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Phytochemicals and antioxidants in leaf extracts of *Ginkgo biloba* with reference to location, seasonal variation and solvent system

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ABS T RA CT

*Aims:*To determine the influence of location, seasonal variation and solvent system in production of phytochemicals and antioxidants from ginkgo leaves.
Methods: Total phenolic and flavonoid contents and antioxidant activity in ginkgo leaf extracts were estimated spectrophotometrically. Factorial analysis was performed to correlatetheinfluenceoflocation,seasonandsolventonproductionofphytochemicals and antioxidants.
*Results:*Totalphenolicandflavonoidcontentsaswellastheantioxidantswereestimated maximum in autumn. Among solvents, acetone/water extracts gave best results for phenolic and flavonoid contents while methanolic extracts were best for antioxidants. Phenolic content, the predominant indicator of phytochemicals, showed significant cor- relation with antioxidant activity.
*Conclusion:*Factorial analysis among location, season and solvent with respect tothe phytochemicals and antioxidants, was found to be statistically significant. Presence of phytochemicalsalongwiththeprotectivefeatureintheformofantioxidantsisindicative of the importance of this species in pharmacological industry.

Keywords:Ginkgobiloba(ginkgo) Phytochemicals PhenolicsAntioxidantsIndianHimalayanRegion(IHR)

1. Introduction

Medicinal plants are known potential source of many phenolic compounds and antioxidants. Among these, poly- phenols in particular, have been recognized for antioxidant activity and many other health benefits.[1]Phenolic and fla- vonoids,asnaturalantioxidantsandfreeradicalscavengers, haveinvolvedsubstantialinterestduetotheirimportancein food and pharmacological industry.[2]Factors, such as geographic location, age of the plant, season, associated microflora, nutritional status, and environmental stress are known to influence the secondary metabolite profile of a particular plant species. Seasonal variation in trees, for example from dormant to active phase, brings progressive changes in traits like production of phytochemicals.[3]Be- sides,optimizationofmethodswithrespecttosolvent system is important for determination or extraction of the phytochemicals from any plant species.

*Ginkgobiloba*L.(familyGinkgoaceae),commonlyknown as living fossil, harbors many beneficial medicinal proper- ties. Traditionally, it has been used on an extensive basis, either as food or medicinal component, almost all over the world.Theleafextractofginkgocontainspharmaceutically imperative flavonoids, glycosides and ginkgolides which expand blood flow, act as antioxidant and mainly used as memory enhancer and anti-vertigo.[4]The present study is focused on the evaluation of phytochemicals and antioxi- dantsinleafextractsofginkgoalongwiththefactorial analysis among locations × seasons, seasons × solvents and locations × solvents.

2. Materialsandmethods

Plantmaterial

Ginkgo leaves were collected in three seasons fromfive different locations referred as GB1 (Kalika, Almora), GB2 (Chaubatia,Almora),GB3(Snowview,Nainital),GB4(High court,Nainital)andGB5(Glenthorn,Nainital)inUttarakhand, India.[5]The leaves, dried at room temperature, were grounded to fine powder and stored at 4 °C for further analysis.

Preparationofextracts

Dried leaf powder (10 g) was mixed with 25 ml methanol (ME), ethylacetate(EA),n-butanol(n-B),acetone/water(AW)(3:2) and

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water (aqueous/WE), separately. The leaf extract was stirredcontinuouslyfor24handthenfiltered.Thefiltratewas centrifugedat10,000rpmfor10minandthesupernatant,was stored at 4 ˚C prior to use (within 2 days).

Determinationoftotalphenolicandflavonoid contents

Total phenolic and flavonoid contents were determined by FolineCiocalteu's and aluminum chloride calorimetric methods,respectively[6,7]followingquantificationonthebasisof standardcurveofgallicacidandquercetin.Resultsarepresentedinmilligrams(mg)gallicacid(GAE)andquercetin(QE)equiva- lent,respectively,pergramofleafsampleondryweightbasis.

Determination of antioxidant activity (radical scavenging (ABTS), antiradical (DPPH) and reducing power (FRAP) assays)

Totalantioxidantactivitywasmeasuredby ABTS,DPPHand FRAP assays following methods of Cai et al[8]and Amarowicz etal[9,10]Standardcurveofarangeofconcentrationsofascorbic acid was prepared for quantification of antioxidant potential. Resultswereexpressedinmilligram(mg)ascorbicacidequiv- alent (AAE) per gram of leaf sample on dry weight basis.

Statisticalanalysis

Determination of total phenolic and flavonoid contentsand antioxidantcapacitybyABTS,DPPHandreducingpower

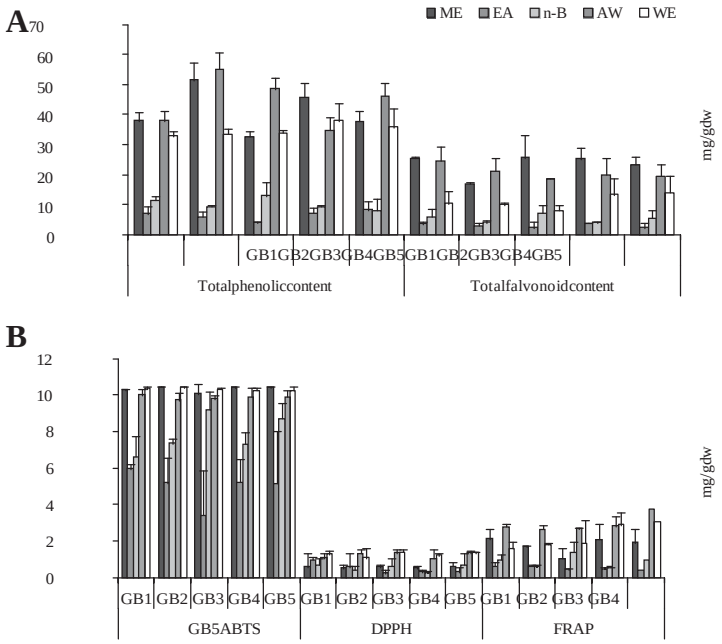
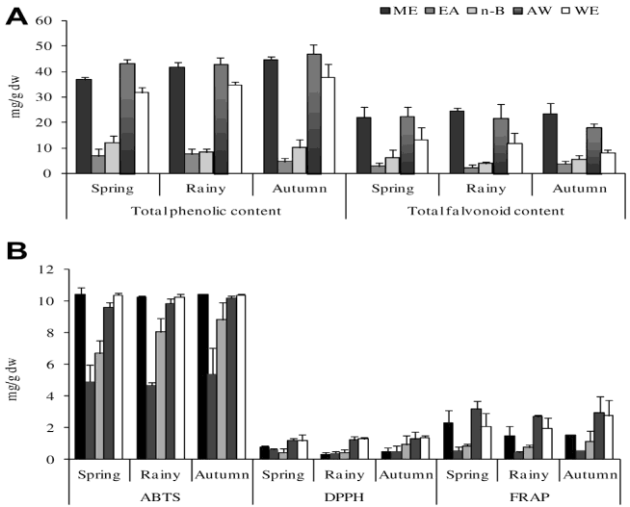


Fig. 1 e Phytochemicals and antioxidants in ginkgo leafextracts at different locations. (A) total phenolic and flavonoid contents, and (B) antioxidant activity (ABTS, DPPH, FRAP).

assay was conducted in triplicates. The value for each sample was calculated as the meanSD. Factorial analysis of vari- ance and



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significant difference among means were tested by twowayANOVAinreplication.Correlationcoefficientswere calculated using Microsoft Excel 2007.

Fig. 2 e Phytochemicals and antioxidants in ginkgo leafextracts in different seasons. (A) total phenolic andflavonoid contents, and (B) antioxidant activity (ABTS, DPPH, FRAP).

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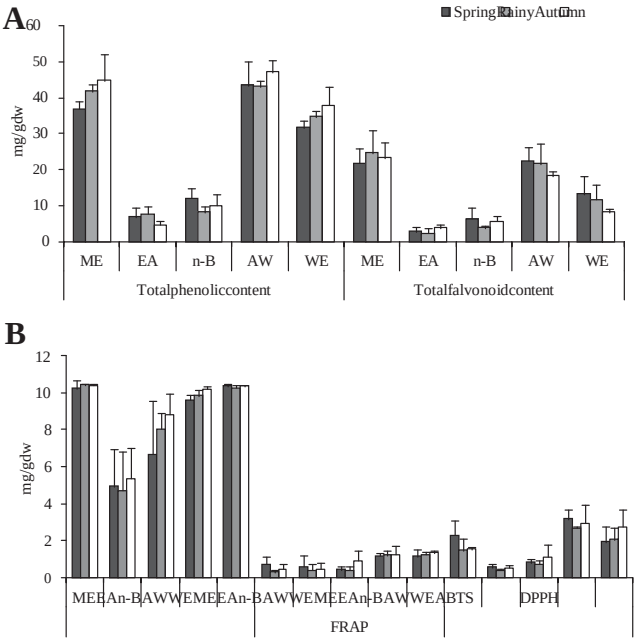

Fig. 3 e Phytochemicals and antioxidants in ginkgo leafextracts in different solvents (A) total phenolic and flavonoid contents, and (B) antioxidant activity (ABTS, DPPH, FRAP).

extracts of GB3 and GB4, respectively. In WE, maximum con- tent was for GB4 and minimum for GB1. GB3 gave maximum valueforn-BandGB5forEAfortotalphenoliccontent (Fig. 1A). Total flavonoids were higher in GB3 in ME and n-B, respectively, in comparison to GB2 and GB4. Higher flavonoid content was in EA for GB4 and in WE for GB5 (Fig. 1A). Anti- oxidant activity in ABTS was higher in ME and WE for GB2, respectively. Subsequently, GB1 gave higher antioxidant ac- tivity in EA and AW, respectively, while GB3 showedmaximum antioxidants in n-B. Based on DPPH assay, GB3 exhibited highest values for antioxidants in n-B, AW and WE, respectively. ForGB1and GB5,highest valueswererecordedin EA and ME, respectively. In FRAP assay, GB5 showed higher activityinAWandWE,respectively;GB3inn-B;GB2inEAand GB1 in ME (Fig. 1B).

Variationsinphytochemicalsariseduetothespecific environmental conditions, including both bioticand abiotic.[11,12]Generally, with increase in altitude, environ- mental conditions such as UV radiation, temperature, rainfall, moisture, etc., changes occur rapidly. The biochemicals measuredinginkgoleafextracts,inthepresentstudy,areon thehighersideascomparedtotheearlierreportsfromother countries.[13,14]The 5 locations in the present study, falling between 1742 and 2260 m altitude representing temperate climaticconditions,arelikelytobeassociatedwiththe higher contents of phytochemicals and antioxidants. Findings on productionofpolyphenolsandantioxidants,inrespecttion environmentalstress,havebeenlinkedtothedefense mechanism.[15]

3. Resultsanddiscussion

Phytochemicalsandantioxidantsinginkgoleaves at different locations

Significant variations (p<0.05) were observed inphyto- chemicals and antioxidants in leaf extracts of different loca- tions in different solvents. In ME and AW, GB2 gave higher phenoliccontent,whilelowervalueswererecordedinEA

Phytochemicalsandantioxidantsinginkgoleaves in different seasons

Total phenolic content in ginkgo leaf extracts variedsignifi- cantly with respect to season and organic solvent, being maximum in autumn (Fig. 2A). Phenolic content was excep- tionallyhigherinrainyandspringseasoninEAandn-B.Total

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Table 1 eAnalysis of variance for determining effect of solvents, seasons and their interaction with phytochemicals and antioxidant activity in ginkgo leaf extracts (given as f value).

Location	Sourceofvariation	df	Phytochemicals		Antioxidantactivity		
			TPC	TFC	ABTS	DPPH	FRAP
GB1	Solvent(S)	4	691,936.67***	405,216.72***	90,893.87***	13,656.23***	12,737.04***
	Season(S)	2	51,039.16***	8289.85***	24,412.81***	3616.33***	1129.70***
	S×S	8	53,096.14***	13,357.34***	21,752.97***	993.04***	491.58***
GB2	Solvent(S)	4	2,025,206.05***	444,923.81***	211,154.29***	28.89***	4065.47***
	Season(S)	2	33,178.64***	9466.39***	5331.77***	3.96***	48.73***
	S×S	8	14,617.20***	10,601.75***	5790.39***	12.78***	36.30***
GB3	Solvent(S)	4	1,031,256.71***	483,295.95***	243,030.95***	20,906.01***	3732.14***
	Season(S)	2	94,933.48***	16,824.75***	8065.06***	4389.47***	1641.31***
	S×S	8	116,135.97***	27,375.31***	15,780.63***	910.08***	595.57***
GB4	Solvent(S)	4	888,494.27***	593,733.35***	162,822.13***	10,388.55***	77,975.80***
	Season(S)	2	44,739.59***	56,428.10***	10,839.66***	808.30***	3861.01***
	S×S	8	53,242.26***	25,616.70***	3396.54***	1331.49***	6377.25***
GB5	Solvent(S)	4	1,195,793.77***	272,908.46***	160,479.36***	10,078.51***	468,226.40***
	Season(S)	2	4423.19***	12,862.07***	14,936.57***	2754.08***	42,617.60***
	S×S	8	29,119.36***	15,369.38***	21,628.06***	1519.68***	24,587.54***

dfedegreeoffreedom;dwedryweight;TPCeTotalphenoliccontent;TFCeTotalflavonoidcontent;Levelofsignificance: ***ep<0.001.

Table2eCorrelationmatrixbetweentotalphenolicand flavonoid contents and antioxidant activity in ginkgo leaves from 5 locations using different assays.

	TPC	TFC	ABTS	DPPH	FRAP
TPC	1				
TFC	0.737**	1			
ABTS	0.7167**	0.639**	1		
DPPH	0.376*	0.1924	0.507**	1	
FRAP	0.734**	0.614**	0.639**	0.602**	1

TPCeTotalphenoliccontents;TFCeTotalflavonoidcontents; Level of significance: * e p <0.05; ** e p <0.01.

flavonoid content was higher in spring in 3 solvents, AW, WE and n-B, during rainy season in ME and during autumn in EA (Fig. 2A). Antioxidant activity performed by three assays showed significant variation with respect to the seasons, maximumbeinginABTSandDPPHinautumn(Fig.2B).Incase of FRAP, higher activity was recorded during spring followedby autumn (Fig. 2B).

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Importanceofseasonalvariationinaccumulationoftotal phenolic and flavonoid contents and antioxidants has been recognized.Althoughaclearandregulartrendduetoseasonal variation was not observed in the present study, the total phenoliccontentwasrelativelyhigherinautumn.Kobusetal[13]reportedhigherlevelofpolyphenolsinOctoberascomparedto August.Besides,higheraccumulationofphenolicandflavo- noidsduringwinterislikelytobeattributedtothestresscon- ditionssuchastemperatureandplantgrowthstage.Ingeneral, thesecondarymetabolitesremainatlowlevelinginkgoduring springandsummerwhicharetheinitialstagesforthegrowthof shootsandleaves.Afterward,towardsautumnandwinter,as thegrowthandmetabolismbecomeslower,thephytochemi- cals tend to accumulate in higher amounts.

Optimization of solvent systems for extraction of phytochemicals and antioxidants

Theoptimizationexperimentsconducted forpreferenceof solventrevealedthatAWwasthebestsolventforextracting

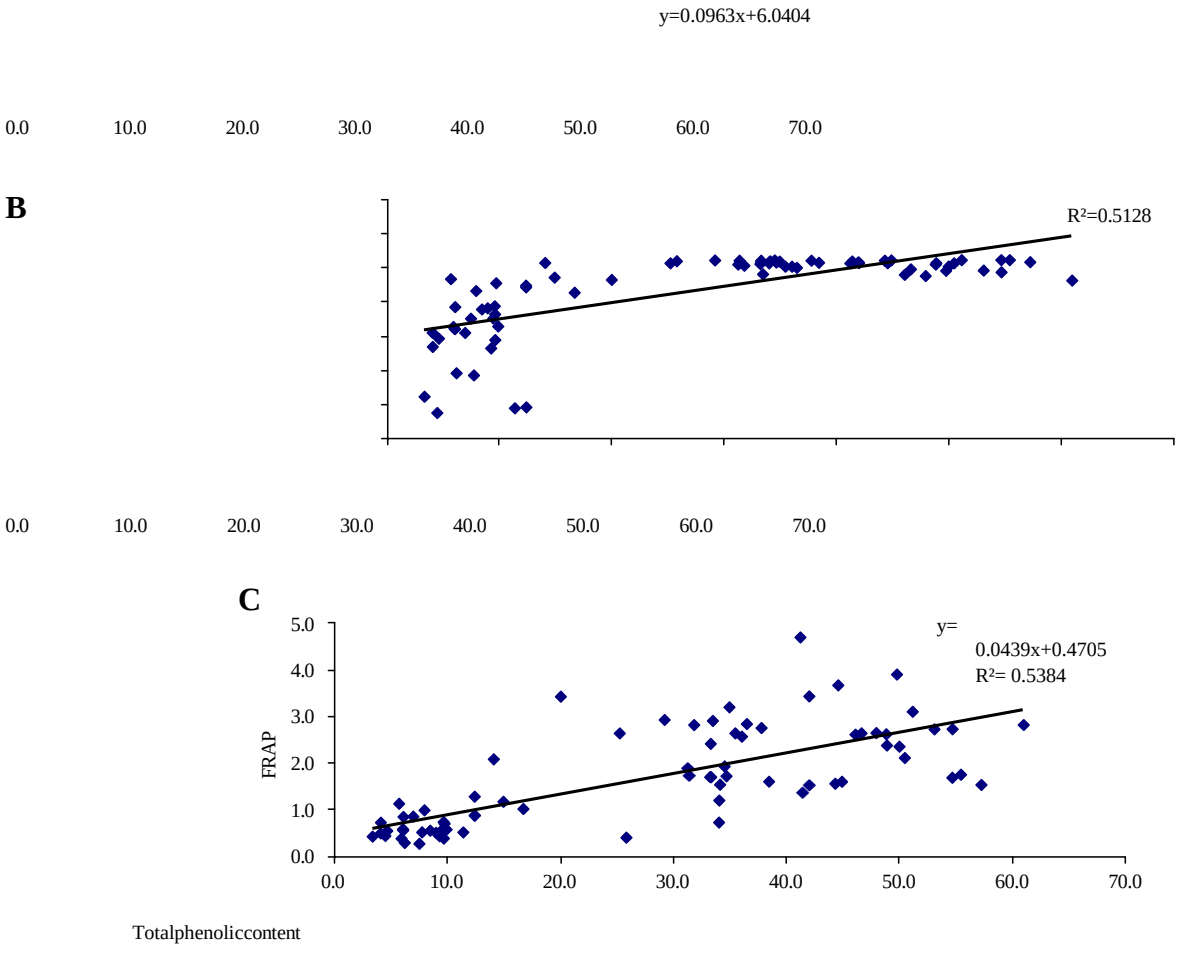
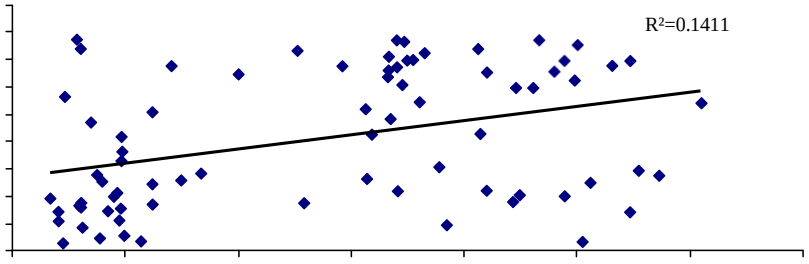


Fig. 4 eLinear relationship between total phenolic contents and antioxidants measured by (A) ABTS (B) DPPH and (C) FRAP in ginkgo leaf extracts.



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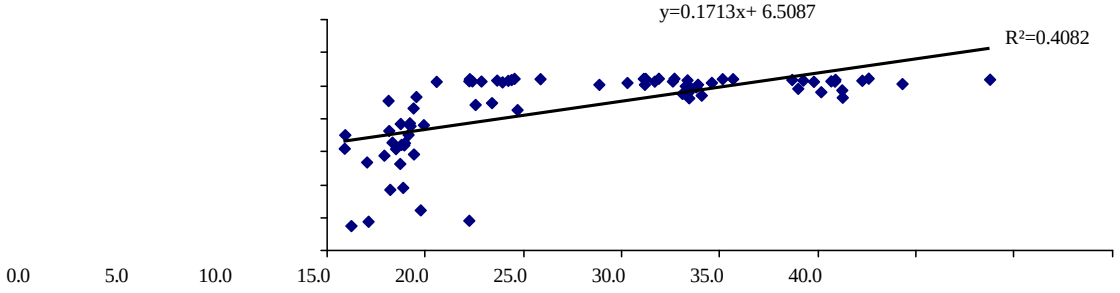
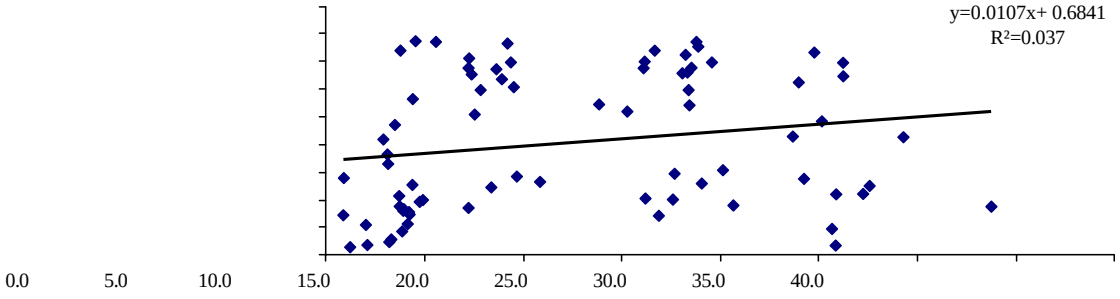
phenoliccontentinallthethreeseasons;followedby ME >WE >n-B >EA. Similarly, total flavonoid content was recordedhighestinAWduringrainyandautumnfollowedby
ME during spring (Fig. 3A). Different solvent systemsalso influenced the extraction of antioxidant activity in different seasons. Antioxidant activity measured by ABTS assay was highest in ME in rainy and autumn and in WE in spring. In DPPHassay,theactivitywasrecordedhighestinWEinallthe seasons. Also, the reducing power assay showed higher anti-oxidantactivityinAWduringalltheseasons(Fig.3B).Facto-
rial analysis exhibited that the solvents andseasons individually and their interaction significantly (p<0.001) influencedtheaccumulationofphytochemicalsandantioxi-
dantactivity(Table1).Thebestresultsobtainedforextraction ofphytochemicalsandantioxidantsinAWandMEareinline with the earlier reports.[13,16]Phenolic compounds are often linked with other biomolecules, such as polysaccharides, proteins, etc., therefore, an appropriate solvent system is required for their extraction. Polarity of different solvents is likely to have significant consequence on polyphenolic con- tentandantioxidantactivityaswell.[17]Importanceofsolventsystem has also been reported in determination ofantimi- crobial activity[5]in ginkgo leaf extracts.

Among the three assays used for determination ofanti- oxidant activity in the present study, ABTS gave best results followed by DPPH and FRAP. ABTS is soluble in both aqueous and organic solvents and having reducing properties of 2, 2- azinobis-(3-ethylbenzoline sulphonate) radical, in which the antioxidantactivitycanbepre´cisedduetothehydrophilicand lipophilicnatureofthecompound.DPPH,possessingabilityto get dissolved only in organic solvent, ethanol in particular,can be predicted as an imperative restriction while inter- preting the role of hydrophilic antioxidants. Previous studies have also indicated the merits of using ABTS assay in assessing antioxidant potential of plant extracts.[18]With re- gard to the FRAP, the antioxidants reduce the ferric ion/ferri- cyanide complex to the ferrous form, the Perl's Prussian blue complex. The reducing power is related to the presence of the compounds, which apply their action by flouting the free radical chain through donating hydrogen atom compounds.[19]The reducing power of extracts prepared from ginkgo leaves has been reported.[20]





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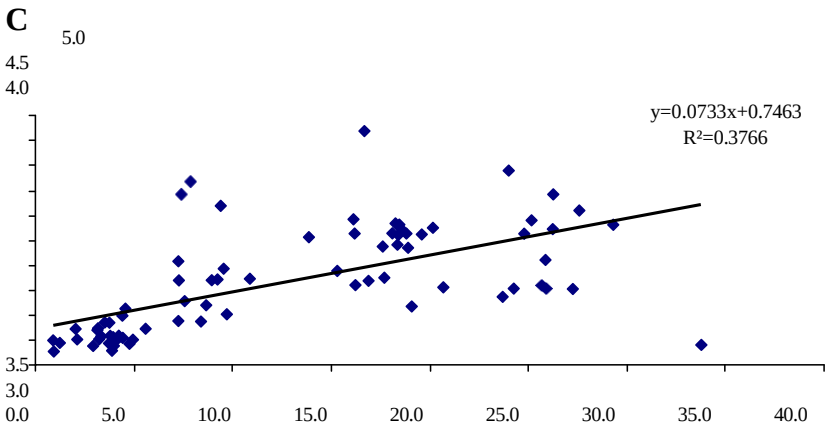

Fig.5eLinearrelationshipbetweentotalflavonoidcontentsandantioxidantsmeasuredby(A)ABTS(B)DPPHand(C)FRAP in ginkgo leaf extracts.

Correlations among total phenolic andflavonoid contents and antioxidant activity

Correlation matrix exhibited significant positive relationship between total phenolic and flavonoid contents and the antiox- idant activityperformedbyall the three assays(Table 2). Linear regression analysis revealed that total phenolic content con-tributes14.1e51.2%ofradical scavengingproperty(r^2¼0.141for DPPHand0.512forABTS)and53.8%ofreducingproperty (r^2¼0.538)(Fig.4AeC).Likewise,totalflavonoidcontentcon-tributes3.7e40%ofradicalscavengingproperty(r^2¼0.037for DPPH and 0.408 for ABTS) and 37% of reducing property(r^2¼0.376)(Fig.5AeC).Similarfindingshavebeenreportedin otherHimalayanspeciesaswellwheretotalphenoliccontent andantioxidantactivitycorrelatepositively.[18]

4. Conclusion

TheIHRharborsplethoraofmedicinalplants.Whilethenat- ural habitat of ginkgo is in China, Japan, and Korea, some established trees have been reported from the hilly areas of IHR, maximum being in the state of Uttarakhand. Ginkgo possesseshighamountsofphenoliccontentsandhighlevels ofgallicacidequivalents.Ginkgotrees,beinginlimitednum-berandgrowingunderlowtemperatureclimaticconditions, extend opportunity to make use of these trees for under-standingthephysiologicalaspects,suchasaccumulationof phytochemicals,productionofantimicrobials,withemphasis on propagation and conservation of the species.[5,21,22,23]

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