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GC/MS CHARACTERIZATION, ANTIOXIDANT AND FREE RADICAL SCAVENGING CAPACITIES OF METHANOLIC EXTRACT OF *OXALIS CORNICULATA* LINN: AN AYURVEDIC HERB

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ABSTRACT
A well known medicinal herb *Oxalis corniculata* is called as Indian sorrel, creeping wood sorrel, Ambatee, Ambutee grown as weeds throughout tropical parts of India. The main objective in the present study was aimed to verify the phytochemical characterization in the methanolic extract through GC/MS analysis and to reveal the antioxidant and antiradical capacities of *Oxalis corniculata* by analyzing *in vitro* antioxidant assays. Phytochemical investigation was carried out to identify the possible components from *Oxalis corniculata* fresh leaves by GC/MS. It has revealed twelve peaks of GC/MS chromatogram. Squalene is generally reported for anticancer activity while Vitamin E is considered as source of antioxidant. Methanolic extract of *Oxalis corniculata* was compared with antioxidant references such as gallic acid, rutin, ascorbic acid for *in vitro* antioxidant assays. From the results, it is firstly reported that vitamin E and squalene present as antioxidant bioactive in methanolic extract of *Oxalis corniculata* fresh leaves. Methanolic extract of *Oxalis corniculata* exhibited significant effect in the antioxidant and free radical scavenging activities may be due to presence of vitamin E and other bioactive constituents.
Keywords: GC/MS, *Oxalis corniculata,* antioxidant, free radical, methanolic extract

INTRODUCTION

Oxalis corniculata Linn is a well-known most versatile medicinal herb in India having a wide spectrum of biological activities belonging to family Oxalidaceae. [1] It has various synonyms like *Oxalis foliosa*, *Oxalis monadelpha*, *Oxalis repens*, *Oxalis villosa* and has common names in different languages like Ambutee, Ambatee (Marathi), while Indian sorrel, creeping wood sorrel, yellow oxalis (English); Chaangeri (Sanskrit and Hindi). The plant was identified taxonomically as small, annual, erect twigs. Leaves are roundly ovate with three foliolate leaflets and yellow flowers.[2] Sour in taste due to acidic constituents like oxalic, ascorbic, and tartaric, citric acids, and essential fatty acids are present in the herb.[3] Vitexin (8-β-D glucopyranosyl apigenin) found commonly in *Oxalis corniculata* Linn, a plant of good repute in Ayurvedic system of medicine. [4]

In according to state-of-the-art review, it has been reported the various activities of *Oxalis corniculata* linn such as anti-inflammatory, anxiolytic, anticonvulsant, antifungal, antiulcer, antinociceptive, anticancer, antidiabetic, hepatoprotective, hypolipidemic, abortifacient, antioxidant, diuretic, antimicrobial and wound healing properties.[1] On reviewing the studies on the herb *Oxalis corniculata* Linn till the date, Gas Chromatography-Mass Spectrometry (GC/MS) analysis of the plant has not been revealed.

Phytochemical investigation was carried out to identify the possible components from *Oxalis corniculata* fresh leaves by GC/MS. By analyzing the different novel *in-vitro* antioxidant capacity of the leaves of *Oxalis corniculata* may helpful for understanding the exact nature. Methanol has been chosen as an

extracting solvent for herb *Oxalis corniculata* linn as it provides good yield with highest antioxidant and antiradical potencies among different solvents.[5] Antioxidative abilities like phosphomolybdenum assay (PMA), cupric ions reducing antioxidant capacity (CUPRAC) and ferric ions reducing ability power (FRAP) methods while free radical scavenging activities such as hydroxyl, DPPH, and anti-peroxidation like thiobarbituric acid reactive substance (TBARS), metal chelation capacity (MCC) were assayed as *in-vitro* models. Methanolic extract of *Oxalis corniculata* was ascertained in the novel antioxidant activities by comparing with antioxidant references such as gallic acid, rutin, ascorbic acid and EDTA.

EXPERIMENTAL

Material and Methods

Chemicals

Ammonium persulphate, Thiobarbituric acid, Folin & Ciocateu's phenol reagent, Aluminum chloride, Gallic acid, Neocuproine, Cupric chloride, Ferric chloride, Nitro B.T., Ammonium molybdate, Trichloroacetic acid, Ferrous chloride, Ferrous sulphate, Potassium phosphate, Sodium phosphate, Potassium ferricyanide, Sodium carbonate, Sodium nitroprusside, Sodium acetate, Sodium salicylate, Butan-1-ol were purchased from Loba chemicals. Griess reagent, Diphenyl-1-picrylhydrazyl (DPPH), Potassium persulfate was purchased from Sigma Aldrich. Ferrozine SP was purchased from Hi-Media.

Collection of herb

Plant material was collected in the month of July 2014. Fresh leaves of *Oxalis corniculata* Linn methanolic extract was prepared and collected in a suitable container in the previous study.[6]

GC/MS analysis

GC/MS analysis[7] of methanolic extract of *Oxalic corniculata* Linn was performed using a GC Clarus 500 Perkin–Elmer system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25µm ID × 0.25µm df). For GC/MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2µl was employed (a split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

Identification of phytochemical constituents

GC/MS mass-spectrum was interpreted by using the database of National Institute Standard and Technology (NIST). Mass spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library.

Total phenolics content

Phenolics content assay was performed in the method of Blainski *et al*.[8] Methanolic extract of *Oxalis corniculata* in the different concentration range from 100µl to 500µl were added to each test tube containing of 900µl to 500µl distilled water respectively. 500µl of Folin-Ciocalteu reagent solution, 500µl of 100mg/ml sodium carbonate were added after 5min. These tubes were kept aside for 2 hours. Absorbance was measured at 765nm. The concentrations of phenolic compounds in *Oxalis corniculata*

extract were expressed as gallic acid equivalents (GAEs). All assays were conducted in triplicate and its mean was calculated.

Total flavonoids content

Aluminum chloride colorimetric method was used for flavonoids determination.[9] Methanolic extract of *Oxalis corniculata* in the different concentration range from 100µl to 500µl were added to each test tube containing of 900µl to 500µl distilled water respectively.100µl of 20% aluminum chloride in ethanol; 100µl of 5% sodium acetate and 800µl of distilled water were transferred to these tubes and kept incubated at room temperature for 30 minutes. Absorbance was measured at 415nm. The concentrations of flavonoids in *Oxalis corniculata* extract were expressed as rutin equivalents (REs). All assays were conducted in triplicate and its mean was calculated.

Phosphomolybdenum assay

Total antioxidant activity was estimated by phosphomolybdenum assay in methanolic extract of *Oxalis corniculata* as described by method.[10] 1ml each of 0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate were added in 20ml of distilled water and made up volume to 50ml by adding distilled water. Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with 1ml of Molybdate reagent solution and incubated at 95°C for 90min. After cooling the absorbance of the reaction mixture was measured against at 695nm.

Cupric ion reducing antioxidant capacity assay

Cupric ion reducing capacity was measured in methanolic extract of *Oxalis corniculata* in accordance to the method of CUPRAC.[11] 1ml 10mM cupric chloride ($CuCl_2$), 1ml 7.5mM neocuprione and 1ml 1M ammonium acetate buffer (pH 7) solutions were added to test tubes. Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with reaction mixture independently. These reaction mixtures were incubated for half hour at room temperature and measured against blank at 450nm.

Ferric reducing ability power

Ferric ions reducing power was measured according to the method of FRAP[12] in methanolic extract of *Oxalis corniculata* with a slight modification. Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with 1ml of 20mM phosphate buffer and 1ml potassium ferricyanide (1%, w/v) and incubated at 50°C for 30 min. 1ml of TCA (10%, w/v) and 0.5ml ferric chloride (0.1%, w/v) were added to the reaction mixture and absorbance was measured at 700nm.

Hydroxyl free radical scavenging activity

The scavenging ability of the extracts on hydroxyl radicals was determined in methanolic extract of *Oxalis corniculata* according to the method.[13] 0.041gm of $FeSO_4$ and 0.32gm of sodium salicylate was mixed to 100ml of distilled water. 4µl of $H_2O_2$was dropped to it, vortexed for uniform mixing and labeled as "Smirnoff Reagent". Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with 1ml of Smirnoff reagent and incubated about 30min at 37°C. Absorbance of the reaction mixtures was read at 562nm. The scavenging ability on hydroxyl radicals was calculated by use of given equation. The percentage of scavenged OH- of extract was calculated using the following formula: Scavenged OH- % = [(Ac-Ae)/Ac x 100] where, Ac = absorbance of control and Ae = absorbance of extract.

DPPH free radical scavenging activity

The capacity of extracts to scavenge the stable DPPH free radical was measured in methanolic extract of *Oxalis corniculata* by the method.[14] Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with 1ml of 0.1mM DPPH and kept incubated in dark room at normal temperature for 30min. After incubation, optical density of these incubated tubes was measured at 517nm. Control was prepared by mixing 10µl of ethanol in place of extract with 3ml of ethanol and 1ml of 0.1mM DPPH and absorbance

was determined immediately. The percentage of scavenged DPPH of extract was calculated using the following formula: Scavenged DPPH % = [(Ac-Ae)/Ac x 100] where, Ac = absorbance of control and Ae = absorbance of extract.

Thiobarbituric acid reactive substance assay

Lipid peroxidation assay was performed according to modified protocol of TBARS[15] in methanolic extract of *Oxalis corniculata* to measure the lipid peroxide formed using egg yolk homogenates as lipid-rich media. In the assay of lipid peroxidation, malondialdehyde (MDA) was detected by presence of pink color.

Egg Yolk Homogenate Preparation

In accordance of Vasudewa *et al*[16] egg yolk was separated from the albumen and the yolk membrane was removed. 10ml of egg yolk solution was added in 1.15 gm of NaCl in 100ml of distilled water. The solution was homogenized for 30seconds and ultrasonicated for 5 min. In (A+TBA) set, each test tube containing 1ml of extract and ascorbic acid, 100µl of diluted egg homogenate was transferred. To induce lipid peroxidation, 50µl of 0.07M $FeSO_4$ was added. These mixture tubes were kept for 30min for incubation. To stop lipid peroxidation, 50µl of 1.2M trichloroacetic acid (TCA) was added and following 0.8% thiobarbituric acid (TBA) and 3.5M acetic acid in amount of 0.5ml each were added to it and vortexed well. These resultant tubes were placed in the incubator at 95°C for 60min. To eliminate this non-MDA interference, another (B–TBA) set of extracts was treated in the same way as above mentioned set (A+TBA) by excluding TBA. The absorbance of (B–TBA) was subtracted to the absorbance of (A+TBA) for yielding the absorbance for extract (E). After cooling it, 5ml of butan-1-ol was added to each tube and vortexed for 5min. The absorbance of upper organic layer was measured at 532nm. Percentage of lipid peroxidation inhibition was calculated by following formula. Antioxidant index (AI) was calculated using the following equation: AI= (1-E/C) ×100 where, E = absorbance of extract [E = (A+TBA)-(B-TBA)], C = absorbance of fully oxidized control. All values are based on the anti-oxidant index whereby the control is completely peroxidized and each extract providing a degree of improvement, indicated as percent protection.

Metal ion chelating capacity assay

The chelating ability of the extracts on ferrous ions was determined in methanolic extract of *Oxalis corniculata* according to the method of metal ion chelating assay.[17] Methanolic extract of *Oxalis corniculata* and ascorbic acid were mixed with 50µl of solution of 2mM $FeCl_2.4H_2O$ and incubated about 30min at 37°C. The reaction was initiated by the addition of 200µl of 5mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10min. The chelating activity measured by measuring the disappearance of purple color in absorbance of solution at 562nm. Different concentration range of EDTA as standard was prepared in correspondence to the sample. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following formula: Chelating % = [(Ac-Ae)/Ac x 100] where, Ac = absorbance of control and Ae = absorbance of extract.

Statistical analysis

Results were given as mean ± standard deviation of 3 replicates. The results are expressed as mean values and Standard Deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α= 0.05. This treatment was carried out using SPSS v.16.0 (Statistical Program for Social Sciences) software.

RESULTS AND DISCUSSION

GC/MS chromatogram analysis of the methanolic extract of *Oxalis corniculata* in fig. 1 showed twelve peaks indicating the presence of phytochemical constituents. In accordance to reference mass spectra of the constituents available in the NIST library, the peaks of GC-MS spectrum revealed that squalene,

vitamin E, glucose and various fatty acids were identified. Table 1 shows the list of identified phytoconstituents with peak area. Out of these compounds, the most current compounds were squalene; a triterpine compound and vitamin E have been reported for various activities.

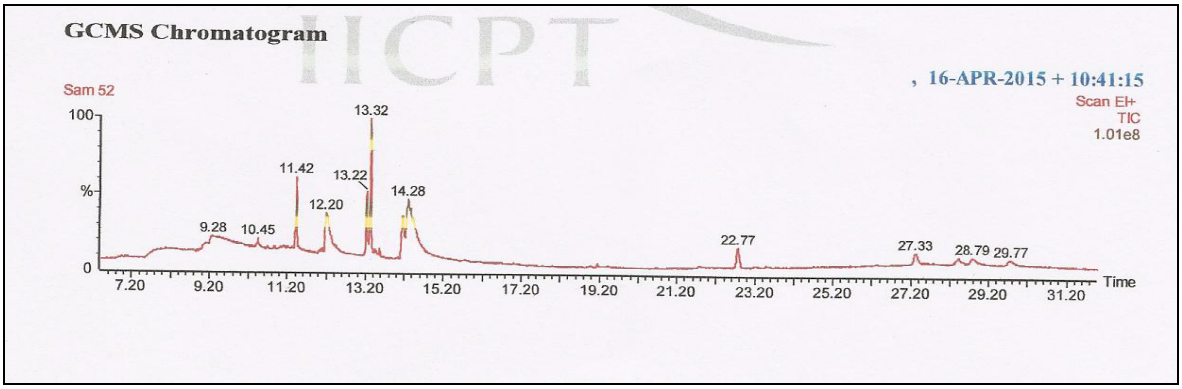

Fig.-1: GC/MS chromatogram of methanolic extract of *Oxalis corniculata*

Table-1: Phytochemical constituents of methanolic extract of *Oxalis corniculata* by GC/MS

No.	RT	Name of the compound	MF	MW	PA %
1	9.28	D-Glucose, 4-o-α-D-glucopyranosyl	$C_{12}H_{22}O_{11}$	342	27.82
2	10.45	α-D-glucopyranoside, O-α-D-glucopyranosyl (1.fwdarw.3) -α-D-fructofuranosyl	$C_{18}H_{32}O_{16}$	504	3.89
3	11.42	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	5.29
4	12.20	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	10.19
5	13.22	9,12-Octadecadienoic acid, methyl ester, (E,E)	$C_{19}H_{34}O_2$	294	2.60
6	13.32	9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	280	6.19
7	14.28	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	$C_{19}H_{32}O_2$	292	26.93
8	22.77	Squalene	$C_{30}H_{50}$	410	2.48
9	27.33	Vitamin E	$C_{29}H_{50}O_2$	430	4.54
10	28.41	9,12-Octadecadienoic acid (Z,Z)-, phenyl, methyl ester	$C_{25}H_{38}O_2$	370	3.31
11	28.79	1b, 5, 5, 6a-Tetramethyl-octahydro-1-oxa-cyclopropa[a]inden-6-one	$C_{13}H_{20}O_2$	208	3.77
12	29.77	7-Oxabicyclo [4.1.0]heptanes,1-methyl-4-(2-methyloxiranyl)-	$C_{10}H_{16}O_2$	168	2.99

Where, MF-Molecular Formula, MW-Molecular Weight, PA%-Peak Area Percentage

Total Phenolics Content Estimation

Different concentration range of gallic acid was assayed in the Folin Ciocalteu Reagent assay for estimating phenolics content. y=0.155x-0.003, (R^2=0.994) was found to be equation of gallic acid. TPC was calculated from the following equation: TPC=C x V/M where, T=Total Phenolic Content (mg/g) of extract as GAE, C=Concentration of GA established from the calibration curve in (mg/ml), V=Volume of the extract solution in ml {0.1-0.5ml} & M= weight of extract in g {0.2-1.0g}. It was used x=(y + 0.003)/0.155 as a reversed formula.

Total Flavonoids Content Estimation

Different concentration range of rutin was assayed in the aluminum chloride method for estimating total flavonoid contents. y=0.014x, (R^2=0.982 was found to be equation of rutin standard). Total Flavonoid Content was calculated from the following equation: TFC=C x V/M where, T= Total Flavonoid Content (mg/g) of extract as RT, C= Concentration of RT established from the calibration curve in (mg/ml), V=

Volume of the extract solution in ml {0.1-0.5ml} & M= weight of extract in g {0.2-1.0g}. It was used x=y/0.014 as a reversed formula.

Table-2: Percentage yield, total phenolics content and total flavonoids content

Percent yield	Total Phenolics Content			Total Flavonoids Content		
MOC	MOC	GA	GAE	MOC	RT	RTE
0.18%	0.212	0.314	0.693	0.09	0.029	2.07

Where, MOC- methanolic extracts of Oxalis corniculata, GA-gallic acid, GAE-Gallic acid Equivalents, RT-rutin, RTE- rutin Equivalents

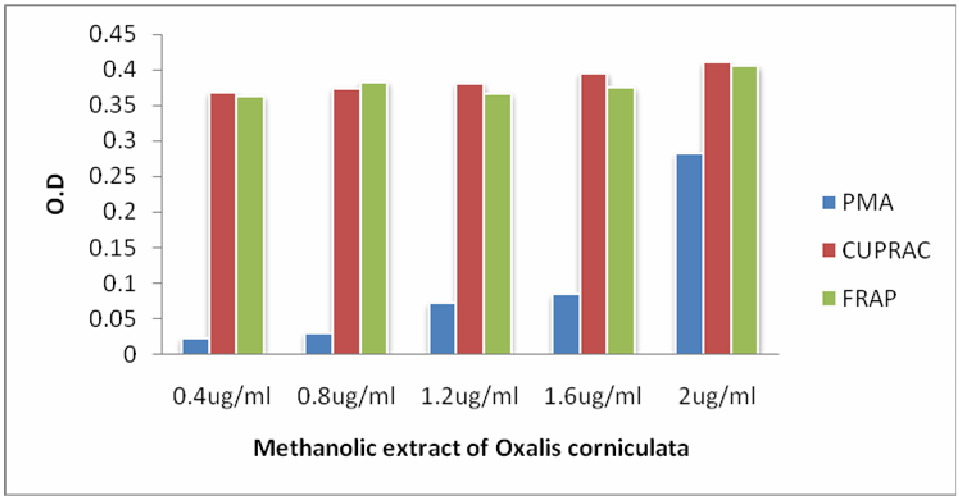

Fig.-2: Antioxidant capacity assay of methanolic extract of *Oxalis corniculata*

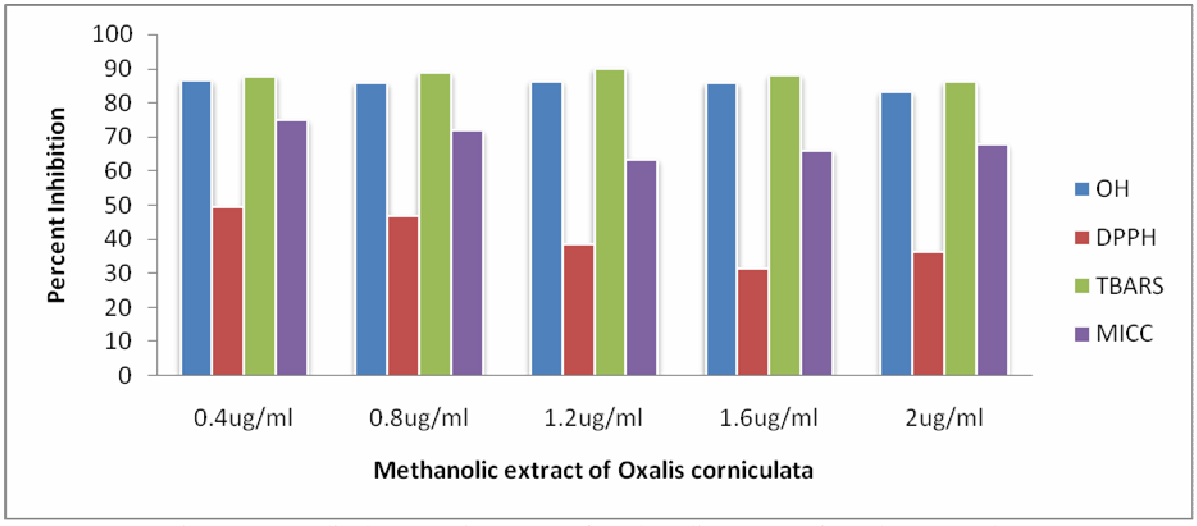

Fig.-3: Free radical scavenging assay of methanolic extract of *Oxalis corniculata*

Many sources of many plants can often be identified from the peak pattern of the chromatograms obtained directly from headspace GC/MS analysis. Squalene is a triterpine generally found in latex and resins of plants and useful in defense against diseases and reported to have anticancer, anti-oxidant and other pharmacological activities.[18] Vitamin E was identified in this plant as a source of oil soluble antioxidant

substance. Certain classes of fatty acids were identified which acts as antioxidant and anti-inflammatory agents.

Phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a bluish green colored phosphate/Mo (V) complex with absorbance at 695 nm.[10] It provides reduction capacity quantitatively through the reduction reaction rate among antioxidant, oxidant and molybdenum ligand by thermally generating auto-oxidation during prolonged incubation period at higher temperature. PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It gives a direct estimation of reducing capacity of antioxidant.

CUPRAC assay is based on the complexometric and redox reaction between copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent.[11] CUPRAC involves both of complexometric and redox reactions. The redox reaction giving rise to a colored chelate of Cu (I)-Nc is relatively not affected by many parameters such as air, sunlight, solvent type, and pH. CUPRAC reagent is reasonably selective, stable, easily accessible, and sensitive.[11]

FRAP assay includes the simultaneous use of ferricyanide and ferric ions as chromogenic oxidants.[12] High absorbance indicates the more reducing power of different extracts. FRAP assay include the simultaneous use of ferricyanide and ferric ions as chromogenic oxidants supplied more favorable redox conditions for a greater variety of antioxidants. Mostly intracellular iron is in ferrous (+2) ions form, superoxide ions can convert it to the ferric (+3) ions form to take part in Fenton reaction. Fenton reaction involves the creation of free radicals by transition-metal ions such as iron and copper that are present in vivo by donating or accepting free electrons via intracellular reactions. [12]

Hydroxyl radical is the most reactive among the oxygen radicals inducing severe damage to proteins, DNA and lipids by crossing cell membranes and leads to lipid peroxidation.[13] Hydroxyl free radicals generated that may attack on the sugar of DNA which leads to fragmentation of DNA strand.[19]

DPPH is a stable free radical which has been excessively used for assessment of scavenging activity of natural products. The ability of the investigated extracts to act as donors of hydrogen atoms or electrons in transformation of $DPPH^*$ radicals into its reduced form DPPH-H was measured.[14]

Egg yolk lipids undergo rapid non-enzymatic peroxidation when hatched in the presence of ferrous sulfate. Malondialdehyde (MDA) is the end product in the egg-lipid peroxidation process. During oxidative degeneration by free oxygen free radicals which give pink color as indicator in the presence of thiobarbituric acid.[15]

Metal ions catalyze the rate of free radicals formation. Ferrozine chelates with Fe^{+2}. In the presence of chelating properties of spices, the complex formation is disrupted, leading to a decrease in the red color of ferrous ion and ferrozine complex.[16] Metal-Catalyzed Oxidation (MCO) systems catalyze the reduction of intracellular iron which is in the form of Fe (III) to Fe(II) mostly through superoxide ions and of O2 to H_2O_2. These products react at metal-binding sites on the protein to produce active oxygen species (viz; OH, ferryl ion) which alters the nature of proteins at the metal-binding site and cause DNA and protein damage. [20, 21]

Many researchers have been reported the antioxidant and free radical scavenging potencies of alcoholic extract of medicinal plants such as *Lagenaria siceraria*[22], *Bacopa monniera*[23] and *Jasminum auriculatum*[24] and *Kalanchoe pinnata*[25,26] in our previous studies have been exhibited significant capabilities to quench free radicals and inhibiting the oxidative reaction. Most of natural products are enormous antioxidant sources available in food including spices[27-29] which having significant antioxidative and antiradical potencies. Thus, it is advisable, better and safe to use naturally occurring products as antioxidant supplements instead of utilizing synthetic antioxidants as preventive measure for rising prevalence of cancer risks.

CONCLUSION

From the results, it is firstly reported that vitamin E and squalene present as bioactive compounds in methanolic extract of *Oxalis corniculata* fresh leaves. Methanolic extract of *Oxalis corniculata* Linn exhibited significant effect in the antioxidant and free radical scavenging activities may be due to presence of vitamin E and other bioactive constituents like flavonoids and phenolics. Isolation of

phytochemical components present in the methanolic extract of *Oxalis corniculata* and its characterization should be further investigated.

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