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Antioxidant Potentiality and HR-LCMS Profiling of Drought Tolerance Inducing Compounds from Groundnut Root Extracts

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ABSTRACT

Background: In crop plants drought tolerance is influenced by the activation of acclimation programs, genotype-specific traits and the antioxidant system's response to stress intensity. The objective of the present study was to identify drought tolerance eliciting and antioxidant metabolites of the groundnut (genotype GKVK-5) roots sequential extracts by performing *in vitro* antioxidant assays.

Methods: The soxhlet method was used to prepare groundnut root extracts sequentially. After that, a phytochemical examination both qualitative and quantitative was conducted. HR-LCMS analysis was performed and the compounds were identified by comparing their retention time, mass, abundance area and m/z cloud best match in the Metlin Library. *In vitro* antioxidant activity was determined using assays for total antioxidant capacity, reducing power, DPPH radical scavenging, ABTS radical scavenging, metal chelating ability and nitric oxide radical scavenging.

Result: HRLCMS analysis of ethyl acetate extract (REE) revealed antioxidant phenolic components, including gallic acid, quinic acid, azelaic acid, ferulic acid and phenylalanine. Methanol extract (RME) was shown to include bioactive compounds such as kaempferol 7-o-glucoside, naringenin 7-o-glucoside, resveratrol and L-2-amino-3-methylenehexanoic acid. Significant total antioxidant activity (194 µg/mg ascorbic acid) and high percentages of oxidative free radical inhibition in DPPH (63.69±2.85%), ABTS (71.31±2.09%) and metal chelating ion radical scavenging (66.31±5.56%) assays were demonstrated by the REE and these results are consistent with its total phenolic content (173.08 µg/mg). In accordance with its total flavonoid concentration (159.71 µg/mg), the RME showed remarkable reducing power (464 µg/mg) and efficient reduction of hydroxyl oxide radical (51.07±3.19%) and nitric oxide radical scavenging (67.08±5.97%). This study suggests that the drought tolerance characteristic of groundnut genotype GKVK-5 may be influenced by the combined effects of antioxidants and the total reductive capacity of metabolites.

Key words: Antioxidant metabolites, Drought tolerance, Groundnut, Root extracts.

INTRODUCTION

Arachis hypogaea L., commonly known as groundnut, is a cash crop and essential oilseed that is grown in semiarid zones (Ravi *et al.,* 2023). Drought stress is the major abiotic constraint affecting groundnut productivity and quality worldwide. Drought stress is a significant environmental challenge that leads to crop yield reductions, with the extent of the loss influenced by factors such as timing, intensity, duration and local conditions like temperature, salinity and light intensity (Zhen *et al.,* 2022). Heat stress poses a significant challenge to legume cultivation, leading to substantial yield losses (Manonmani *et al.,* 2024; Mahantesh Sujata *et al.,* 2018). Elevated temperatures induce a cascade of detrimental effects, including disruptions to plant morphology, anatomy, physiology and biochemistry. These changes ultimately hinder growth and development. Photosynthesis and respiration are particularly sensitive to heat stress, resulting in a decline in yield across many crop species (Bita and Gerats, 2013; Rehman *et al.,* 2021; Zahra *et al.,* 2023). To mitigate heat stress, plants employ various adaptive mechanisms includes maintaining membrane stability, scavenging reactive oxygen species (ROS), producing antioxidants, accumulating and adjusting compatible solutes, activating mitogen-activated protein, kinase (MAPK) and calcium-dependent protein kinase (CDPK) signaling pathways, modulating chaperone signaling and transcriptional activation. These mechanisms collectively enable plants to cope with the adverse effects of high temperatures (Wahid *et al.,* 2007).

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Heat stress can also cause oxidative stress and the production of activated oxygen species (AOS), such as singlet oxygen (1-O_2), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), which are signs of cellular damage brought on by high temperatures (Liu and Huang, 2000). Heat stress can also cause tissue dehydration. Numerous researchers have assessed the groundnut pods nutritional value and phytochemical composition. There is little information available about the medicinal properties and bioactive components of its roots, especially in relation to treating cancer and liver problems. The traditional practitioners of Challakere, Chitradurga District, Karnataka State are using the tap root of groundnut to cure early problems of liver cancer. Further, to discover the functionally effective bio-actives and make it easier to comprehend their impact on the target, it is essential to decipher the intricate chemistry of bioactive crude extracts utilizing HRLC-MS techniques (Barba-Ostria *et al.,* 2022).

Previously, we investigated the effect of sowing windows *viz.,* II fortnight of June, I fortnight of July, II fortnight of July and I fortnight of August, 2017, on the growth and yield of groundnut genotypes *viz.,* GKVK-5, GPBD-4, G2-52 and TMV-2 cultivated in drought conditions (Raagavalli *et al.,* 2019). The experimental results revealed that, the genotype GKVK-5 recorded significantly higher pod yield (16.73 q ha^{-1}), shelling percentage and kernel yield. While, the crop sown during II fortnight of June recorded significantly higher pod yield, shelling percentage and kernel yield compared to delay in sowing. The evaluation of heat use efficiency and identification of drought tolerance eliciting metabolites were also identified in the leaf methanol extract of genotype GKVK-5 (Raagavalli *et al.,* 2024). The results showed that GKVK-5 had a total dry matter of over 18.75g per plant, with an increased HUE of 14.56 $g^oC\ day^{-1}$ at 90 days after sowing. Higher HTUE (2.258 q ha^{-1} HTU^{-1}) resulted in more pod output (16.73 q ha^{-1}) and haulm yield (26.99 q ha^{-1}) compared to TMV 2 (10.48 q ha^{-1} and 15.07 q ha^{-1}). The increase in HUE demonstrates GKVK-5's tolerance to drought stress. HR-LCMS profiling of leaf methanol extract revealed the identification of drought tolerance-eliciting phytochemicals, such as 2- Amino-3-methylhexanoic acid, 6-Deoxyfagomine Maltoxazine and 2-Furanyl pyrrolidine, in cation mode. Antioxidant flavonoids Astragalin 7-rhamnoside, Naringenin 7-O-glucoside and Phenolics, Rutin, Gallic acid and quinic acid were detected in anion mode with high DB hits and retention time (Raagavalli *et al.,* 2024). The aim of this investigation was to evaluate in vitro antioxidant activities and to identify antioxidant metabolites and drought tolerance-inducing components in sequential root extracts of groundnut (genotype GKVK-5).

MATERIALS AND METHODS

Cultivation of groundnut crop

The groundnut genotype namely, GKVK-5, seeds were sown at a depth of 5 cm with 30 × 15 cm spacing and the field experiment was conducted under rain-fed conditions during the *Kharif* season on sandy loam soils of the University of Agricultural and Horticultural Sciences, Shivamogga (UAHS), Bavikere, University of Agricultural and Horticultural Sciences, Shivamogga, Karnataka State, India (13°42′ latitude and 75°51′ longitude and 695 m above MSL). The experiment was laid out in a randomized complete block design with a factorial concept reported by (Gomez and Gomez 1984). The recommended dose of fertilizers (25: 50: 25 kg N: P_2O_5: K_2O) was applied as a basal dose (50% N).

Preparation of root extracts

Groundnut root material was collected during the pod harvesting, cleaned under running water, allowed to air dry in the shade, manually pulverized and 250 g was utilized for serial soxhlet extraction over the course of 48 hours using the solvents petroleum ether, ethyl acetate and methanol. A rotary flash evaporator (Buchi, Flawil, Switzerland) was used to remove the solvent under vacuum. The extracts that were left behind were labeled RPE (Root Petroleum Ether Extract), REE (Root Ethyl Acetate Extract) and RME (Root Methanol Extract) and the yield was expressed as a percentage of the weight of the powdered root.

Qualitative phytochemical screening

The desiccator dried residue obtained from each of the extracts were resuspended in Milli-Q water used for qualitative phytochemical examination to identify alkaloids, flavonoids, terpenoids, glycosides and phenols using established standard tests (Kokate *et al.,* 1999).

Quantitative determination of total phenols and flavonoids

The total phenolic content of RPE, REE and RME was determined using the method described by Matthaus (2002). The total flavonoid content was determined according to the modified method of (Zhishen *et al.,* 1999).

HR-LCMS analysis of root sequential solvent extracts

The profiling of bioactive compounds was analyzed using a high-resolution liquid chromatograph mass spectrometer (HR-LCMS) instrument from Agilent Technologies. A needle wash was used to inject 5 µl of RPE, REE and RME into an Agilent ultra-high performance liquid chromatography system equipped with a Hypersil Gold column (C18 100 × 2.1 mm^{-3} MICRON). For the MS analysis, a capillary voltage of 3,500 V, nebulizer pressure of 35 psi, gas temperature of 250°C and drying gas flow of 13 l/minute were utilized. The chromatography method employed ESI 10032014_ MSMS.m for 30 minutes. The HR-LC-MS analysis was conducted at the SAIF, Indian Institute of Technology, Mumbai, India, using both positive and negative ionization modes of the ESI. The compounds were identified by comparing their retention time (RT), mass, abundance area and m/z cloud with the stored Metlin Library available at IIT Bombay. Agilent Mass Hunter software was used for the mass spectrometric analysis.

In vitro antioxidant activity

Determination of total antioxidant and reducing power

The total antioxidant activity was determined by the Phosphomolybdenum method (Prieto *et al.,* 1999). The total reduction capability was determined according to the method of Oyaizu, (1986).

In vitro free radical scavenging assays

The *in vitro* free radical scavenging activity of extracts (RPE, REE and RME) and the leaves sequential solvent extracts (LPE, LEE and LME) were measured using the following methods:

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) by the method of Blois, (1958). Metal chelating activity was determined according to the method of Dinis *et al.* (2016). ABTS (2, 2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) radical scavenging activity according to the method of Arnao *et al.* (2001). Hydroxyl radical scavenging activity was determined according to the method of Klein *et al.* (1981). Nitric oxide radical scavenging activity was determined according to Marcocci *et al.* (1994). The % inhibition of radicle scavenging activity was calculated using the formula:

$$\frac{[(A_{control} - A_{test})}{A_{control}]} \times 100$$

Where,

$A_{control}$ = Absorbance of the control reaction.

A_{test} = Absorbance of the extract reaction.

The IC_{50} value was calculated using the formula:

$$IC_{50} = \frac{\Sigma C}{\Sigma I} \times 50$$

Where

ΣC = Sum of extracts and pure compound concentrations used to test.

ΣI = Sum of the percentage of inhibition at different concentrations.

Satstical analysis

The statistical analysis was performed by one-way ANOVA in Graphpad prism Software. The results were expressed as mean ± SEM.

RESULTS AND DISCUSSION

Quantitative determination of total phenols and flavonoids

It was found that the yields of the root extracts in methanol and ethyl acetate were 76.5 g and 48.5 g/kg of the dried mass, respectively. Alkaloids, flavonoids, phenolics and terpenoids were among the many elements found in groundnut roots and leaves sequential soxhlet extracts, according to phytochemical analysis (Table 1). The results of the quantitative screening of root extracts indicated that REE contained a high concentration of total phenols (173.08 µg/mg). On the other hand, RME had a higher total flavonoid concentration (159.71µg/mg). Gundaraniya *et al.,* (2020) have examined the metabolomic profiling of genotypes of susceptible and drought-tolerant peanuts (*A. hypogaea* L.) in response to drought stress.

HRLCMS profiling of groundnut root sequential extracts

The antioxidant metabolites RPE, REE and RME are profiled by HRLCMS in both positive and negative ionization modes of spectra (Fig 1). Table 2 displays the compounds that show the best match with the stored Metlin library in both cation and anion mode in terms of retention time, mass, abundance area and m/z cloud. In human liver cancer cells, sedimentanolide induces autophagy through the PI3K, p53 and NF-κB signaling pathways (Hsieh *et al.,* 2015). Sedanolide expressed with best match m/z cloud findings in cation mode spectra of RPE reported for antioxidant property (Momin and Nair, 2002). In Challakere, Chitraduga District, Karnataka, we also observed that the traditional healer was using groundnut root extract to treat the early stages of liver cancer.

L-phenylalanine,N-(1-Deoxy-1-fructosyl) phenylalanine and soyasaponin III were expressed with the highest m/zcloud values in the cation mode spectra of REE. According to Ramzan, (2023) phenylalanine is an important and useful amino acid that can mitigate the negative effects of drought stress by modifying plant development, photosynthesis and the antioxidant defense system. According to Diverekar *et al.* (2022) it functions as the precursor to several compounds that are important for plant development, reproduction, defense against abiotic and biotic stressors,

Table 1: Qualitative analysis and quantitative determination of phytochemicals.

	Qualitative tests			Quantitative determination (µg/mg)		
	RPE	REE	RME	RPE	REE	RME
Alkaloids	-	+	+	-	82.41	42.79
Flavonoids	-	+	+	-	127.59	159.71
Phenolics	-	+	+	-	173.08	142.28
Terpenoids	+	+	-	89.82	34.06	-

RPE-Roots petether extract; REE- Root ethyl extract; RME- Roots methanol extract; (+) indicate the presence; (-) the absence of the respective phytochemical classes.

including anthocyanins, salicylate, lignin and phenyl propanoids. The phenolic molecules gallic acid, quinic acid, azelaic acid and ferulic acid, as well as the flavonoid antioxidants formononetin and quercetin, were shown to have highly abundant m/z values in the anion mode spectra of REE antioxidants.

The cation mode spectra of RME revealed the presence of drought tolerance, eliciting metabolite L-2-amino-3-methylenehexanoic acid and a polyphenol compound resveratrol in repeated folds indicating its synthesis in the groundnut genotype GKVK-5, during drought stress conditions. L-2-Amino-3-methylenehexanoic acid is an endogenous (2S, 3S)-α-amino acid that occurs naturally in several plant species and has potent physiological activity in addition to enhancing resistance against extreme temperature stresses (Yang *et al.,* 2023). Groundnut roots are the source of the polyphenol molecule resveratrol, which has been shown to possess anti-inflammatory and antioxidant qualities that guard against conditions including diabetes, Alzheimer's disease and cancer. Resveratrol has anti-inflammatory properties that make it a useful treatment for skin inflammation and arthritis (Tuyen *et al.,* 2013). The methanol extract contained the phenolic acids isoferulic acid and quinic acid, as well as the well-known antioxidant flavonoids quercitin, kaempferol, kaempferol 7-o-glucoside and naringenin 7-o-glucoside. The drought-tolerant eliciting metabolites of RME and REE, as well as their structure and MS spectra, are displayed in Fig 2 ABCD and EFGH, respectively and were captured from the HRLCMS Metlin library.

Determination of total antioxidant and reducing potentialities

One of the main processes that produces free radicals in food, medications and even living systems is oxidation. Numerous studies (Ru *et al.,* 2019; Zhang *et al.,* 2018) have found a linear relationship and strong association between the antioxidant activity of plant extracts and their phenolic acid concentration. Fig 3A and 3B demonstrate the groundnut root extracts' total antioxidant and reducing potential. The total oxidant potentiality was higher in REE (194 µg/mg ascorbic acid), according to the data and this could be because phenolic acids were present in higher concentration (173.08 µg/mg). Conversely, RME had a higher overall reducing power (464 µg/mg ascorbic acid), which could be attributed to the higher flavonoid concentration (159.71 µg/mg).

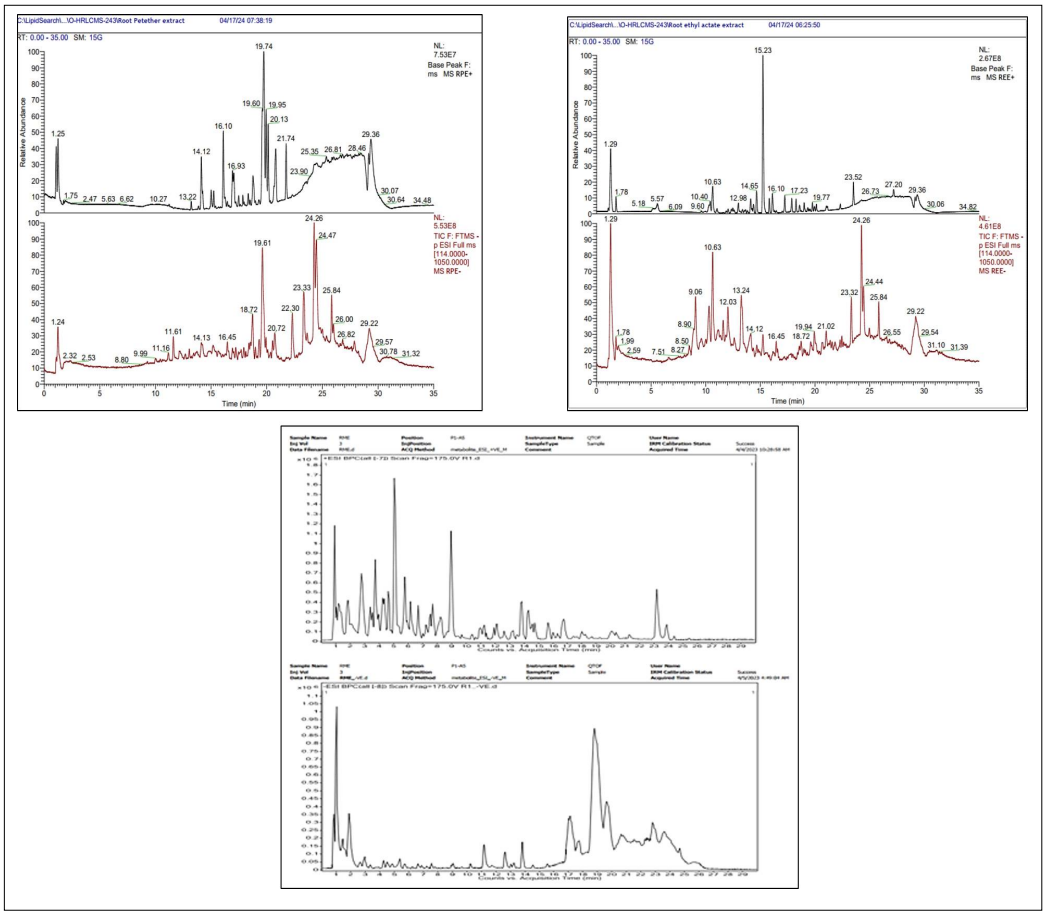

Fig 1: HRLCMS analysis of RPE, REE and RME showing positive and negative ion modes of spectra.

Table 2: Identification of cation and anion compounds from HRLCMS analysis of groundnut root sequential extracts.

Sequential soxhlet extracts	Compound name	Formula	Calc. MW	RT [min]	Area (Max.)	Chem spider results	Mz cloud best match
			Cation compounds				
Petroleum ether	Sedanolide	$C_{12}H_{18}O_2$	194.13	19.60	25406167	149	46.9
	Erucamide	$C_{22}H_{43}NO$	337.30	24.52	58789452	13	41.8
	Calcitriol	$C_{27}H_{44}O_3$	416.32	19.92	5510988	23	40.3
Ethyl acetate	L-Phenylalanine	$C_9H_{11}NO_2$	165.08	10.64	111024898	20	88.4
	N-(1-Deoxy-1- fructosyl) phenylalanine	$C_{15}H_{21}NO_7$	327.12	2.961	111024898	20	88.4
	Damascenone	$C_{13}H_{18}O$	190.14	17.38	6358613	8	79.3
Methanol	Tranexamic acid	$C_8H_{15}NO_2$	157.11	1.39	1148132	26	99.9
	Tranexamic acid	$C_8H_{15}NO_2$	157.11	1.39	452074	14	98.5
	L-2-Amino-3-methylene hexanoic acid	$C_7H_{13}NO_2$	143.09	1.86	265992	11	96.7
	L-2-Amino-3-methylene hexanoic acid	$C_7H_{13}NO_2$	143.09	1.86	65992	7	89.9
	Zapotin	$C_{19}H_{18}O_6$	342.11	1.44	54922	6	88.5
	(Z)-Resveratrol 3-(3″ sulfoglucoside)	$C_{20}H_{22}O_{11}S$	470.08	23.90	36928	3	86.7
	Soyasaponin III	$C_{42}H_{68}O_{14}$	796.46	15.63	21230629	11	86.1
			Anion compounds				
Petroleum ether	Octadecanedioic acid	$C_{30}H_{48}O_4$	472.35	19.19	1682182039	10	98.3
	Traumatic acid	$C_{18}H_{30}O_3$	294.22	18.49	10297557	19	94.4
	Oleic acid	$C_{18}H_{34}O_2$	282.26	17.24	9165094	35	54.4
Ethyl acetate	Formononetin	$C_{16}H_{12}O_4$	268.07	14.65	107891426	34	99.7
	Gallic acid	$C_7H_6O_5$	170.02	2.41	69359020	3	99.6
	Azelaic acid	$C_9H_{16}O_4$	188.10	11.60	38762651	56	99.3
	Quercetin	$C_{15}H_{10}O_7$	302.04	12.65	22978168	5	98.9
	Ferulic acid	$C_{10}H_{10}O_4$	194.05	9.01	10048461	50	66.6
Methanol	Kaempferol	$C_{15}H_{10}O_6$	286.04	0.975	770400	32	91.4
	Epicatechin gallate	$C_{22}H_{18}O_{10}$	442.09	10.38	153121	10	86.6
	Kaempferol 7-O-glucoside	$C_{21}H_{20}O_{11}$	448.10	17.98	149209	10	84.7
	Naringenin 7-O-glucoside	$C_{21}H_{22}O_{10}$	434.12	5.35	125623	9	83.1
	Astragalin 7- rhamnoside	$C_{27}H_{30}O_{15}$	594.15	6.16	353645	4	83.1
	Quinic acid	$C_7H_{12}O_6$	192.06	1.39	9960	4	82.7

Table 3: *In vitro* antioxidant potentials of groundnut root sequential extracts.

Antioxidant activity	Extracts µg/mL			% of Inhibition			IC_{50} Value			
	RPE	REE	RME	RPE	REE	RME	RPE	REE	RME	Std
										Ascorbic acid
	100	100	50	11.69±1.21	19.69±3.84	30.15±1.00	551.62	405.20	197.622	124.87
	200	200	100	15.21±1.72	28.00±1.31	39.22±2.25				
DPPH	300	300	150	27.83±2.54	36.75±3.74	44.47±2.43				
	400	400	200	33.52±2.58	44.83±1.78	48.63±2.71				
	500	500	250	47.81±2.72	63.69±2.85	54.55±1.19				
	25	25	10	8.48±0.98	13.19±1.87	11.34±1.23	131.36	93.020	46.2751	11.93846
	50	50	20	13.07±1.48	24.77±3.67	19.60±3.59				
ABTS assay	75	75	30	25.61±2.25	38.39±2.45	31.30±0.93				
	100	100	40	35.07±2.68	51.16±3.97	44.49±3.50				
	125	125	50	45.96±2.92	71.31±2.09	54.09±4.50				
Hydroxyl radical	100	100	50	2.96±0.28	3.01±0.75	3.19±0.53	642.26	586.83	253.183	164.80
scavenging assay	200	200	100	7.91±1.08	9.76±1.39	15.21±1.21				
	300	300	150	18.98±1.55	21.39±1.81	29.98±1.97				
	400	400	200	25.32±2.75	29.84±2.61	37.08±2.27				
	500	500	250	44.09±2.99	47.02±3.09	51.07±3.19				
	200	200	200	11.27±0.99	14.09±1.21	19.28±1.15	1014.97	943.74	706.36	317.61
Nitric oxide radical	400	400	400	19.51±1.71	22.51±1.33	31.97±1.01				
scavenging activity	600	600	600	27.39±3.29	30.65±2.76	47.61±3.66				
	800	800	800	39.44±3.01	41.59±2.09	53.69±3.18				
	1000	1000	1000	50.43±4.38	53.91±3.28	67.08±5.97				
										EDTA
Metal chelating	250	250	200	9.32±1.18	20.46±1.10	12.56±0.85	1280.74	824.63	1113.38	406.45
	500	500	400	20.27±2.04	34.51±1.94	26.51±3.01				
	750	750	600	29.04±2.95	51.44±4.45	33.78±3.03				
	1000	1000	800	42.38±3.03	62.47±3.20	48.20±1.92				
	1250	1250	1000	46.29±4.03	66.31±5.56	52.53±4.06				

* RPE-Roots petroleum ether Extract; REE- Root ethylacetate extract; RME- Roots methanol extract.

Standard reference used- Ascorbic acid for DPPH, ABTS, Hydroxyl radical and nitric oxide radical assays; EDTA for metal chelating assay. Values are represented as mean± standard error, N=3.

Free radical scavenging activities

Reactive oxygen species (ROS) are byproducts of regular cell metabolism that function as both useful and harmful organisms (Valko *et al.,* 2006). The physiological functions of ROS in cellular responses to hypoxia, such as defense against pathogenic agents and the development of a mitogenic response to biotic and abiotic stressors, are among the beneficial effects of ROS that occur at low to moderate concentrations. The RPE, REE and RME in vitro free radical scavenging experiment was conducted in a dose-dependent fashion. Table 3 displays the percentage of free radical inhibition as well as the IC_{50} values. The results of our tests indicate that among the three consecutive extracts of groundnut root tested, REE demonstrated significant quenching of free radicals in DPPH radical scavenging (63.69±2.85%), ABTS radical scavenging (71.31%±2.09%) and Metal ion chelating (66.31±5.56) activities. This was followed by RME, which showed significant inhibition of free radicals in the above assays, with percentages of 54.55±1.19%, 54.09%±4.50% and 52.53±4.06%, respectively. The percentage of inhibition of free radical was significant in the RPE tested assays. Our

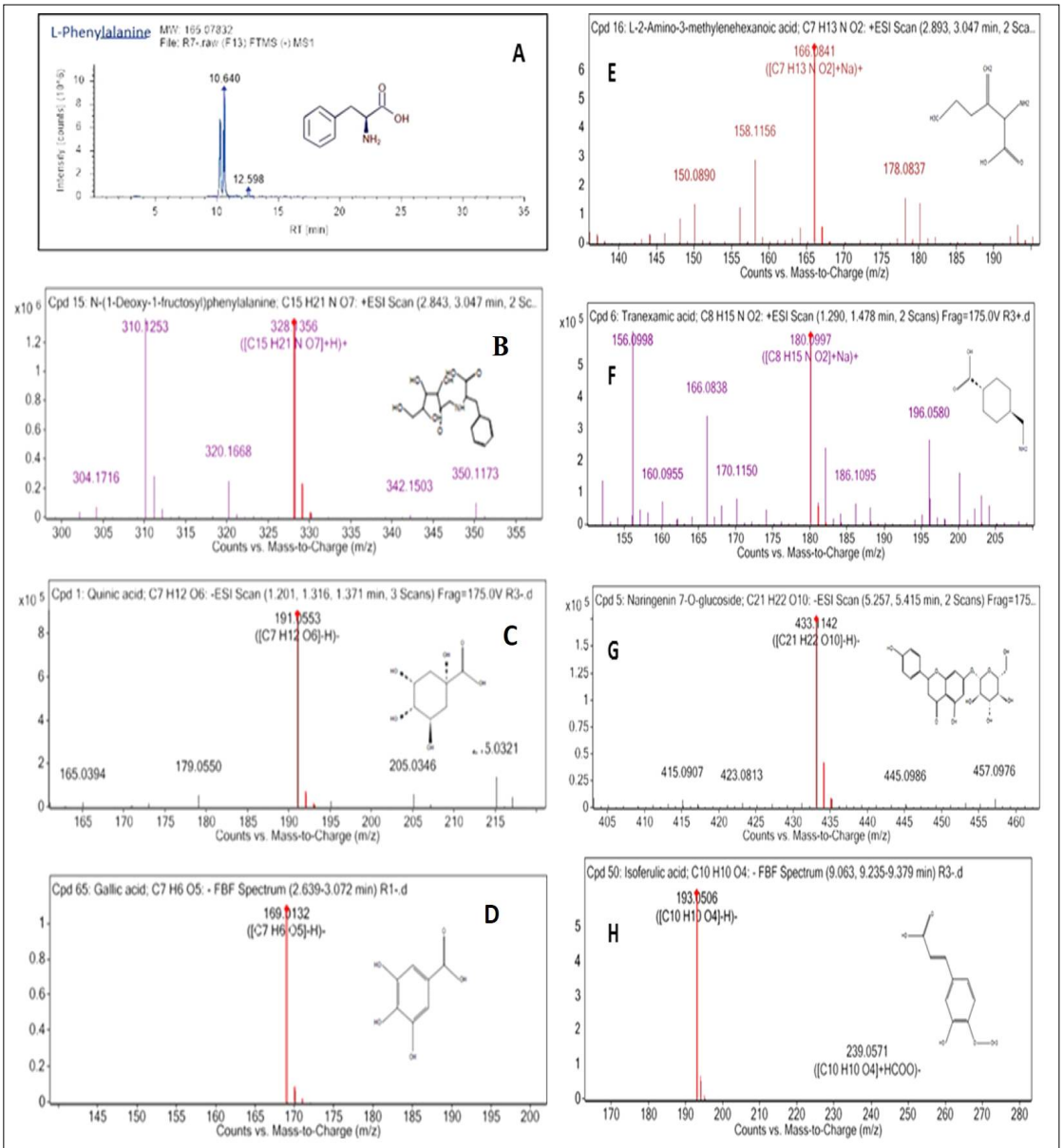

Fig 2: MS spectrum and structure of antioxidant and drought stress tolerant eliciting molecules of groundnut (genotype GKVK-5) roots.

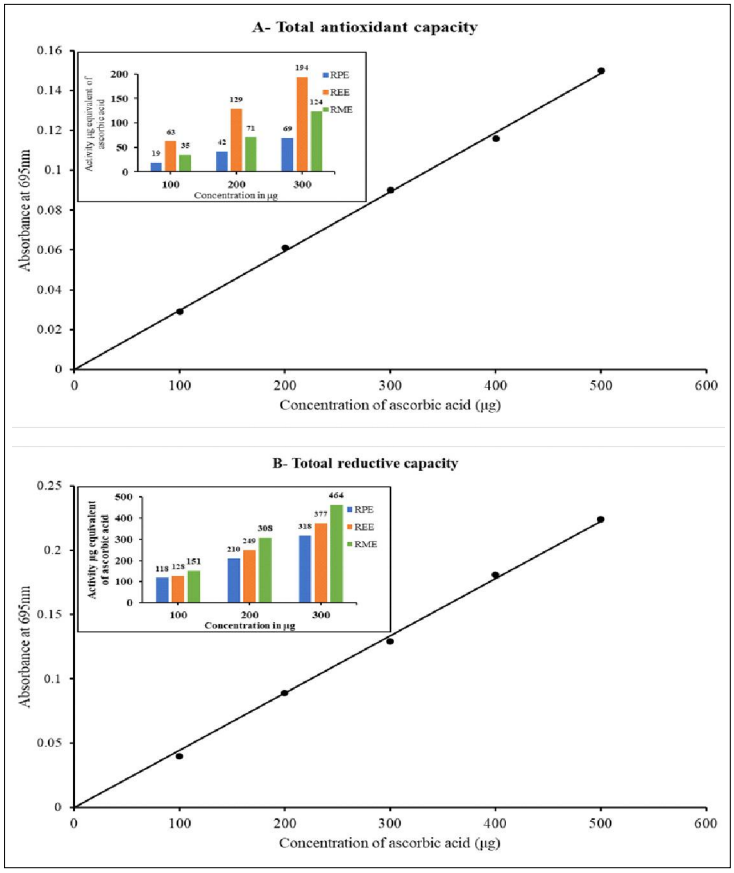

Fig 3: Total antioxidant and total reductive capacity of RPE, REE and RME of groundnut.

findings indicate that REE exhibited antioxidant scavenging activity. According to Kessler *et al.* (2003), flavonoid compounds play a role in free radical chelating or scavenging, while phenolic compounds serve as free radical terminators. A number of processes have been linked to antioxidant activity, including reductive capacity, chain initiation prevention, binding of transition metal ion catalysts, peroxide breakdown, inhibition of ongoing hydrogen abstraction and radical scavenging (Pisoschi *et al.,* 2021). Reactive nitrogen and oxygen species (ROS) and their formation are enhanced by drought-induced metabolic dysregulation, which in turn impacts the cell's redox regulatory state.

CONCLUSION

The findings of this study indicate that the groundnut genotype GKVK-5 possesses significant drought tolerance, which is likely due to the presence and combined effects of various antioxidant and bioactive metabolites. The ethyl acetate extract (REE) and methanol extract (RME) of the groundnut roots demonstrated substantial antioxidant activities and free radical scavenging abilities, attributed to their high phenolic and flavonoid contents, respectively. These findings suggest that antioxidant properties may play a crucial role in enhancing the stress tolerance mechanisms of genotype GKVK-5.

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Conflict of interest

The authors declare that this publication does not involve any conflicts of interest.

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