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A Study of Polyphenolic Compounds and *in vitro* Antioxidant Activity of *Trianthema portulacastrum* Linn. Extracts

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ABSTRACT

Background: *Trianthema portulacastrum* Linn. is an exotic plant growing as weed in Africa, Southeast Asia and tropical America. Chemical fingerprinting under the present study was undertaken to characterize the polyphenolic compounds and antioxidant activities present in the plant.

Methods: Several *in vitro* antioxidant assays were performed to evaluate the antioxidant activities in aqueous, alcoholic and hydro-alcoholic extracts. Characterization of polyphenolic compounds was done by HPLC and GCMS analyses.

Result: Total phenols, flavonoids and total antioxidant activity as assessed by DPPH, ABTS and total antioxidant capacity assays was found to be maximum in hydroethanolic extract of the plant. HPLC analysis confirmed the presence of gallic acid, protocatechuic acid, catechin hydrate, vanillic acid, epicatechin, p-coumaric acid, rutin, salicylic acid, myricetin, quercetin and trans-cinnamic acid in plant extracts being maximum in hydroethanolic followed by aqueous, hydromethanolic, ethanolic and then methanolic extracts. Catechin, epicatechin, rutin, myricetin and quercetin were main flavonoid compounds in most of the plant extracts. GCMS analysis also confirmed the presence of several other antioxidants. Based on the findings of the phytochemical analyses and *in vitro* antioxidative studies, it can be clearly established that *Trianthema portulacastrum* has rich amounts of potent polyphenolic and flavonoid compounds having antioxidant properties.

Key words: ABTS, Antioxidant activity, DPPH, Flavonoids, GCMS, HPLC, Polyphenols, *Trianthema portulacastrum*.

INTRODUCTION

Antioxidants are chemical substances derived from natural or synthetic origin, which protect the body from oxidative stress. Studies suggest that antioxidants possess anti-inflammatory, antimutagenic and anti-carcinogenic activities (Hamid *et al*., 2010).

Trianthema portulacastrum Linn. (Aizoaceae family) commonly known as horse purslane is an exotic plant growing as 'weed' in Africa, Southeast Asia and tropical America. The plant has been reported to possess a variety of pharmacological actions, including analgesic, antipyretic, antioxidant, anti-inflammatory, hypolipidemic, hypoglycaemic, antibacterial, antifungal and anticancerous activities.

Polyphenolic compounds are important antioxidants which exhibit chemopreventive actions like antioxidant, anticancer, antimutagenic and anti-infiammatory effects (Huang *et al.,* 2010). Polyphenol rich foods and beverages help to increase plasma antioxidant capacity, hence, consumption of antioxidants is associated with reduced levels of oxidative damage. Oxidative damage to cells is the main pathological disturbance of numerous chronic diseases. Minimizing oxidative damage can be one of the most important approaches to the primary prevention of chronic diseases and ageing-associated health problems. *Trianthema portulacastrum* Linn. displays excellent oxidative stability suggesting the possible presence of phenolic compounds that act as antioxidants.

The present study was aimed to determine and quantify the polyphenolic compounds present in aqueous, ethanolic, hydroethanolic, methanolic and hydromethanolic extracts of *Trianthema portulacastrum* using high performance liquid chromatography (HPLC), to identify the unknown compounds by gas chromatography- mass spectrometry (GCMS) and to estimate the antioxidant properties of different extracts by various methods.

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MATERIALS AND METHODS

Plant material and preparation of extracts

The aerial parts of *T. portulacastrum* were collected before flowering stage from nearby fields of Pantnagar, Uttarakhand

during the months of January to April 2016. The plant was taxonomically identified and authenticated by the Botanical Survey of India (BSI), Dehradun and the voucher specimen of the plant has been deposited in herbarium of BSI, Dehradun with Accession No. 116105. The study was conducted in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture and Technology, Pantnagar.

Five extracts *viz.* aqueous, ethanolic, hydroethanolic, methanolic and hydromethanolic were prepared by extraction in water, ethanol, hydroethanol (1:1), methanol and hydromethanol (1:1), respectively. The extracts were prepared by cold maceration method (Handa *et al*., 2008). The dried extracts were kept in air tight glass containers in deep freeze at -20°C.

Determination of polyphenolic profile of plant extracts by HPLC

A modified method (Zhang *et al.,* 2013) was standardized for detection and quantification of 16 polyphenolic and flavonoid compounds present in plant extracts in a single run. HPLC System (Shimadzu Corporation, Japan) consisting of binary pumps along with UV detector was used. Reverse phase C18 column along with guard column were used. Column temperature was set at 25°C. Loop size of 20 µl was used. Mobile phase consisted of A (3% acetic acid) and B (acetonitrile) with gradient elution at total flow rate of 0.5 ml/min. Gradient time programme for pumps consisted of 0% B up to 11.5 min, 5% B from 11.5 to 19.5 min, 10% B from 19.5 to 32.5 min, 19% B from 32.5 to 45.0 min and 37% B from 45.0 to 60.0 min. Total run time was 60 min.

Pure analytical standards of polyphenolic and flavonoid compounds purchased from Sigma Aldrich, USA were used as reference standards. Working standard solutions (0.001-10 $\mu g.ml^{-1}$) were prepared in mobile phase A. The dissolved plant extracts were filtered through 0.20 µm filters (Millex, 13 mm, nylon) before direct injection into the HPLC system.

Chemical fingerprinting of plant extracts using gas chromatography mass spectrometry (GCMS)

Gas chromatograph mass spectrometer, model GCMS-QP2010 Ultra (Shimadzu Corporation, Japan) equipped with autosampler, autoinjector and Supelco SP-2560 fused silica capillary column was used for the study. The software GC-MS solution ver. 4 was used to analyze mass spectra and chromatograms. The results of mass spectra were compared by making similarity search using NIST11.lib and Wiley8.lib spectral library search programs linked with GC-MS instrument.

Determination of total phenols

Total phenols in plant extracts were quantified using Folin-Ciocalteau reagent method (Singleton and Rossi, 1965) with modifications.

Determination of total flavonoids

Total flavonoids in plant extracts were quantified by aluminum chloride colorimetric method (Zhishen *et al.,* 1999) with modifications.

DPPH radical scavenging assay

DPPH radical scavenging assay was assessed according to the previously described method (Burits and Bucar, 2000) with some modifications.

ABTS radical cation ($ABTS^{\bullet+}$) scavenging assay

The assay was performed as per the previously described method (Re *et al*., 1999) with some modifications.

Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity (TAC) of plant extracts were evaluated according to a previously described method (Prieto *et al*., 1999).

Statistical analysis

Results of the study were analyzed applying one way ANOVA and expressed as Mean±SEM. The differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Fresh plant materials of *Trianthema portulacastrum* after complete drying at 37°C yielded 11.71% dry matter. There was wide variation in percent yield of different extracts ranging from 2.96%, 7.49%, 12.53%, 16.01% and 20.88% as in case of TPE, TPM, TPA, TPHM and TPHE, respectively. It may be due to varying degree of solubility of phytochemicals present in plants extracted in different polarity solvents used in the extraction process. As per a previous study, phytochemical composition, antioxidant and reducing activities of the extracts are positively associated with the use of organic solvents during the extraction process (Ganguli *et al.,* 2018). In our study, the higher yield of hydroethanolic and hydromethanolic extracts supports the findings.

Determination of polyphenolic profile of plant extracts by HPLC

All 16 compounds were eluted within a run time of 60 min in gradient flow (Fig 1). The retention time (RT), coefficient correlation (R^2), limit of detection (LOD) and maximum absorbance wavelength (λ_{max}) have been presented in Table 1. The standardized method for determination of polyphenolic compounds presents good validation parameters like linearity (R^2, 0.999), precision (consistent RT), range and LOD.

Gallic acid, protocatechuic acid, catechin hydrate, vanillic acid, epicatechin, p-coumaric acid, rutin, salicylic acid, myricetin, quercetin and trans-cinnamic acid were estimated to be present in plant extracts being maximum in hydroethanolic followed by aqueous, hydromethanolic, ethanolic and then methanolic extracts. Catechin, epicatechin, rutin, myricetin and quercetin were main flavonoid compounds in most of the plant extracts.

Apart from identified peaks (Fig 2), a good number of peaks were detected with reasonable peak areas indicating presence of a large number of other unknown phytochemicals too, in extracts. Hydroethanolic extract of *T. portulacastrum* contained 1231.97 µg catechin, 245.18 µg epicatechin,

683.62 µg rutin, 1992.14 µg myricetin and 165.37 µg quercetin per gram of extract which was highest among other extracts. A previous study (Al-Sherif and Gharieb, 2011) reported *para*-hydroxybenzoic, vanillic, ferulic, *o*-coumaric, pyrogallic, protocatechuic and *trans*-cinnamic acids in methanolic extract of *T. portulacastrum* which is in accordance with the findings of our study. Another study (Jabbar *et al.,* 2019) supports our findings who reported the presence of five important compounds including caffeic acid (3.17 ppm), gallic acid (3.22 ppm), quercetin (4.11 ppm), cinnamic acid (11.81 ppm) and chlorogenic acid (16.11 ppm) in 70% ethanolic extract of *T. portulacastrum.*

Polyphenolic and flavonoid compounds have been reported to possess antioxidant properties which can help in ameliorating oxidative stress (Ganguli *et al.*, 2018). Plant derived phenolic compounds are safer and promising sources of antioxidants which can be utilized for therapy of various diseases. *Trianthema portulacastrum* Linn. displays excellent oxidative stability suggesting the possible presence of phenolic compounds that act as antioxidants.

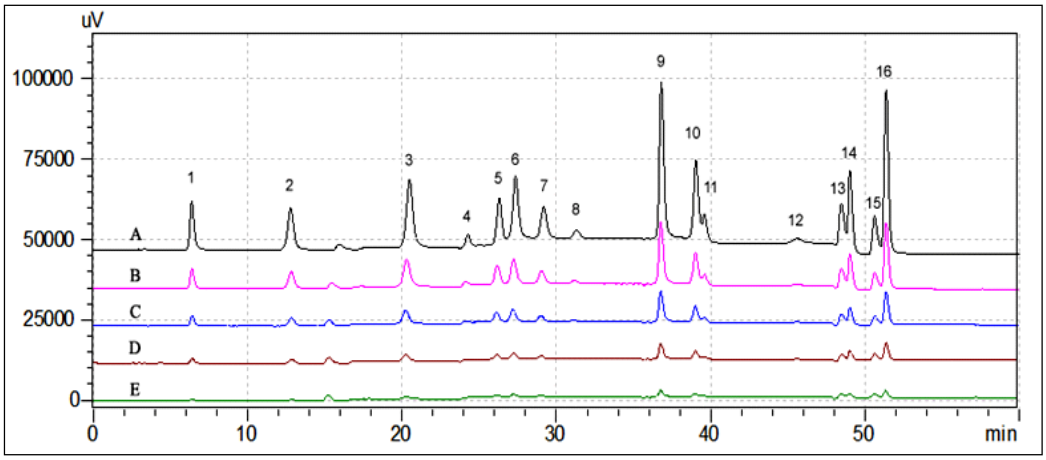

Fig 1: Comparison of HPLC chromatograms (in baseline drifted view) of 2.5, 1, 0.5, 0.25 and 0.10 µg/ml concentrations of 16 polyphenol standards.

1. Gallic acid, 2. Protocatechuic acid, 3. *p*-Hydroxybenzoic acid, 4. Catechin hydrate, 5. Vanillic acid, 6. Caffeic acid, 7. Syringic acid, 8. Epicatechin, 9. *p*-Coumaric acid, 10. *trans*-Ferulic acid, 11. Rutin, 12. Salicylic acid, 13. Myricetin, 14. Resveratrol, 15. Quercetin and 16. *trans*-Cinnamic acid. Order of chromatograms from top to bottom: A - 2.50, B - 1.00, C - 0.50, D - 0.25 and E - 0.10 µg/ml.

Table 1: Retention time (RT), limit of detection (LOD), maximum absorbance wavelength and linearity of 16 standards of polyphenols.

Polyphenols	Peak	RT (min)	λ_{max} (nm)	R^2	LOD (ng/ml)
Phenolic acids					
Gallic acid	1	6.418±0.007	271	0.9999	10
Protocatechuic acid	2	12.844±0.016	260	0.9999	10
p-Hydroxybenzoic acid	3	20.319±0.046	256	1.0000	5
Vanillic acid	5	26.187±0.032	260	0.9982	25
Caffeic acid	6	27.271±0.032	324	0.9998	25
Syringic acid	7	29.058±0.033	275	0.9952	25
p-Coumaric acid	9	36.782±0.008	310	0.9978	5
trans-Ferulic acid	10	39.015±0.009	324	0.9988	10
Salicylic acid	12	45.372±0.144	304	0.9996	100
trans-Cinnamic acid	16	51.356±0.008	278	0.9998	25
Flavonoids					
Catechin hydrate	4	24.150±0.031	278	0.9998	25
Epicatechin	8	31.191±0.032	280	0.9992	50
Rutin	11	39.586±0.007	255	0.9995	10
Myricetin	13	48.474±0.006	374	0.9950	10
Resveratrol	14	49.012±0.007	306	0.9961	25
Quercetin	15	50.610±0.005	370	0.9970	25

Mean±S.E. (n=5).

Chemical fingerprinting of plant extracts using GCMS

The GCMS mass spectral analysis of ethanolic and methanolic extracts of *T. portulacastrum* (Fig 3, 4) revealed the presence of various bioactive compounds like β-sitosterol, stigmasterol, squalene, n-hexadecanoic acid, hexadecanoic acid trimethylsilyl ester, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-, oleic acid, octadecanoic acid, mome inositol, phytol and cis-9-hexadecenal.

Phytosterols are potent antioxidants. These directly inhibit tumor growth by slowing cell cycle progression, inhibition of tumor metastasis and induction of apoptosis (Bradford and Awad, 2007). Squalene, a triterpene which is a key intermediate in the synthesis of plant and animal steroids, possesses antioxidant, chemopreventive activity against colon carcinogenesis (Rao *et al.,* 1998) and skin cancer. Dodecanoic acid, tetradecanoic acid, n-pentadecanoic acid, 9,12-octadecadienoic acid (z,z)- and n-hexadecanoic acid (synonym: palmitic acid) have antioxidant and antimicrobial activities. 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (synonym: α-linolenic acid) which was present in all the extracts in very high amounts is a proven hypocholestaemic agent which reduces the risk of cardiovascular diseases. Studies regarding GCMS spectral analysis of various extracts of *T. portulacastrum* are very less.

Determination of total phenols and flavonoids of *T. portulacastrum* extracts

The total phenol content ranged from 54.43±3.14 to 106.51±3.09 mg GAE/g extract with lowest in aqueous and highest in hydroethanolic extract. Hydromethanolic, methanolic and ethanolic extracts also exhibited significant phenol content. The total flavonoid content varied from 4.24 ±0.33 to 20.93 mg rutin equivalent/g extract. Aqueous extract exhibited significantly ($p<0.05$) lower content, moderate in ethanolic and methanolic with highest in hydroethanolic extract.

Several phenolic compounds identified in our study have been reported to possess strong antioxidant activity. Gallic acid induces apoptosis which is associated with ROS mediated oxidative stress, mitochondrial dysfunction and an increased intracellular Ca^{2+} level (Inoue *et al.,* 2000). It is a powerful antioxidant and has been considered a useful phytochemical for cancer chemoprevention. It is used as a reference standard for determining total phenol content in plant extracts and other analytes. Protocatechuic acid, vanillic acid, catechins, rutin, quercetin and myricetin have been reported to contain excellent antioxidant properties. Majority of the flavonoids exhibit strong antioxidant activity (Al-Sherif and Gharieb, 2011).

DPPH radical scavenging assay of plant extracts

DPPH radical scavenging activity of different extracts of *T. portulacastrum* expressed in terms of ascorbic acid equivalents has been presented as Table 2. The antioxidant activity measured in ascorbic acid equivalents for TPA, TPHE, TPHM, TPE and TPM extracts were found to be 44.45±0.58, 92.68±2.65, 54.64±0.84, 25.52±0.90 and 39.26±0.79 mg AAE/ g of extract, respectively, which differed significantly ($p<0.05$) among themselves.

$ABTS^{\bullet+}$ scavenging assay of plant extracts

$ABTS^{\bullet+}$ scavenging activity of different extracts of *T. portulacastrum* expressed in terms of ascorbic acid equivalents has been presented as Table 2. IC_{50} value was found to be 17.06±0.35 µg/ml for ascorbic acid. IC_{50} values for TPA, TPHE, TPHM, TPE and TPM extracts were found to be 122.16±2.12, 76.97±1.16, 87.29±0.70, 190.51±5.39 and 131.18±1.74 µg/ml, respectively, which differed significantly ($p<0.05$) among themselves. Lowest IC_{50} value of 76.97±1.16 µg/ml was obtained for TPHE whereas highest was for TPE (190.51±5.39 µg/ml) extract. AAE for TPA, TPHE, TPHM, TPE and TPM extracts were found to be 139.82±5.29, 221.77±6.64, 195.50±5.44, 89.60±1.41 and 130.10±3.26 mg AAE/g extract.

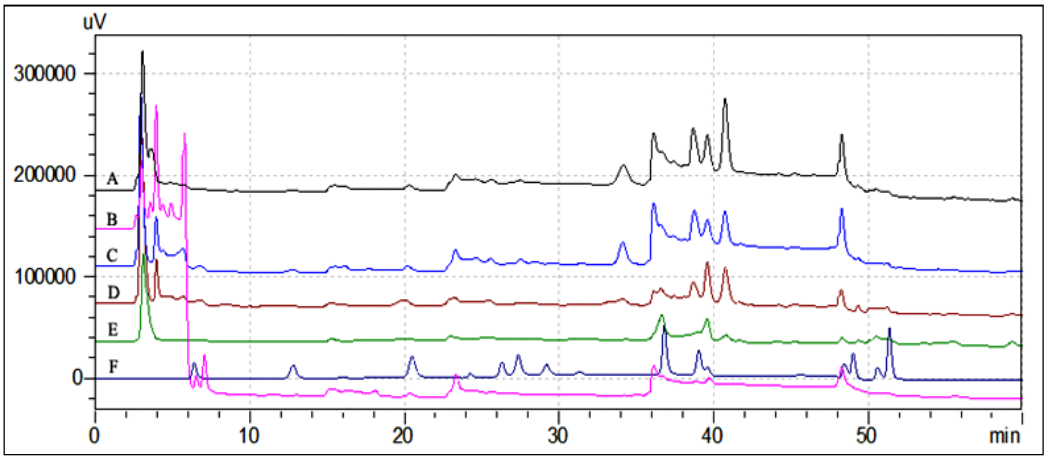

Fig 2: Comparison of HPLC chromatograms of different extracts of *Trianthema portulacastrum* and standards of 16 polyphenols. Order of chromatograms from top to bottom: A - TPHE, B - TPA, C - TPHM, D - TPM, E - TPE and F - standards of 16 polyphenols.

Total antioxidant capacity (TAC) of plant extracts

Total antioxidant capacity (TAC) determined by phosphomolybdenum method for different extracts of *T. portulacastrum* has been presented in Table 2. The antioxidant capacity was expressed in terms of milligram of ascorbic acid equivalent (AAE) per gram of extract.

TAC for various extracts of *T. portulacastrum* ranged from 56.79±2.94 to 103.51±4.50 mg AAE/g extract with highest value in hydroethanolic and least in aqueous extract. Hydromethanolic, ethanolic and methanolic extracts accounted moderate activity of 85.84±3.35, 61.39±2.22 and 57.61±1.83 mg AAE/g extract, respectively.

There is direct relationship of AAE with the antioxidant capacity whereas, there is inverse relationship between IC_{50} value and antioxidant activity. IC_{50} value of ascorbic acid with DPPH assay in the present study was found to be 4.49 ±0.08 µg/ml which is similar to the IC_{50} value of 5.94±0.28 µg/ml as reported by a previous study (Chludil *et al.,* 2008). IC_{50} values for TPA, TPHE, TPHM, TPE and TPM extracts were found to be 101.05±1.63, 48.51±1.00, 82.20±0.74, 176.24±4.08 and 114.45±1.85 µg/ml, respectively. Lowest IC_{50} value was obtained for TPHE whereas highest was for TPE extract indicating that hydroethanolic extract was having best antioxidative activity whereas ethanolic extract proved the least. A previous study (Badmanaban *et al.,* 2010) reported similar IC_{50} values of 97.89 and 166.67 µg/ml for methanolic and aqueous extracts, respectively of *T. portulacastrum.* Another study (Yaqoob *et al.,* 2014) reported the antioxidant activities of *T. portulacastrum* hydrolysates and found maximum activity in shoot followed by root and leaves which supports the selection of aerial (shoot) parts of the plant in our study.

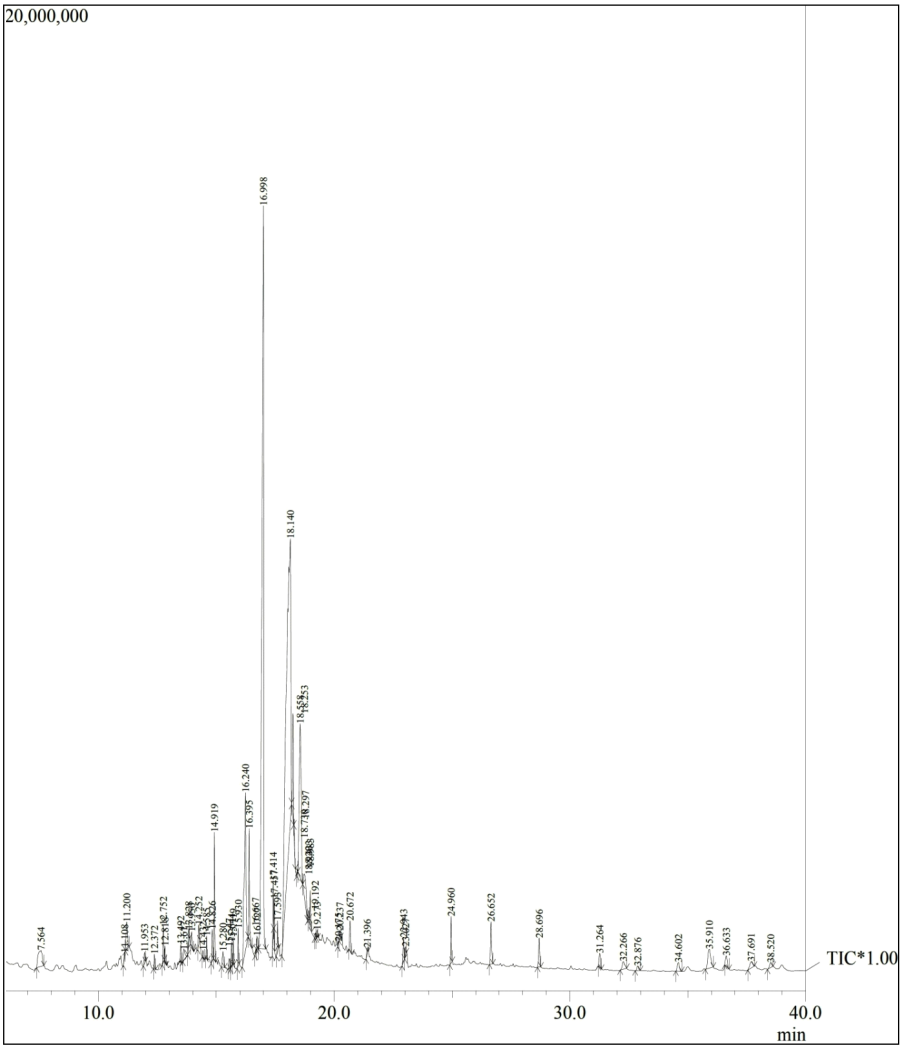

Fig 3: GCMS total ion chromatogram (TIC) of ethanolic extract of *Trianthema portulacastrum*.

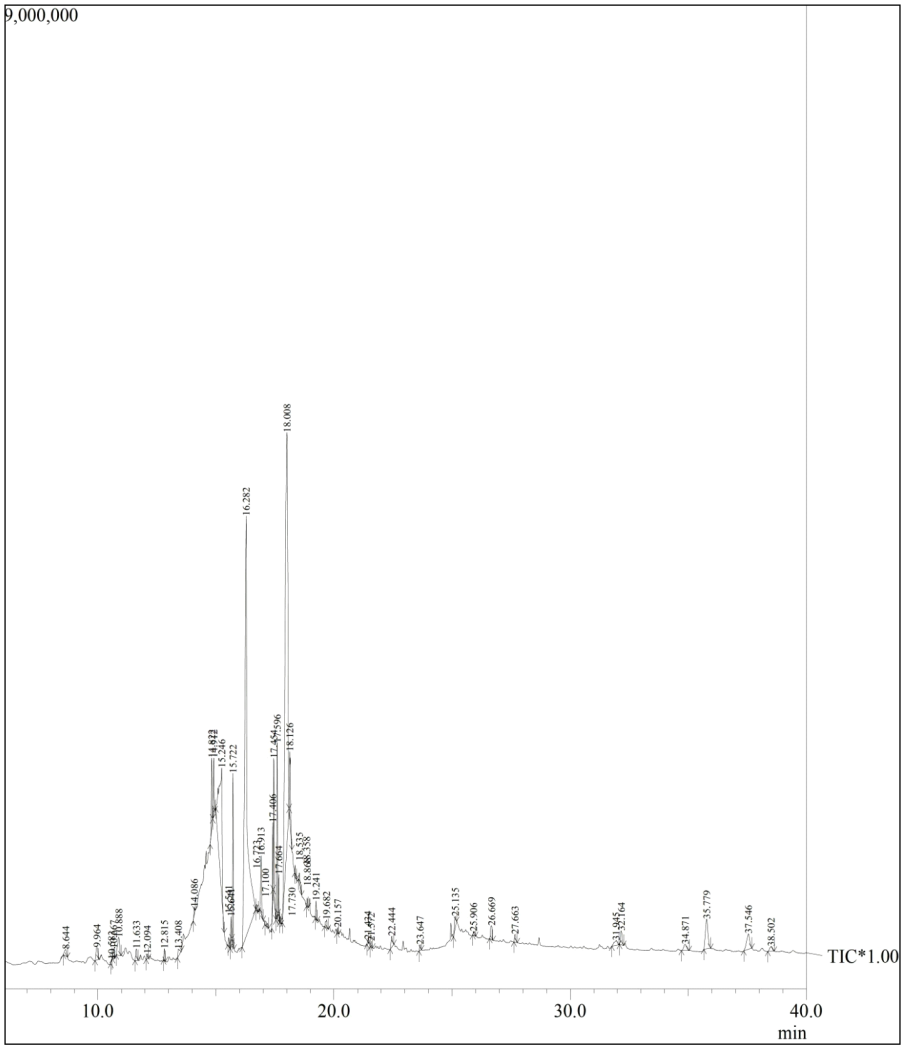

Fig 4: GCMS total ion chromatogram (TIC) of methanolic extract of *Trianthema portulacastrum*.

Table 2: Antioxidant properties, total phenols and flavonoids content of Aqueous (TPA), Ethanolic (TPE), Hydroethanolic (TPHE), Methanolic (TPM) and Hydromethanolic (TPHM) extracts of *Trianthema portulacastrum*.

Samples	DPPH	$ABTS^{\cdot+}$	TAC	Total phenols	Total flavonoids
	mg AAE/g E	mg AAE/g E	mg AAE/g E	mg GAE/g E	mg RE/g E
TPA	44.45±0.58^{a}	139.82±5.29^{a}	56.79 ±2.94^{a}	54.42±3.14^{a}	4.24±0.33^{a}
TPE	25.52±0.90^{b}	89.60±1.41^{b}	61.39±2.22^{a}	62.48±2.43^{a}	8.01±0.44^{b}
TPHE	92.68±2.65^{c}	221.77±6.64^{c}	103.51±4.50^{b}	106.51±3.09^{b}	20.93±0.75^{c}
TPM	39.26±0.79^{a}	130.10±3.26^{a}	57.61±1.83^{a}	76.74±2.60^{c}	10.00±0.61^{b}
TPHM	54.64±0.84^{d}	195.50±5.44^{d}	85.84±3.35^{c}	81.88±2.31^{c}	13.31±0.82^{d}

Mean±SEM (n=3) values bearing different superscripts differ significantly within column ($p<0.05$).

mg AAE/g E= mg Ascorbic acid equivalent/gm of plant extract.

mg GAE/g E= mg Gallic acid equivalent/gm of plant extract.

mg RE/g E= mg Rutin equivalent/gm of plant extract.

CONCLUSION

Based on the findings of the phytochemical analyses and *in vitro* antioxidative studies, it can be clearly established that *Trianthema portulacastrum* has rich amounts of potent polyphenolic and flavonoid compounds having antioxidant properties. The presence of important polyphenols and flavonoids like rutin, gallic acid, protocatechuic acid, quercetin and myricetin in different extracts further affirms strong antioxidant potential. Hence, there is need to sub-fractionate the hydroethanolic extract of the plant for identification of novel compounds having distinct pharmacological properties.

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Conflict of interest

The authors declare that they have no conflict of interests.

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