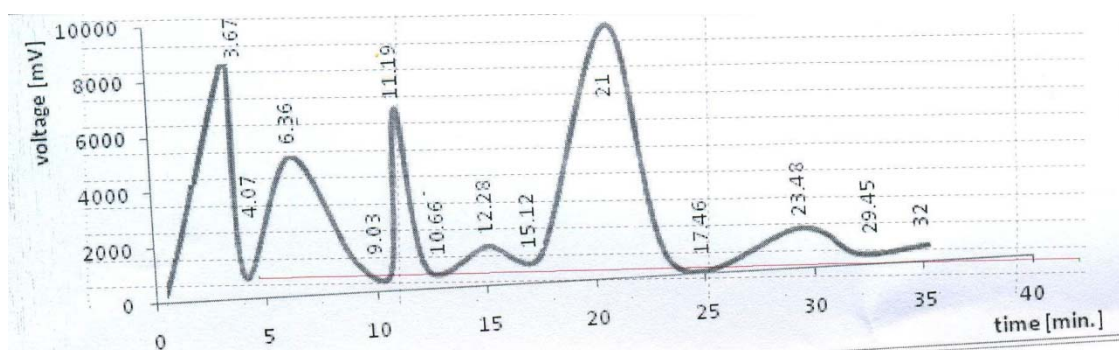


Figure 3. HPLC Chromatogram for analysis of *Convolvulus arvensis* methanol extract.

phytochemicals is capturing interests very rapidly. These chemical constituents include flavonoids, polyphenols, carotenoids, terpenoids and tannis which have been obtained from our daily dietary agents. They reverse the carcinogenesis and inhibit the development of persistent

tumor (Sengupta et al., 2004). When tumor initiator, DMBA and tumor promoter, Croton oil (active constituent: 12-O-tetradecanoylphorbol-13-acetate) is applied on the mice skin, reactive oxygen species are formed. These ROS including O_2^- , OH^- , H_2O_2 have ability to move from

site of formation of the other healthy cells. DMBA with its active metabolites cause mutation of healthy cells via diol epoxide induction (Rubin, 2001). Increase ROS disturb the balance of oxidation/reduction reaction, oxidative stress parameters and take part in chemical carcinogenesis by changing the gene expression and destructing the cellular components. TPA along with ROS, increases the epidermal ornithine decarboxylase, COX-2 and nitric oxide synthase level (Shakilur et al., 2008).

Enzymatic oxidative stress parameters including SOD and CAT and non enzymatic GSH help to play important role in the enzymatic defense system and their lower level promote the tumor in healthy cells. Reduced glutathione helps to protect the body from xenobiotics, toxic metabolites and ROS (Lu, 1999). SOD and CAT capture the reactive oxygen species and minimize their carcinogenic and mutagenic potential, balance the hydrogen/oxygen peroxide level by causing alteration in O₂ and H₂O₂ radicals (Dasgupta et al., 2004). In carcinogen control group, level of GSH, SOD and CAT were significantly decreased and LPO increased along with the tumor incidence, tumor yield and tumor burden due to the presence of increased ROS. The whole plant of *C. arvensis* methanol extract decreased the tumor incidence, tumor yield, tumor burden, cumulative number of papilloma and lipid peroxidation level as compared to the carcinogen control group. This plant extract by two-folds increased the level GSH, SOD and CAT level in a continuous group in which plant extract was applied throughout the experimental period (16 weeks) at 300 mg/kg/b.wt. The phytochemical analysis had shown the presence of flavonoids, saponins, tannis and terpinoids and HPLC confirmed the quercetin, that is flavonoid. Previous literatures have proved that quercetin has ability to capture the ROS, superoxide anions, hydroxyl and lipid peroxy radicals, inhibit cyclooxygenase, lipooxygenase, monooxygenase, phospholipase A₂, protein kinase and NADH-oxidative pathways (Morton et al., 2000).

It has been reported and proved that the major constituent as tumor suppressor is flavonoids. Its cytotoxic activity is due to its unique structure. Activity of flavonoids is monitored by quantity, location and substitution of OH group or groups on A and B rings and double bond at C2-C3 level. Its activity is also connected with DNA topoisomerase II, which induce the destruction between DNA double stands and helps to reconnect these which performed various important functions in the cell mechanisms (Cao et al., 1996). Studies depicted that quercetin, an important flavonoid having significant anti carcinogenic activity by inhibiting the over expression of tumor causing genes, functions of topoisomerase II, arrest the uncontrolled cell growth at G1, S, G2 and M in all phases of cell cycle and maintains its balance. Along

with these it indirectly increases the level of tumor suppressor genes and their related genes and protein expression (Parmar et al., 2009). It is concluded that all those agents which have the ability to capture the free radicals *in vivo* may have cytotoxic potential against cancer.

Conclusion

C. arvensis methanol extract in 300 mg/kg has shown significant cytotoxic properties due to the presence of flavonoids and tannins against DMBA induced skin carcinogenesis. This investigation has introduced new phytochemicals for cancer prevention and will open new ways in this research era.

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Conflict of interest

There is no conflict of interest as regard this study.

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