Antioxidant Evaluation and Chemo-Profiling of Non-conventional Curcuma Species as Conservation of Drug Targeting Molecule

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**Antioxidant Evaluation and Chemo-Profiling of Non-conventional *Curcuma* Species as
Conservation of Drug Targeting Molecule**

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Abstract

The objective of the present study was to determine and compare antioxidant activities of different species of *Curcuma* i.e. *Curcuma longa*, *Curcuma caesia* and *Curcuma aromatic* and their chemoprofiling for conservation of highest yield plants. The functional group analysis of the plants extract were determined by FTIR and structural features of phyto-constituents and Chemo-profiling of ethanol fractions of all targeted *Curcuma* species showing highest activity were analyzed by GC–MS/MS. Ethanolic extract of *C. aromatica* (ECA) was exhibited to determine high radical scavenging activity against DPPH (92.12%), lipid Peroxidation ($85.13 \pm 0.23\%$), nitric oxide scavenging, total reducing power ($700.83 \pm 8.14 \mu\text{g}/\text{mg}$) and ferric reducing power assay. In ABTS $\cdot^+$ and phosphomolybdate assay *Curcuma* species showed a significant difference ranging from 67.53 – 98.57%. Furthermore, the amount of total phenolic and flavonoid content ranged from 3.63 ± 1.06 – $545.77 \pm 2.76 \mu\text{g}/\text{mg}$ eq GAE and 21.18 – 101.29 $\mu\text{g}/\text{mg}$ eq QUE respectively. The results indicated that the ethanolic extracts showed highest *in vitro* antioxidant activity. It can be conclude from the study that *Curcuma* species may serve as an important bioresource as an antioxidant drug.

Keywords: *C. Longa*, *C. caesia*, *C. aromatica*, FTIR, antioxidant, curcumin.

1. Introduction

Plants produce potential sources of natural bioactive compounds such as secondary metabolites, many of which have been subsequently exploited by humans for their beneficial roles in a diverse array of biological functions [1]. Cellular system has developed many endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, and nonenzymatic antioxidants like vitamin E and C are neutralises the free radicals [2-3]. Many researchers have investigated that the increase levels of antioxidants present in plants are believed to decrease the oxidative damage and its harmful effects [4]. This *Curcuma* species possess three main diverse phytoconstituents, which have lots of pharmaceutical importance. Chemical constituents of turmeric have been extensively investigated by a long time. Phenols including diarylheptanoids and diarylpentanoids, phenylpropene and terpenoids like monoterpenes, sesquiterpenes, diterpenes, triterpenoids, sterols, flavonoids and some alkaloidal compounds were identified in *Curcuma*.

Antioxidants, which are the substances to prevent oxidation of other compounds or neutralize free radicals. Curcumin is well known systemic markers of oxidative stress it can significantly reduce the production of reactive oxygen species such as superoxide anions, hydrogen peroxide, and nitrite radical production. Curcumin is considered as a chain-breaking antioxidant. In addition, it is a lipophilic compound, which makes it an efficient scavenger of peroxy radicals, like vitamin E [5]. Curcumin can be inhibiting the activity of ROS-generating enzymes such as lipoxygenase/cyclooxygenase and xanthine hydrogenase/oxidase [6] to modulate the activity of GSH, catalase, and SOD enzymes due to the neutralization of free radicals [7-8].

Approximately, 100 species of rhizomatous herbs belong to genus *Curcuma*, family Zingiberaceae are widely distributed in tropical and subtropical regions of the world and mostly cultivated in Asian countries, out of these Turmeric (*C. longa* Linn), black turmeric (*C. caesia* Roxb.) and wild turmeric (*C. aromatica* Salisb.) have high traditional importance. *C. longa* is termed as “Indian Saffron” because of its golden color. *C. longa* is the most common traditional drug of Indian and Chinese medicines used as anti-inflammatory, wound healing antibacterial, antifungal, anticancer, antioxidant, anti-HIV, immunomodulatory activities, relieve colic and chest pain, and menstrual difficulties [9]. The effects of *C. longa* are ascribed to the presence of diarylheptanoid compounds collectively known as curcuminoids, which includes curcumin, demethoxycurcumin, and bisdemethoxycurcumin [10]. Effects of curcumin are mediated through

the regulation of various transcription factors growth factors, inflammatory cytokines, and protein kinases.

C. longa is a well-known species of *Curcuma*; several researches have performed for this plant but the two uncommon species i.e., *C. caesia* and *C. aromatica* need to explore completely for its biological importance. *C. caesia* rhizome is a bluish black in color. The inner part of the rhizome emits a characteristic sweet smell due to the presence of essential oil. In India black turmeric have been used to treat fever, stomach problems, allergic, diarrhea, jaundice, liver ailments, snake bite, heart burn, chronic cough, asthma, inflammation, sprains muscle relaxant, haemorrhoids, leprosy, cancer, epilepsy, fever, wound, vomiting, menstrual disorder anthelmintic, aphrodisiac, and gonorrhoeal discharges [11]. *C. aromatica* stimulate immunomodulatory properties and enhance the health conditions by arresting ageing. It compounds related an antibacterial activity against various microbial infections. In addition used as a tonic, carminative, in sprains, snakebite, skin eruptions, infections and very efficiently improve complexion since long time ago, promoting blood circulation to remove blood stasis and used as cancer preventive drug [12-14]. Hence, the present study aimed to evaluate the antioxidant property of non-conventional *Curcuma* species and compare to identify the best one. The aim of this study was to evaluate the antioxidative properties of the various extracts of *Curcuma* species and find the best extracts, which possesses highest antioxidant potential at last, examine the chemical composition of most prominent extracts by GC–MS/MS.

2. Materials and methods

2.1 Materials and reagents

Gallic acid, ascorbic acid, potassium ferricyanide (III), DPPH (2,2-diphenyl-1-picrylhydrazyl), 2,2'-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS.+), 2,4,6-tripyridyl-s-tiazine (TPTZ), tannic acid, quercetin, aluminium chloride, ferric chloride (FeCl₃), sodium nitroprusside, sulphanilic acid, Naphthyl ethylene dianilinedihydro chloride (NEDD), sodium phosphate, ammonium molybdate, streptomycin were purchased from Sigma-Aldrich (USA). Folin–Ciocalteu's phenol reagent, trichloro acetic acid (TCA), solvents ethanol, methanol, ethyl acetate, chloroform, dichloromethane and acetone were of analytical grade.

2.2. Collection, Authentication and Extraction of different species of *Curcuma*

The rhizome of *Curcuma* species; *C. longa* (Bargaon-CL), *C. caesia* (Acholi-CC) and *C. aromatica* (Jamsarar-CA) was collected from different villages of Rajnandgaon district Chhattisgarh, India. It was authenticated by the Department of Botany, Guru Ghasidas Vishwavidyalaya and voucher specimen was deposited in the herbarium of the Institute. The samples were dried and ground to fine powder. The powders were subjected to extraction using different solvents i.e. Chloroform, dichloromethane, acetone, ethyl acetate, methanol and ethanol, successively of increasing polarity by soxhlet extraction unit. The extracts were evaporated to dryness and concentrated using a vacuum evaporator for further use. The dried extract was insoluble in petroleum ether, so the remaining solvent extracts were used for analysis.

2.3. Determination of polyphenols

Total polyphenolic content (TPC) of the extracts was determined using Folin Ciocalteu method as described by Singleton VL et al. 1999 with slight modification [15]. Folin Ciocalteu reagent is a heteropoly acid complex formed of orthophosphoric acid and molybdotungustic acid there by producing reduced species having characteristic intense blue color exhibiting absorbance at 738 nm. Briefly, in a test tube containing 0.4 mL of plant extract (1 mg/mL conc.), 2 mL of 10% Folin Ciocalteu's reagent and 1.6 mL of 20.25% Sodium carbonate was shaken properly and kept in dark for 2 h. The absorbance was determined using spectrophotometer (UV-1800, Shimadzu, Japan) at 738 nm. TPC was calculated with the help of gallic acid standard curve and expressed as $\mu\text{g GAE/ mg sample}$.

2.4. Total flavonol Content

The total flavonol content was determined according to the aluminium chloride (AlCl_3) method described by Rajan et al. 2011 in which AlCl_3 forms acid stable complex with the C_4 keto group or C_3 or C_5 hydroxyl group of flavones and flavonols [16]. In this method, 0.5 mL (1 mg/mL conc.) plant extract was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The absorbance was measured at 425

nm after 30 min at room temperature. The total flavonol content was calculated with the standard curve of Quercetin and expressed the value as µg/mg Quercetin equivalent.

2.5. Determination of total flavonoids

Total flavonoids spectrophotometric method was performed according to *Dewanto et al. 2002* [17]. In this method, 250µL of extracts (1mg/mL) was mixed with 1.25mL of distilled water and 75µL of 5% NaNO₂ solution followed by addition of 150µL of 10% AlCl₃ solution after 5 min. Further, 500µL of 1M NaOH and 275µL of distilled water were added to the mixture after 6 min. The solution was mixed well and the absorbance was read at 510 nm. Flavonoids content was calculated with the calibration curve of Quercetin and results were expressed as µg of Quercetin Equivalents per mg of extract (QUEs).

2.6. Total reducing power assay

The total reducing power (TRP) of plant extract was determined according to the method of *Priya et al., 2012* [18]. Extracts of *Curcuma* rhizome 1mg/mL were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min followed by addition of 2.5 mL of 10% TCA (trichloro acetic acid) and then mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1%) and then absorbance was measured at 700 nm. Gallic acid standard curve was used to calculate the TRP of plant extracts. Higher absorbance of the reaction mixture indicates greater reducing power.

2.7. DPPH radial Scavenging Activity

The DPPH free radical scavenging assay was performed by the method of *Williams et al. 1995*, and *Aadil et al. 2014*, with minor modifications [19-20]. The stock solution of DPPH was prepared by 12 mg DPPH in 50 mL methanol and the working solution was diluted by mixing 15 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.05 at 517nm (control). A volume of 2800 µL aliquot of this solution was mixed with 200 µL of 500 µg/mL of plant extracts. The reaction mixture was mixed thoroughly and incubated in dark for 2 h at room temperature. The absorbance was measured at 517 nm, taking ascorbic acid as standard. The scavenging activity was calculated by the formula as follows:

$$\text{Scavenging activity (\%)} = \frac{(A_c - A_s)}{A_c} \times 100$$

Where A_c was the absorbance of the control and A was the absorbance of sample

2.8. FRAP (Ferric reducing power assay)

Ferric reducing power assay of *Benzie and Strain, 1996* with minor modification was used to check the antioxidant property of rhizome extracts of *Curcuma* [21]. The stock solutions of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM anhydrous $FeCl_3$ solutions were prepared. The working solution was freshly prepared by mixing 10:1:1 ratio of acetate buffer, TPTZ and $FeCl_3$, respectively. Plant extracts, 200 μ L (500 μ g/mL) were allowed to react with 2800 μ L of the FRAP solution for 30 min in dark. Absorbance of colored product (Ferrous tripyridyltriazine complex) was taken at 593 nm and result was expressed in terms of μ M of ascorbic acid.

2.9. ABTS $\cdot^+$ radical scavenging activity

The ABTS $\cdot^+$ assay was performed according to method of *Arnao et al. 2001* with some modifications [22]. The ABTS $\cdot^+$ radical was produced by reacting 7.4 mM ABTS $\cdot^+$ stock solution with 2.6 mM potassium persulfate and allowing the mixture to stand in dark at 4°C for 12-16 h before use. A volume of 1 mL ABTS $\cdot^+$ solution was diluted with 50 mL methanol to $A_{734} = 0.7$. Plant extracts 200 μ L (500 μ g/mL) were allowed to react with 2800 μ L of the ABTS $\cdot^+$ solution for 2h in dark. The absorbance was taken at 734 nm using tannic acid as standard.

$$\text{ABTS scavenging activity \%} = \frac{(A_c - A_s)}{A_c} \times 100$$

Where, A_c was the absorbance of the control and A_s was the absorbance of sample.

2.10. Nitric oxide scavenging assay

The free radical scavenging activity was measured by a nitric oxide assay, adopted from *Garraat DC, 1964* with minor modification [23]. The reaction mixture containing the plant extracts in 500 μ L (1mg/mL), sodium nitroprusside (10 mM, 2 mL) and phosphate buffer saline (PBS, pH 7.4 500 μ L) was incubated at 25°C for 150 min. After incubation, 0.5 mL of reaction mixture from each reaction vessel was separately taken to which 1.0 mL sulphanilic acid was added and allowed to stand for 5 min followed by addition of 1.0 mL of Naphthyl ethylene dianilinedihydrochloride (NEDD) then incubated for 30 min at 25°C. A pink colored chromophore was formed in

diffused light. The absorbance of the solution was measured at 540 nm against the corresponding blank.

2.11. Phosphomolybdate assay for evaluation of antioxidant capacity

To check the antioxidant activity of plant extracts, phosphomolybdate assay given by *Prieto et al. 1999* and *Aadil et al. 2014* was performed [24, 20]. An aliquot of 200 µL of plant extracts (1.0 mg/mL) were mixed with 1.0 mL of test reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a water bath at 70°C for 90 min. Samples were cooled at ambient temperature and the absorbance was measured against a blank at 695 nm. Antioxidant activity of each sample was expressed in terms of ascorbic acid equivalent to mM/100 mg of sample.

2.12. Lipid peroxidation by TBA assay

This method was adopted from *Cunn A et al. 2007* [25]. Lipid peroxidation is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with two molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 532 nm. The plant extracts in 0.5 mL volume (1.0 mg/mL) were taken in vial followed by addition of 0.5 mL homogenate of chicken liver prepared in ice cold 0.15 M KCl. Further, 100 µl of 0.2 mM FeCl₃ was added in the mixture and incubated at 37°C for 30 min then 2.0 mL (0.25N) ice cold HCl containing 15% TCA, 0.38% TBA, and 0.5% BHT were added and heated at 80°C for 60 min. The absorbance was measured at 532 nm.

$$\text{Inhibition of lipid peroxidation (\%)} = \frac{1 - \text{sample OD}}{\text{blank OD}} \times 100$$

2.13. UV–visible spectral analysis

UV–visible spectroscopy is the most commonly used technique for the determination of curcumin and curcuminoids like compounds. In order to check the purity and preliminary characterization of phyto-constituents, absorption spectra were recorded by UV–visible spectroscopy (UV-1800, Shimadzu, Japan) from 200 to 500 nm. The extracted phyto-constituents of three targeted *Curcuma* samples were dissolved in their respective solvents (1 µg/mL) that were used for extraction before measuring the absorbance.

2.14. FTIR analysis

FTIR spectra for plant extracts of *Curcuma* species were obtained between 4000 and 500 cm⁻¹ with a resolution at 4 cm⁻¹. Spectra including standard were recorded using Thermo fisher scientific, OMNIC-9 FTIR spectrophotometer. Extracts of all three *Curcuma* species (1 mg) were mixed with 100 mg KBr and properly grounded before analysis.

2.15. GC-MS/MS analysis of most active extract

On the basis of primary screening the extracts showing highest antioxidant activity i.e, *C. longa* (ECL), *C. caesia* (ECC), *C. aromatica* (ECA) were selected for GC-MS/MS analysis. For sample derivatization 50 mg of concentrated ethanol extracts were redissolved in 200 µl of respective solvent vortexed properly and filtered through 0.22 mm filter, then added 100 µl BSA+TMCS and incubated at 80°C for 30 minute.

Instrumentation and chromatographic conditions for GC-MS/MS analysis was carried out on a GCMS-QP2010 Ultra, USA, coupled with a mass selective detector (MSD). For the GC-MS/MS analysis the samples were injected in split less mode in a column HP5 phenyl methyl siloxane [25 µm (film thickness) × 320 µm (internal diameter) × 30 m (length of column)]. The gas chromatograph was interfaced to a mass spectrometer instrument employing the following conditions viz. Column Oven Temp. :70 °C Injection Temp. :260°C, Injection Mode: Splitless Sampling Time: 1 min. Start Time: 6 min, end Time: 49.98min, Start m/z: 40.00 End m/z: 650.00.

Identification of putative components based on mass spectra of GC/MS was done using the NIST/EPA/NIH Mass Spectral Database, with NIST MS search program [National Institute Standard and Technology (NIST), Scientific Instrument services, Inc., NJ, USA]. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

2.16. Statistical analysis

The experiments were performed in triplicates. The data were recorded as mean ± SD and analyzed by Graph pad prism-5. One way ANOVA was performed by standard procedures.

Significant differences between means were determined by Dunnett's tests and $p < 0.05$ was regarded as significant and $p < 0.0001$ was very significant.

3. Results and Discussion

3.1. Estimation of total phenol content

The phenolic compounds acts as an antioxidant and radical scavenging agents by breaking free radical chain reaction through a hydrogen atom donation [26]. The content of total phenolics in each fractions were determined as gallic acid equivalents (GAE) using standard curve equation, $y = 0.0067x + 0.095$, $R^2 = 0.9984$. Significantly, higher ($P < 0.0001$) TPC was obtained in the ethanolic extract of *C. longa* (ECL) 545.77 ± 2.76 $\mu\text{g}/\text{mg}$ GAE followed by *C. caesia* (ECC) 430.77 ± 1.16 and *C. aromatica* (ECA) 411.07 ± 5.36 $\mu\text{g}/\text{mg}$ GAE. The range of phenolic content of different extracts varied between 3.63 ± 1.06 to 545.77 ± 2.76 μg of GAE per mg of sample. The order of phenolic content was as follows: ECL > MCL > ECC > ECA > MCA > MCC > ACL > ACA > EaCL > EaCA > DCL > CCL > EaCC > ACC > DCA > DCC > CCC > CCA (Table 1). The key role of phenolic compounds as scavengers of free radicals has emphasized in several literatures [27]. Plants produce many phenolic antioxidants due to their secondary metabolic activity. The antioxidant activity plays an important role in chelating transitional metals and scavenging free radicals [28]. The above results indicated that the phenolic content was high in polar extracts of all the three species of *Curcuma*. This confirms the fundamental role of polar phenolics in evaluation of free radical scavenging. The total antioxidant activities of the non-polar extracts may be due to the presence of volatile components. The relationship between phenol content and its antioxidant activity has already reported [29-30].

3.2. Total flavonols

Flavonoids are also polyphenolic compounds that are ubiquitous in nature and according to their chemical structure categorized into catechins, anthocyanidins, chalcones, flavonols, flavones, flavanones and isoflavones [31]. The total flavonol content varied from 3.54 ± 2.69 to 525.70 ± 7.69 $\mu\text{g}/\text{mg}$ QUE. Maximum flavonol content was found in the acetone extract of *C. aromatica* as shown in Table 1.

3.3. Total flavonoids

Flavonoids play significant role as scavengers of various oxidizing species i.e. superoxide anion, hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen. Therefore, many plants containing flavonoid attributed as antioxidants [32]. The pharmacological activities of flavonoids were closely related to their functional group. Moreover, flavonoids may exert their cell structure protection through a variety of mechanisms. One of their potent effects may be through their ability to increase levels of glutathione, a powerful antioxidant [33]. The total flavonoids content of *Curcuma* species ranging from 18.14 ± 2.61 $\mu\text{g}/\text{mg}$ QUE to 101.29 ± 1.07 $\mu\text{g}/\text{mg}$ QUE. Highest flavonoids content was found in the methanolic extract of *C. longa* followed by ethyl acetate extract of *C. aromatica* (Table 1). Similar result was reported previously that *C. longa* possess high flavonoid content as compared to *C. caesia* [34]. Extracts with polar solvents have a higher flavonoid content compared to extracts with non-polar solvents. In general, the antioxidant activity of *Curcuma* is due to the presence of flavones, isoflavones, flavonoids and carotenoids [35]. In present study, flavonoid content of *C. aromatica* and *C. longa* was high therefore it exhibited maximum antibacterial as well as antioxidant activity compared to *C. caesia*.

3.4. Total reducing power assay

The reducing capacity of compound serves as a significant indicator of its potential antioxidant activity [36]. It is measured as the transformation of Fe^{3+} to Fe^{2+} in presence of antioxidant compound [37]. The total reducing power of all plant extracts (Table 2) were evaluated and compared with the standard gallic acid. The highest reducing power was recorded in ethanolic extract of *C. aromatica* (700.83 ± 8.14 $\mu\text{g}/\text{mg}$ gallic acid equivalent) and lowest with chloroform extract of *C. aromatica* (25.83 ± 5.51 $\mu\text{g}/\text{mg}$ gallic acid equivalent). *C. aromatica* showed highest reducing power activity compared to other two species. It has reported that *C. longa* possess significant antioxidant activity [38]. However, in this study we have found other two species of *Curcuma* that possess antioxidant potential to scavenge the free radicals i.e. *C. caesia* and *C. aromatica*. The reducing power of *Curcuma* is generally associated with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom [39].

3.5. DPPH radical scavenging activity

DPPH is a stable free radical and widely used to estimate the free radical scavenging activity of antioxidants. DPPH interact with antioxidants, either transfer of e- or hydrogen atoms to DPPH, therefore neutralize the free radicals and color of reaction mixture of DPPH changes from purple to yellow [40]. DPPH radical scavenging activity of all the organic solvent extracts of rhizome of *C. longa*, *C. caesia* and *C. aromatica* exhibited good scavenging activity ranging from 31.80% to 92.12% as shown in Table 2. The radical scavenging activity of the extracts was equivalent to ascorbic acid, which was used as standard. The ethanolic extract of *C. aromatica* exhibited highest scavenging activity (92.12%) among all the extracts of three species with lowest EC₅₀ value (54.18 µg/mL). However, the chloroform extract of *C. aromatica* showed lowest scavenging activity (32.80%) and highest EC₅₀ (153.8 µg/mL). It is known that lower EC₅₀ value indicates higher antioxidant potential. Comparatively, polar extracts exhibited stronger affinity to give H- ion to DPPH and neutralize than non-polar extracts. Lowest to highest EC₅₀ value was ranged from 54.18 µg/mL to 153.8 µg/mL. *C. longa* and *C. caesia* scavenging activity is lower in some extent as compared to *C. aromatica*, extracts activity support the FTIR data due to C=OH, ketone or O-H groups appeared in FTIR spectrum. The entire functional groups present in the FTIR spectra provide stability to the compound and possess great potential to scavenge the antioxidants. It has reported methanolic extract of *C. longa* showed strong antioxidant activity against the DPPH radical with percentage inhibition in the range of 86–92% [41]. The antioxidant activity in DPPH assay (86.91%) in ethanolic fraction of *C. caesia* reported by Devi et al is much similar to the present study [42].

3.6. FRAP (ferric reducing power assay)

Antioxidant compounds reduce the ferric tripydyltriazine (Fe III TPTZ) complex to ferrous form and produce intense blue color, which can be measure at 593 nm. Different plant extracts exhibited varying antioxidant potential expressed in terms of µM as showed in (Table 2). FRAP activity was considerably varied from 58.75 ± 15.6 to 809.16 ± 10.4 µM. The highest FRAP value exhibited in ethanolic extract of *C. aromatica* (809.16 ± 10.4 µM) and lowest in the chloroform extract of *C. caesia* (58.75 ± 15.6 µM). All the values were significant (P< 0.0001) as compared to highest FRAP value. The order of FRAP value of different solvent extracts was: ECA > ECC>EaCA> MCA > ECL>EaCC> ACA > ACL> MCC> MCL>EaCL> DCL> CCL> DCC> DCA > ACC> CCA > CCC. All the extracts of *C. aromatica* exhibited great potential to reduce the ferric complex to ferrous form in comparison to *C. longa* and *C. caesia* extracts.

Similarly, the polar extract showed highest activity compared to non-polar extracts. Literature revealed that the rhizome oil of *Curcuma* species contains antioxidant compounds, which reduce the ferric complex to ferrous form [43]

3.7. ABTS^{•+} radical scavenging assay

ABTS^{•+} forms a relatively stable free radical, which decolorizes in its non-radical form [44]. The scavenging of ABTS^{•+} radical cation, a blue green chromogen produced by a reaction between ABTS^{•+} and potassium per sulphate, which is converted to colorless ABTS^{•+} in the presence of antioxidant reductant and quantified in terms of inhibition percentage [45]. The ABTS^{•+} decolorization assay of all the extracts of *Curcuma* species (Table 2) showed significant difference to each other and exhibited an inhibition range of 67.53% to 98.57%. Tannic acid was used as a standard showed EC₅₀ 12.05 ± 0.38 µg/mL. *C. longa* ethyl acetate extract showed lowest EC₅₀ 50.79 µg/mL and highest EC₅₀ was exhibited by methanolic extract of *C. aromatica*. *C. longa* extracts exhibited great potential to decolorize the ABTS^{•+} radical as compare to *C. aromatica* and *C. caesia*. The EC₅₀ value of different plant extracts were ranged from 50.79 µg/mL to 72.26 µg/mL, ethyl acetate extract of *C. longa* and *C. aromatica* showed much similar antioxidant potential in ABTS^{•+} radical. The order of scavenging potential EC₅₀ value of all the plant extracts was as follows: EaCL < EaCA < ECL < DCL < MCL < ACL < MCC < DCC < EaCC < CCL < ACA < DCA < CCC < ECC < ACC < ECA < CCA < MCA. Curcumin is an effective ABTS^{•+} radical scavenger in a concentration dependent manner, scavenging effect of curcumin on ABTS^{•+} at the concentration of 45 µg/mL [46]. Results of recent studies also supported ABTS^{•+} scavenging potential of *Curcuma* extracts.

3.8. Nitric oxide scavenging assay

Nitric oxide interacts with oxygen to nitrite ions, which is measured at 540 nm absorbance on spectroscopy. It is generated from sodium nitroprusside in aqueous medium at physiological pH. Nitrous acid generated from nitric oxide forms purple azo dye that reflect its extent of scavenging with decrease in the amount of nitrous acid.

Nitric oxide radical plays multiple roles in a variety of biological processes, which includes as an effector molecule, neuronal messenger, vasodilator, antimicrobial agent, etc. [47]. Curcumin and curcuminoids present in *Curcuma* species reduced the amount of nitrite formed by the reaction between oxygen and nitric oxide generated from sodium nitroprusside and scavenge

the free radicals [48]. The nitric oxide scavenging activity was highest in ethanolic extract of *C. aromatica* i.e. 85.71% and minimum scavenging activity was observed in the methanolic extract of *C. aromatica* (MJA-13%). Curcumin was used as a standard and its value to scavenge NO was 162.96 ± 9.81 μ g. Ethanol, acetone and ethyl acetate extract of all the three species of *Curcuma* showed higher scavenging activity. The EC₅₀ value of NO assay ranged from 116.70 ± 0.76 μ g to 678.70 ± 10.51 μ g/mL (Table 2). The EC₅₀ value or order of activity was as follows: ECA > DCL > ACL > EaCA > CCL > CCA > ACA > CCC > MCL > ACC > ECL > DCC > EaCC > EaCA > MCC > ECC > EaCL > MCA.

3.9. Phosphomolybdate assay for evaluation of antioxidant capacity

Reduction of molybdenum (VI) to molybdenum (V) by the antioxidants and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH show a maximum absorbance at 695 nm [24]. Table 2 represents varying phosphomolybdate activity of different plant extracts of all the *Curcuma* species. Methanolic extract of *C. aromatica* exhibited the highest antioxidant power (271.3 ± 0.95) μ g/500 μ g eq to ascorbic acid while lowest antioxidant effect was showed chloroform extract of *C. aromatica*. Methanolic fractions of all the three species of *Curcuma* showed the highest antioxidant capacity in phosphomolybdate (PM) assay. Based on the results the order of antioxidant capacity of plant extracts was MCA > MCL > MCC > DCA > ECL > ECC > CCC > DCC > EaCC > ACA > EaCA > ECA > ACL > CCL > DCL > EaCL > ACC > CCA. The reports indicate that the antioxidant capacity of *C. caesia* is better than *Curcuma amada* in phosphomolybdate assay where its value range from 2457.33 and 4534.33 mM/100mg AAE (Reenu J et al, 2015).

3.10. Lipid peroxidation by TBA assay

Lipid peroxidation (LPO) is used as an indicator of oxidative stress in cells and tissues. LPO is measured by thiobarbituric acid reactive substances (TBARS) method. It is a well-established method for assessment of cellular injury in plant and animals. The decomposition of lipid peroxides produces several reactive compounds. The measurement of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) generates from polyunsaturated fatty acid peroxides are indicators of lipid peroxidation [49].

MDA is a marker of oxidative stress [50]. In this study, the potential of all extracted samples of *Curcuma* species was measured to inhibit the lipid peroxidation in chicken liver

homogenate induced by the $\text{FeCl}_2 - \text{H}_2\text{O}_2$ system. Table 2 shows varying range of inhibition potential of all the extracts of *Curcuma*. Highest LPX inhibition was found in ethanolic extract of *C. aromatica* ($85.13 \pm 0.23 \%$) and lesser extent in chloroform extract of *C. caesia* ($26.18 \pm 0.50\%$). Ethanol and dichloromethane extract of all the three species showed great LPX inhibition and protected cell damage at $85.13 \pm 0.23\%$ and most of the extract showed greater than 70% inhibition. The result supported that turmeric as a natural antioxidant can be used to prevent the oxidative deterioration of fat containing food systems or reactive oxygen species mediated tissue damage in biological systems [18].

3.11. Characterization of *Curcuma* extracts

3.11.1. Spectral analysis

The UV visible spectra of all the plant extracts of three targeted *Curcuma* species is shown in Fig. 1. The characteristic peaks of different extracts were observed in the range of 230-400 nm. *C. longa* extracts absorption spectra in range between 230-425 nm and the maximum absorption shown at 412-425 nm, similar to curcumin which confirms presence of curcumin in *C. longa* extracts. For curcumin identification *C. longa* showed absorption EaCL at ($A_{412\text{nm}} - 0.361$), ACL ($A_{414\text{nm}} - 2.115$), ECL ($A_{412\text{nm}} - 2.407$) likewise. *C. caesia* and *Curcuma aromatica* fractions showed varying spectrum as compare to *C. longa* extracts and in this case, spectrum is compared to camphor, present in those species. Most of the absorption of *C. caesia* extracts has ranged from 260-300 nm, much more similar to camphor. Ethyl acetate extract of *C. caesia* have showed maximum absorption at 276 nm and *C. aromatica* (EaCA) at 289 nm.

3.11.2. FTIR analysis of plant extracts

FTIR spectra of all the successive extracts of *Curcuma* species showed typical bands and variations corresponding to different functional groups (Fig. 2). Results revealed variations in band intensities of different extracts of *Curcuma* species. Functional groups were strongly affected by the extraction methods as well as the type of solvent used. Different intensities for aromatic -OH groups were found in all the extracts of *Curcuma* at $3385\text{-}3509\text{ cm}^{-1}$. Methyl (CH_3) and methylene ($-\text{CH}_2$) symmetric and assymetric vibration found in-group of curcuminoids were ranging from $2800\text{-}3000\text{ cm}^{-1}$ [51]. C=O stretching of carboxyl and ketone group appeared in all the extracts at $1652\text{-}1750\text{ cm}^{-1}$, aromatic skeletal vibration was found at

1500-1600 cm⁻¹. The peaks at 1521-1616 cm⁻¹ associated with ring conjugated C=C stretch and benzene ring bending vibration were observed in all the extracts of *Curcuma* [52]. The stretching peaks at 1360-1576 cm⁻¹ related to C=C medium, weak, multiple bands of aromatic ring or aromatic skeleton vibration and O-CH₃, C-H deformation. The vibration mode at 1027-1270 cm⁻¹ related to C-C, C-O and C-N groups observed in different intensities. The vibration modes at 1512-1570 cm⁻¹ and 1340-1379 cm⁻¹ showed N-O stretching of strong or two-band intensity of extracts of *Curcuma*. The band at 1000-670 cm⁻¹ appeared mainly due to aromatic C-O, C-H, C=O, alkyl halide.

3.11.3. GC-MS/MS analysis of active extract

The amount of phyto-constituents in turmeric rhizomes vary due to different varieties, locations, agroclimatic zones, sources and cultivation condition. GC-MS/MS chromatograms of ECL (Fig. 3), ECC (Fig. 4), ECA (Fig. 5) rhizome extracts of *C. longa*, *C. caesia* and *C. aromatica* respectively, showed various peaks indicating the presence of different compounds in the respective extracts.

GC-MS/MS chemometric profile of ECL illustrated the presence of 40 different compounds (Table 3, Fig. 3). Amongst the identified compounds, n-tetracosanol-1 (12.06%), 1-hexadecene (11.48%), 1,6-heptadiene,2-methyl-6-phenyl (10.99%) were found in major amount, whereas curlone (3.85%), turmerone (2.25%), Ar-turmerone (2.03%), Dodecanol (0.84%) etc. were found to be present in trace. ECC illustrated the presence of 34 different compounds, 1-Heneicosanol (19.13%), n-Tetracosanol-1 (8.07%), 1-hexadecene (8.94%), 3-Octadecene, (E)- (6.16%) were found in major amount, whereas Andrographolide (0.63%), (-)-(4R,5S,6R)-4,5,6-TRIS-[[(Tert butyl)dimethylsilyl]oxy}cyclohex- (0.36%), 1,5-Diphenylhex-3-ene (0.55%) etc. identified in minor (Table 4, Fig. 4). Several trace compounds found in ECA were 1-octadecene (12.39%), n-Tetracosanol-1 (15.88%), 1,6-Heptadiene, 2-methyl-6-phenyl- (8.84%) were found in major amount whereas 1-Eicosanol (3.99), Non-adeacyl-pentafluoropropionate (1.66%), 1-(trimethylsilyl) cyclopropanethiol (0.69%) etc. represented in Table 5 and Fig. 5. The distribution of phytochemical constituents and biological activities of active principles present in different rhizome extracts of *Curcuma* species is shown in Table 6 and Table 7.

Most of the compounds identified from rhizome extracts of *Curcuma* were terpenoids, diterpene, triterpene and sesquiterpene. Andrographolide was reported for first time in *C. Caesia* rhizome, which possesses lots of medication. Furthermore, camphor found in *C. caesia*, turmerone, Ar-turmerone, beta- turmerone found in *C. longa* significantly favor the result of *Curcuma* species due to its high pharmaceutical importance. Antimicrobial property of Cyclo octa siloxane hexa decamethyl reported in previous study [53] proved the high antibacterial response of *C. aromatica*. The compounds identified by GC-MS/MS analysis were found to possess a diverse range of pharmacological actions [54]

Conclusion

The plant extracts of all the *Curcuma* species exhibited radical scavenging activity in different *in vitro* assays, therefore, it can be concluded from the study that the selected species of *Curcuma* posses antioxidant activity. *C. caesia* showed highest *in vitro* α -amylase inhibitor activity might be a good source of antidiabetic drug in future. *C. aromatica* had highest potential to inhibit the growth of bacteria due to its highest flavonoid content. Further studies on non-conventional *C. caesia* and *C. aromatica* as antioxidant, antidiabetic and antibacterial in animal models are required to assess their long-term pharmaceutical benefits.

List of abbreviations

Reactive oxygen species = ROS

Butylated hydroxyl toluene = BHT

Butylated hydroxyl anisole = BHA

Total polyphenolic content = TPC

Microbial type culture collection = MTCC

2,4,6-tripyridyl-s-tiazine = TPTZ

Total reducing power = TRP

Ferric reducing power assay = FRAP

494 Gallic acid equivalents = GAE
495 Malondialdehyde = MDA
496 4-hydroxyalkenals = HAE
497 2,2-diphenyl-1-picrylhydrazyl = DPPH
498 2,2'-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid = ABTS
499 Trichloro acetic acid = TCA
500 Quercetin Equivalents per mg of extract = QUEs
501 Nutrient agar media = NAM
502 Mass selective detector = MSD
503 Phosphomolybdate= PM
504 Ferric chloride = FeCl₃
505 Naphthyl ethylene dianilinedihydro chloride=NEDD
506 Lipid peroxidation = LPO

507

508 **Declaration**

- 509 • Ethics approval and consent to participate – No animals and humans were included in the
510 study.
511 • Consent for publication – Not applicable.
512 • Availability of data and materials – It can be made available on request.
513 • Competing interests – There is no competing interest.
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Legends to the Figures:

Figure 1 UV visible spectrum of *C. aromatica*, *C. caesia* and *C. longa* rhizome extracts

Where, CCA = Chloroform extract of *C. aromatica*, DCA = Dichloromethane extract of *C. aromatica*, ACA = Acetone extract of *C. aromatica*, EaCA = Ethyl acetate extract of *C. aromatica*, MCA = Methanol extract of *C. aromatica*, ECA = Ethanol extract of *C. aromatica*, CCC = Chloroform extract of *C. caesia*, DCC = Dichloromethane extract of *C. caesia*, ACC = Acetone extract of *C. caesia*, EaCC = Ethyl acetate extract of *C. caesia*, MCC = Methanol extract of *C. caesia*, ECC = Ethanol extract of *C. caesia*, CCL = Chloroform extract of *C. longa*, DCL = Dichloromethane extract of *C. longa*, ACL = Acetone extract of *C. longa*, EaCL = Ethyl acetate extract of *C. longa*, MCL = Methanol extract of *C. longa* and ECL = Ethanol extract of *C. longa*

Figure 2 FTIR spectra of *C. aromatica*, *C. caesia* and *C. longa* rhizome extracts Where, CCA = Chloroform extract of *C. aromatica*, DCA = Dichloromethane extract of *C. aromatica*, ACA = Acetone extract of *C. aromatica*, EaCA = Ethyl acetate extract of *C. aromatica*, MCA = Methanol extract of *C. aromatica*, ECA = Ethanol extract of *C. aromatica*, CCC = Chloroform extract of *C. caesia*, DCC = Dichloromethane extract of *C. caesia*, ACC = Acetone extract of *C. caesia*, EaCC = Ethyl acetate extract of *C. caesia*, MCC = Methanol extract of *C. caesia*, ECC = Ethanol extract of *C. caesia*, CCL = Chloroform extract of *C. longa*, DCL = Dichloromethane extract of *C. longa*, ACL = Acetone extract of *C. longa*, EaCL = Ethyl acetate extract of *C. longa*, MCL = Methanol extract of *C. longa* and ECL = Ethanol extract of *C. longa*

Figure 3 GC- MS/MS chromatogram of ethanolic extract of *C. longa* rhizome

Figure 4 GC- MS/MS chromatogram of ethanolic extract of *C. caesia* rhizome

Figure 5 GC- MS/MS chromatogram of ethanolic extract of *C. aromatica* rhizome

Figures

Figure 1

UV visible spectrum of *C. aromatica*, *C. caesia* and *C. longa* rhizome extracts Where, CCA = Chloroform extract of *C. aromatica*, DCA = Dichloromethane extract of *C. aromatica*, ACA = Acetone extract of *C. aromatica*, EaCA = Ethyl acetate extract of *C. aromatica*, MCA = Methanol extract of *C. aromatica*, ECA = Ethanol extract of *C. aromatica*, CCC = Chloroform extract of *C. caesia*, DCC = Dichloromethane extract of *C. caesia*, ACC = Acetone extract of *C. caesia*, EaCC = Ethyl acetate extract of *C. caesia*, MCC = Methanol extract of *C. caesia*, ECC = Ethanol extract of *C. caesia*, CCL = Chloroform extract of *C. longa*, DCL = Dichloromethane extract of *C. longa*, ACL = Acetone extract of *C. longa*, EaCL = Ethyl acetate extract of *C. longa*, MCL = Methanol extract of *C. longa* and ECL = Ethanol extract of *C. longa*

Figure 2

FTIR spectra of *C. aromatica*, *C. caesia* and *C. longarhizome* extracts Where, CCA = Chloroform extract of *C. aromatica*, DCA = Dichloromethane extract of *C. aromatica*, ACA = Acetone extract of *C. aromatica*, EaCA = Ethyl acetate extract of *C. aromatica*, MCA = Methanol extract of *C. aromatica*, ECA = Ethanol extract of *C. aromatica*, CCC = Chloroform extract of *C. caesia*, DCC = Dichloromethane extract of *C. caesia*, ACC = Acetone extract of *C. caesia*, EaCC = Ethyl acetate extract of *C. caesia*, MCC = Methanol

extract of *C. caesia*, ECC = Ethanol extract of *C. caesia*, CCL = Chloroform extract of *C. longa*, DCL = Dichloromethane extract of *C. longa*, ACL = Acetone extract of *C. longa*, EaCL = Ethyl acetate extract of *C. longa*, MCL = Methanol extract of *C. longa* and ECL = Ethanol extract of *C. longa*

Figure 3
GC- MS/MS chromatogram of ethanolic extract of *C. longa* rhizome

Figure 4

GC- MS/MS chromatogram of ethanolic extract of *C. caesia* rhizome

Figure 5

GC- MS/MS chromatogram of ethanolic extract of *C. aromatica* rhizome

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