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EXPLORING *IN-VIVO* AND *IN-VITRO OXALIS CORNICULATA* L. FOR PHYTOCHEMICALS USING NON-TARGETED LC-MS APPROACH AND ITS ANTIOXIDANT CAPACITY

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ABSTRACT: *Oxalis corniculata* Linn. (Oxalidaceae) is the nutraceutical important medicinal plant containing essential nutrients like sodium, potassium, calcium, nitrogen and magnesium and also gives health protection due to the presence of secondary metabolites of therapeutic importance. Present study highlights the phytochemical constituents present in the methanolic extract of *in-vivo* and *in-vitro O. corniculata* through non-targeted LC-MS analysis and to reveal the antioxidant capacities of both extracts by analyzing antioxidant assays like DPPH, H_2O_2 radical scavenging, phosphomolybdenum, total phenol content and total flavonoid content. The phytochemical investigation was carried out to identify the possible components from *in-vivo* and *in-vitro* whole plant extract by LC-MS. It has revealed 26 compounds having known biological activity among which embelin is the main compound. Embelin is reported for anticancer, antioxidant, antitumor and anti-inflammatory activity. We have confirmed its presence in both *in-vivo* and *in-vitro* extracts using HPTLC. Both methanolic extracts of *Oxalis corniculata* were compared with antioxidant references such as tannic acid, quercetin and ascorbic acid for antioxidant assays. We are first to report that embelin is present as one of the antioxidant bioactive component in both methanolic extracts of *Oxalis corniculata*. *In-vivo* and *in-vitro* extracts of *O. corniculata* exhibited a significant antioxidant capacity and free radical scavenging activities, probably due to the presence of bioactive constituents and their synergistic action.

INTRODUCTION: *Oxalis corniculata* L. is a small creeper usually grown in moist climates belonging to the family Oxalidaceae. The flowers are yellow in color and the fruits are cylindrical in shape. It is known by different names like Indian Sorrel, Creeping Woodsorrel, Amboti, Amlikaa and has antifungal, antimicrobial, wound healing, cold, refrigerant, appetizing and astringent properties.

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This plant has much more importance in Indian system of medicines such as Ayurveda, Folk, Siddha, and Unani to cure many diseases such as chronic dysentery, jaundice, headache and healing insomnia. The leaves are used as an anthelmintic [1]. It is shown to be rich in niacin, Vitamin C and β-carotene [2]. The whole plant is astringent, depurative and diuretic [3].

Antioxidants protect biological systems against the harmful effects of free radicals which are produced by metabolic processes happening in the human body. This free radicals leads to cause various diseases like, cardiovascular disorder, cancer, neural disfunctions, alzheimer disease, arterio-sclerosis, allergies and aging [4, 5, 6, 7, 8].

To get these reactive free radicals under control, there is need of natural antioxidants which are safe, cheap and easily available. One of the active components from LC-MS profiling obtained was embelin (2, 5- dihydroxy-3-undecyl- 1, 4-benzo-quinone). It is used not only as an anthelmintic but also used in the treatment of lung diseases, pneumonia, heart disease and obesity [9]. The developed HPTLC method for embelin quanti-fication was validated as per ICH guidelines. Literature survey reveals that there are no reports on HPTLC determination of embelin from the *in-vivo* and *in-vitro Oxalis corniculata* L.

Previously the methanolic extract of the whole plant was assessed for its antioxidant and anti-inflammatory activity by *in-vitro* methods and inhibition of lipid peroxidation was studied by thiobarbituric acid reactive substances (TBARS) method on isolated rat liver tissues [10]. Comparison of *in-vivo* and *in-vitro Oxalis corniculata* was not studied for its phytochemicals and antioxidant, so the present study was conducted using a crude methanolic extract of *in-vivo* and *in-vitro* plant material to evaluate total phenolic and flavonoid contents, DPPH free radical scavenging, H_2O_2 radical scavenging, phosphomolybdenum activity and non-targeted LC-MS for phytochemicals.

MATERIALS AND METHODS: The plant material of *Oxalis corniculata* L. was collected from Ratnagiri, Maharashtra India (N 16°53'613" E 7.3°18'784", Accuracy ± 16.4, 40 feet above the sea level). The plant sample was identified by BSI (Botanical Survey of India), Western Regional Centre, Pune and authenticated as BSI/WRC/ Iden/2015/387 and collection number DS 01.

Solvents chloroform, ethyl acetate, acetic acid, and methanol were of analytical grade and purchased from Merck, India. The standard compound, embelin (Purity: 97% w/w) was purchased from Sigma-Aldrich Germany.

FIG. 1: A AND B, *OXALIS CORNICULATA* COLLECTED FROM NATURAL POPULATION; C AND D, *OXALIS CORNICULATA* CULTURE DEVELOPED IN PLANT TISSUE CULTURE LABORATORY USING NODAL SEGMENTS

In-vitro cultures of *Oxalis corniculata* were cultured on MS medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l) [11] using nodal segments as an explant. *In-vitro* cultures initiated from nodal explants **Fig. 1** were taken out from test tubes and cleaned with tap water to remove the medium then air dried and ground to obtain a fine powder with the help of mortar and pestle. Ten gram powder of both *in-vivo* and *in-vitro* plant material was refluxed with 100 ml methanol at 60 °C for 6 h in a Soxhlet extractor. The extract was filtered using Whatman filter paper and concentrated using a Rota evaporator. The concentrated extract and standard embelin were dissolved in methanol to make 1 mg/ml concentration for HPTLC analysis.

For the antioxidant assay, the concentrated methanolic extract was dissolved in 0.1% DMSO to obtain desired concentration and used for the analysis while, ascorbic acid was dissolved in distilled water.

Free Radical Scavenging Ability using a Stable DPPH Radical (2, 2-diphenyl-1picrylhydrazyl): Extract and standard ascorbic acid were taken in the range of 10µg/µl to 50 µg/µl with 1 ml of DPPH solution were added and kept in the dark condition for 30 min. The optical absorbance of the mixture was measured spectrophotometrically at 517 nm using ascorbic acid as standard [12]. IC_{50} values in terms of µg/ml was calculated.

Hydrogen Peroxide Radical Scavenging Activity: A solution of hydrogen peroxide (2M mol/l) was prepared in phosphate buffer (pH 7.4). Plant extract (50 µg/ml) was added to hydrogen peroxide solution (0.6 ml). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a solution containing phosphate buffer of pH 7.4 without hydrogen peroxide as a control [13]. IC_{50} values in terms of µg/ml was calculated.

Phosphomolybdenum Assay: Extracts and standard ascorbic acid were taken at concentration range 10 µg/µl to 50 µg/µl with 1 ml of reagent solution (4 mM ammonium molybdate, 28 mM sodium phosphate, 0.6 M sulphuric acid), kept in water bath at 95 °C for 90 min. After cooling, the optical absorbance was measured at 695 nm and calculated in terms of ascorbic acid equivalents [14].

Determination of Total Phenolics Content (TPC): Total phenolics content in the methanolic extracts of *in-vivo* and *in-vitro Oxalis corniculata* was determined by using modified Folin-Ciocalteau method [15]. Folin phenol reagent was added to the plant extract and standard tannic acid and kept for 30 min incubation in a water bath at 37°C. Absorbance was measured at 765 nm and total phenolic content was expressed as mg/g tannic acid equivalents.

Determination of Total Flavonoids Content (TFC): Sodium nitrate and aluminum chloride were added to plant extract and standard and kept for 10 min at room temperature. The absorbance was measured at 510 nm and total flavonoid content was calculated as quercetin (mg/g) [16].

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis: Non-targeted LC-MS analysis was done using agilent 1260 binary LC system with Agilent Zorbax Extend C18 RRHT column (50 × 2.1 mm, 1.8 µm) using mobile phase of A. Water (0.1% Formic acid): B. Acetonitrile in a gradient system **Table 1**. Methanolic extracts of *in-vivo* and *in-vitro* material of *O. corniculata* were analyzed. Injection volume was 0.2 µl.

All MS acquisitions were performed in the positive electrospray ionization mode. The gas temperature was set at 325 °C. Data were acquired at a scan rate of 2 Hz in the mass range 60-1600 m/z. Further data was analyzed with Mass hunter qualitative software v. B.06.00 and METLIN database.

TABLE 1: MOBILE PHASE GRADIENT

Time (m)	% B	Flow rate
0	5	0.3 mL/m
18	95	0.3 mL/m
27	95	0.3 mL/m
27.1	5	0.3 mL/m
30	5	0.3 mL/m
30	5	0.3 mL/m

HPTLC: Instrument analysis was performed on 10 × 10 cm size precoated silica gel 60 F_{254} TLC plates (E. Merck). Test samples and standard embelin were applied to the plates using Linomat V automatic sample spotter with the help of Hamilton 100 µl syringe. TLC plates were developed in flat bottom twin trough chamber. Densitometry was performed with a TLC scanner III linked to WinCATS software. HPTLC of embelin was validated using Swami et al, 2017 method [17].

Statistical Analysis: Triplicate data from three separate extracts for all assays were generated and subjected to a one-way analysis of variance (ANOVA). Duncan's multiple range test ($P<0.05$) method for significant difference using SPSS 16.0 for windows 10 version. Values are given as a mean ± standard error

RESULTS: DPPH radical scavenging activity was measured spectrophotometrically at 517 nm after incubation of methanolic extracts of *in-vivo* and *in-vitro O. corniculata* with DPPH for 30 minutes in dark. The 50% of DPPH radical scavenging activity of *in-vivo* extract (IC_{50}: 60.654 µg/ml) was significantly more than the *in-vitro* extract (71.652 µg/ml) while, standard ascorbic acid was showing more IC_{50} (27.24 µg/ml) as it is used as standard antioxidant. Extract concentration for 50% H_2O_2 radical scavenging activity was determined. Methanolic *in-vitro* extract showed least IC_{50} value (125.608 µg/ml) as compared to *in-vivo* (143.42 µg/ml). Potent radical scavenging ability of *in vitro* extract is almost equivalent to standard ascorbic acid activity. The phosphomolybdenum assay method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds. The reduction of Mo was highest with methanolic *in-vivo* extract (0.996 µmol AAE/mg of extract) than *in-vitro* extract (1.043 µmol AAE/mg of extract).

Analysis of total phenolic content was done by Folin- Ciocalteau's method and expressed in terms of tannic acid equivalents. Presence of phenolic content in *in-vivo* extract (169.693 mg TAE/g dry wt) followed by *in-vitro* extract (161.815 mg TAE/g dry weight). Total flavanoid content was determined by aluminum chloride method spectrophotometrically. Addition of aluminum chloride and sodium nitrate to the extracts and standard quercetin leads to the formation of yellow color which indicated the presence of flavonoids. *In-vivo* methanolic extract showed maximum flavonoid content (523.33 mg quercetin equivalents / g dry weight) **Table 2**.

TABLE 2: RESULTS OF ANTIOXIDANT ACTIVITY

Activity of *O. corniculata*	*In-vivo* extract	*In-vitro* extract
DPPH (µg/ml)	60.654 ± 0.013 ^{a}	71.652 ± 0.321^{b}
H_2O_2 (µg/ml)	143.42 ± 0.415 ^{bc}	125.608 ± 0.176 ^{a}
Phosphomolybdenum (µmol AAE/mg of extract)	0.996 ± 0.01 ^{bcd}	1.043 ± 0.005 ^{bcde}
TPC (mg TAE/g dry wt)	169.693 ± 0.01 ^{cd}	161.815 ± 0.015 ^{c}
TFC (mg quercetin equivalents/g dry wt)	523.33±0.02 ^{def}	390 ± 0.035 ^{ab}

Means with different letters are significantly different according to Duncan's test ($p < 0.05$).

In non-targeted LCMS profiling, MS data analysis reveals the presence of various primary and secondary metabolites and intermediate compounds which were analyzed using Mass Hunter software *via* METLIN database search. The 26 identified secondary metabolites predominantly belongs to sesquiterpenes, diterpenes, triterpenes, steroids, glucuronides, quinones and phenols. We have given their dominant biological activities in which were available in the literature and research articles. The comparison of the phytochemicals in two different extracts along with their molecular formula, molecular mass, biological activities reported and m/z is shown in **Table 3**.

TABLE 3: COMPARATIVE ACCOUNT OF CHEMICAL COMPOUNDS OBTAINED FROM NON-TARGETED LC-MS

Compound	*in-vivo*	*in-vitro*	RT	Compound Formula	Molecular mass	Activity
Phloridzin	P	A	9.784	$C_{21}H_{24}O_{10}$	436.1363	Antioxidant and antimicrobial
Granisetron	P	P	12.284	$C_{18}H_{24}N_4O$	312.19	Anticonvulsant
Bilirubin	P	A	16.667	$C_{33}H_{36}N_4O_6$	584.26	Antioxidant
Embelin	P	P	11.408	$C_{17}H_{26}O_4$	294.18	Anticancer, antibacterial, antiproliferative, antidiabetic
Quercitrin	P	P	5.957	$C_{21}H_{20}O_{11}$	448.1	Antioxidant
Parthenin	P	A	14.839	$C_{15}H_{18}O_4$	262.12	Antibacterial
Petunidin	P	P	5.956	$C_{16}H_{13}O_7$	317.06	Antioxidant, antimicrobial and anti-proliferative
Octanal	P	A	16.636	$C_8H_{16}O$	128.12	Antioxidant and Anti-fungal
Lecanoric acid	P	A	9.151	$C_{16}H_{14}O_7$	318.07	Antifungal
Dihydrosphingosine	P	A	11.917	$C_{18}H_{39}NO_2$	301.29	Fungicidal
Ophiobolin A	P	P	10.665	$C_{25}H_{36}O_4$	400.26	Anticancer
Phytosphingosine	P	P	11.528	$C_{18}H_{39}NO_3$	317.29	Growth Inhibitor
Methyclothiazide	P	A	13.958	$C_9H_{11}C_{l2}N_3\ O_4S_2$	358.95	diuretic-antihypertensive agent
Cetylpyridinium	P	P	12.164	$C_{21}H_{38}N$	304.3	Antibacterial and anti-biofilm
Epirubicin	P	A	24.016	$C_{27}H_{29}NO_{11}$	543.17	Antileukemic
Dibekacin	P	P	6.467	$C_{18}H_{37}N_5O_8$	451.26	Antibacterial
Dihydroceramide C_2	P	A	17.176	$C_{20}H_{41}NO_3$	343.3	apoptotic activities
Linolenoyl lysolecithin	P	P	12.625	$C_{26}H_{48}NO_7P$	517.31	Insecticidal activity
Methoprene (s)	P	A	20.063	$C_{19}H_{34}O_3$	310.25	insect juvenile hormone activity
Etretinate	P	A	10.669	$C_{23}H_{30}O_3$	354.21	Chemotactic activity
Larixol acetate	P	A	15.923	$C_{22}H_{36}O_3$	348.26	TRPC 6 inhibitor
Sebacic acid	P	A	16.465	$C_{10}H_{18}O_4$	202.12	Antimicrobial
Malvidin	P	A	6.448	$C_{17}H_{15}O_7$	331.08	Anti-inflammatory
Docosanedioic acid	P	A	21.439	$C_{22}H_{42}O_4$	370.3	Catabolic activity
Cosmosiin	P	P	6.45	$C_{21}H_{20}O_{10}$	432.1	Antidiabetic
Rhapontin	P	A	10.924	$C_{21}H_{24}O_9$	420.14	Antibacterial

(P- Present; A- Absent)

HPTLC analysis of both extracts of *Oxalis corniculata* L. showed a characteristic peak of embelin at R_f value of 0.58. Extracts have peaked at same R_f value as that of embelin (0.58), and hence can be said to contain same chemical component embelin **Fig. 2** and **3**.

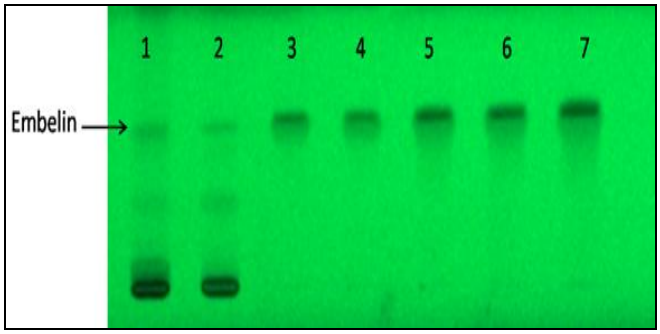

FIG. 2: HPTLC PROFILE AT 254 nm OF METHANOLIC PLANT EXTRACT AND STANDARD EMBELIN
Track 1: O *in-vivo*, Track 2: O *in-vitro*, Track 3-7: Standard embelin concentration of 2 to 10 µg/µl

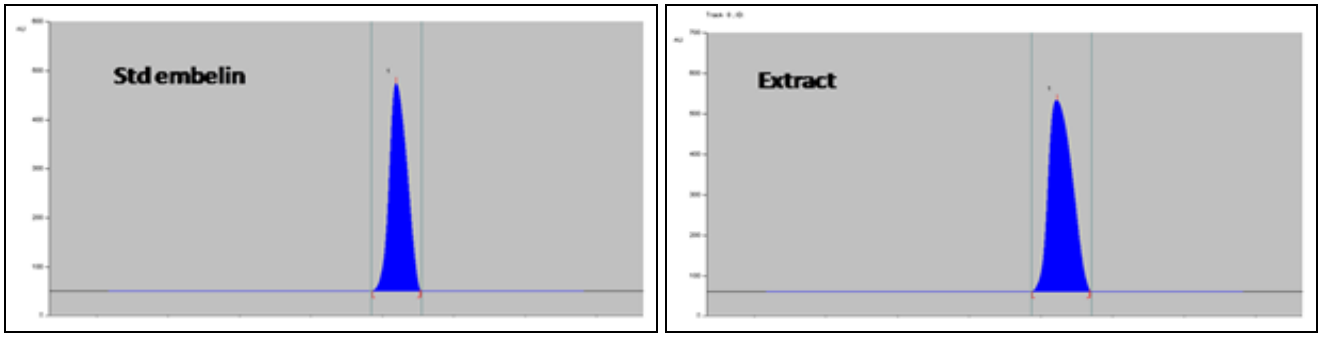

FIG. 3: HPTLC PEAK DISPLAY OF STANDARD EMBELIN AND COMPARATIVE PEAK IN EXTRACTS OF *O. CORNICULATA IN-VIVO* AND *IN-VITRO* EXTRACT AT 254 nm

Quantification of embelin was also done from both extracts **Table 4**.

TABLE 4: CONTENT OF EMBELIN QUANTITATED BY HPTLC DENSITOMETRIC METHOD

Extract	Content of embelin (%)
in -vivo plant material of *O. corniculata*	0.198 ± 0.025 [a]
in-vitro plant material of *O. corniculata*	0.202 ± 0.027 [ab]

(Data represented statistically, mean of three replicate ± standard error, n=7) Means with different letters are significantly different according to Duncan's test (p<0.05).

The developed method has been validated in terms of linearity, precision, specificity, robustness, and accuracy as per ICH guidelines **Table 5**.

DISCUSSION: In present study, both extracts of *Oxalis corniculata* can be considered as potent antioxidants as they possess good antioxidant capacity. To understand the exact nature of oxaliscorniculata, we have used different types of antioxidant assay methods. The DPPH assay is based on scavenging ability of antioxidants towards stable DPPH radical hence, it has been excessively used for assessment of scavenging activity of natural products.

TABLE 5: VALIDATION PARAMETERS: METHOD VALIDATION PARAMETERS FOR THE QUANTITATION OF EMBELIN BY PROPOSED HPTLC METHOD

Parameter	Embelin
Linearity range	2-10 µg
Precision	10 µg
Intraday Precision (% CV)	5.084
Interday Precision (% CV)	4.319
Calibration curve embelin	R sq 0.995
Repeatability (% CV)	
embelin	0.804
O *in-vivo*	12.629
O *in-vitro*	13.781
Robustness	Robust
Specificity	Specific R_f 0.58
LOD (µg)	0.177
LOQ (µg)	0.536
Solvent system	Chloroform: Ethyl acetate: Acetic acid (5:4:1v/v/v)

From our result, it may have possible that some hydrogen donors in antioxidant principles of *in-vivo O. corniculata* reduce the radical when it

reacts with hydrogen donors in antioxidant principles. Hydroxyl radical is the most reactive radical among the oxygen radicals which induces severe damage to proteins, DNA and lipids by crossing cell membranes and leads to lipid peroxidation [18].

The total phenolic content was nearly same in both extracts. Among these two extracts analyzed, a significant phenolic content and antioxidant activity were found, so it can be predicted that the observed antioxidant activity may be due to the presence of total phenolic content in the plant.

The LC-MS profiling of the *in-vivo* and *in-vitro* extracts of *Oxalis corniculata* showed significant number of identified compounds which have various biological activity including the antioxidant activity. The identification of these phyto-constituents will help in isolation of compounds which are natural sources of antioxidant molecules.

As we have done the non-targeted LC-MS profiling, it needs to be validated using another analytical technique. Embelin was available as a standard compound in our lab hence we validated it by co-elution technique using high-performance thin layer chromatography (HPTLC).

Embelin is a plant based benzoquinone which is known for various biological activities like anti-oxidant, antitumor, anti-inflammatory analgesic, antifertility and antimicrobial [10, 19]. It is mainly reported from plants of *Embelia sp.* and *Arsidia sp.* from Myrsinaceae family [20]. It is also reported to present in the hexane and dichloromethane extracts of *Oxalis erythrorhiza* L. from Oxalidaceae family [21]. Identification of embelin from *in-vivo* and *in-vitro* extracts of *O. corniculata* L. is significantly important. This is the first report of quantification of embelin from *O. corniculata* L.

CONCLUSION: The methanolic extract of *in-vivo* and *in-vitro* showed good antioxidant activities. Non-targeted LC-MS approach for identification of phytochemicals from *Oxalis corniculata* has been explored for the first time in this study. Embelin comes as a prominent antioxidant bioactive which was not reported before from *Oxalis corniculata* L.

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CONFLICT OF INTEREST: Authors would like to declare that they have no conflict of interest.

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