Pak. J. Agri. Sci., Vol. 53(4), 941-946x; 2016
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:
http://www.pakjas.com.pk

COMPARATIVE STUDY OF CONVENTIONAL SOLVENT AND SUPERCRITICAL FLUID EXTRACTS OF TURMERIC USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Dietary intrusions emphasize on the dynamic facets of phytonutrients as they exert positive health impact against various metabolic disorders. Amongst, turmeric bioactive ingredients are well known for its strong antioxidant potential. Purposely, turmeric nutraceutic i.e. curcumin was isolated followed by quantification using high performance liquid chromatography (HPLC). For optimal extraction of curcumin, three conventional solvents (aqueous ethanol, -methanol & -acetone), each at 35, 50 and 65 min and supercritical carbon dioxide at varying time intervals; 50, 100 & 150 min were employed. The resultant conventional extracts were tested for total phenolic contents (TPCs), 2,2-diphenyl 1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)/ABTS, ferric reducing antioxidant power (FRAP) and iron chelating assays. Aqueous ethanolic extract showed best results at 65 min; TPC 917.06±10.08 mg GAE/100g, DPPH 65.12±2.87%, FRAP 194.47±8.03 µM Fe^{2+}/g, ABTS 163.14±6.12 µM Trolox/g and iron chelation activity 66.92±2.95%. Furthermore, the best selected conventional solvents and supercritical fluid extracts were quantified via HPLC system. Results revealed highest curcumin yield in supercritical fluid extracts *i.e.* 52.41±2.38 mg/g at 150 min followed by 46.03±2.15 and 33.62±1.24 mg/g at 100 & 50 min, respectively. While, conventional ethanolic, methanolic and acetonic extracts showed values as 31.48±1.35, 28.75±1.09 and 23.19±1.12 mg/g, respectively.
Keywords: Turmeric, supercritical, HPLC, curcumin, antioxidant, total phenolics.

INTRODUCTION

Amongst scientific communities, use of dietary therapies, natural antioxidants from fruits & vegetables and herbs & spices has been rising. Epidemiological studies have exposed that frequent inclusion of natural antioxidants in daily menu is responsible to prevent lifestyle related malfunctions. However, generation of reactive oxygen species (ROS) is an innate phenomenon that damages the integrity of biomolecules in case if balance between free radicals and antioxidants get devastated (Lim and Han, 2016). Polyphenols are the dominant group of phytoceutics present in plant based edibles such as fruits & vegetables, cereal and spices (Sardar *et al*., 2012). The active ingredients in these edibles are associated with health boosting and diseases preventive agents (Suleria *et al*., 2015). In this context, spices are worth considering for their antioxidant potential as corroborated by various efficacy studies with special reference to cinnamon, turmeric, cloves and anise (Kochhar, 2008; Suleria *et al*., 2013). Moreover, there are strong evidences supporting the presence of phytoceutics in spices and their positive health impact besides serving as culinary ingredients (Srinivasan, 2005; Aggarwal *et al*., 2009; Kunwar *et al*., 2011). Basically, spices are seasonings with aromatic properties and often incorporated in the traditional culinary (Suleria *et al*., 2015). Amongst, turmeric is one of the important herbs *i.e.* widely used as spice, culinary additive, medicine, condiment, dye and cosmetic (Lal, 2012).

Turmeric (*Curcuma longa*), botanically belongs to *Zingiberaceae* family and extensively cultivated in China, India, Pakistan, Bangladesh and other tropical regions of South Asia. In Pakistan, it is grown in Kasoor, Lahore, Okara, Bannu and Mirpurkhas; however, its per hectare production is low due to poor agricultural practices (Kiran *et al*., 2013). Turmeric rhizome have plethora of secondary metabolites. The principal bioactive moieties of turmeric rhizome are curcuminoids; complex of three analogues, curcumin I, II & III depending on structural configuration and essential oil. The compositional analysis showed variations from 3-5% in curcuminoids in addition to vitamin C, E and *β*-carotene (Balasasirekha and Lakshmi, 2012).

Phytotherapies based on turmeric bioactives tends to improve the health of the individuals through the scavenging free radicals. Chemical analysis elucidated that *α, β*-unsaturated carbonyl groups of turmeric polyphenols i.e. curcumin are involved in neutralizing nucleophiles. As curcumin exhibits diketone structure, it tends to tautomerize between ketonic and enolic forms that are important for antioxidant potential (Manjunatha and Srinivasan, 2007;

Kelkel *et al*., 2010). The antioxidant power of curcumin was attributed to its functional moieties i.e. *β*-diketone, responsible for free electron transfer. Either it directly interacts with free radical species or propel signal to various targeted molecules (Aggarwal and Sung, 2009). To evaluate antioxidant indices of curcumin, various phytoscreening tests are employed followed by extraction using various organic solvents. Depending on the mode of action, commonly two methods are utilized; one associated with free electron scavenging ability whilst, other based on lipid peroxidation. The former includes DPPH, ABTS, FRAP and ferrous ion chelating (FICA) (Moon and Shibamoto, 2009; Suleria *et al*., 2012).

The reported health claims of curcumin highlighted the need for analytical techniques to isolate this biomolecule. The preliminary step for characterization of curcumin is optimizing extraction conditions to maximize recovery from concentrated source i.e. turmeric. Most of the extraction studies focused on solvent extraction followed by HPLC or spectrophotometric quantification (Imran *et al*., 2015; Osorio-Tobon *et al*., 2016). However, supercritical carbon dioxide extraction technique has acquired wider interest owing to its efficient penetrating power resulting in lowering the degradation of antioxidants (Sticher, 2008). Keeping in view the aforementioned facts, instant project was planned to assess the influence of extraction conditions (solvents & time) on antioxidant capacity of turmeric extracts and comparative analysis of curcumin recovery using conventional and supercritical fluid extraction techniques.

MATERIALS AND METHODS

***Procurement of raw material*:** The study was conducted in the Functional and Nutraceutical Food Research Section of National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad. Fresh rhizomes of turmeric (Kasur) were obtained from Ayub Agriculture Research Institute (AARI), Faisalabad. Turmeric rhizomes were cleaned in order to remove adherent soil. Afterwards, it was oven dried at 60°C and ground for further analyses.

***Preparation of turmeric extracts*:** Various treatments of turmeric extracts were prepared using binary solvent system (50% v/v); aqueous ethanol, -methanol and -acetone each at various extraction times (35, 50 and 65 min) according to method of Bagchi *et al*. (2012). All the extracts were agitated at 300 rpm in the orbital shaker at 25°C for 60 min. Further, they were filtered using Whatman filter paper following concentrating via rotary evaporator. The ensuing samples were kept at 4°C for future analyses.

In Vitro Antioxidant Activity for CSE:

***Total phenolics*:** Total polyphenol contents (TPC) of turmeric extracts were measured through method of Folin-Ciocalteu as mentioned by Himesh *et al*. (2011). Purposely, turmeric extracts (50 µL) was individually added to respective test tubes holding Folin-Ciocalteu's reagent (250 µL) and sodium carbonate solution (750 µL). The final volume was made using distilled water up to 5mL. The optical density (absorbance) was recorded at wavelength of 765 nm after two hours through UV/visible Spectrophotometer against blank containing all reagents apart from turmeric extract. All measurements were performed in triplicate using gallic acid as standard, expressing the results as mg/100g of gallic acid equivalent (GAE). The total polyphenols in each extract were measured by using mentioned expression;

$$C = c \times V / m$$

C = Total phenolic contents (mg GAE/g plant extract); c = Gallic acid concentration (mg/mL); V = Extract volume (mL); m = Sample weight (g)

***Free radical scavenging capacity (DPPH assay)*:** The extracts were screened for DPPH (1,1-diphenyl-2-picrylhydrazyl) free reactive species scavenging ability following the method of Kumar *et al*. (2006). In this context, fresh DPPH solution (3 mL) in relevant extracting medium (0.6 µM) was added in 77 µL sample. Each extract and blank (having solvent and DPPH solution excluding extract) were kept for 15 min in dark. Afterwards, absorbance reduction was noticed in tested extracts at 517 nm using UV/visible spectrophotometer. Blank sample's absorbance was also determined at 517 nm through UV/visible spectrophotometer. The percent inhibition of DPPH radical was calculated as:

$$\text{Absorbance reduction (\%)} = [(A_B - A_A) / A_B] \times 100$$

A_B = Blank sample absorbance at t = 0 min; A_A = Tested extract absorbance at t = 15 min

***Ferric reducing antioxidant power (FRAP assay)*:** The obtained extracts were analyzed for ferric reducing ability by following the guidelines of Asimi *et al*. (2013) with some modifications. As per protocol, fresh FRAP reagent was made by addition of acetate buffer (50 mL), TPTZ (2, 4, 6-tripyridyl-S-triazine) solution (5 mL) in HCL (40 mmol/L) into 5 mL of $FeCl_3.6H_2O$ (20 mmol/L of water solution). Afterwards, 50 µL of turmeric extract was added to FRAP reagent (950 µL) for 4 min. Then, absorbance of blue colored sample was quantified against blank at 593 nm spectrophotometrically. Prepared solutions of $FeSO_4.7H_2O$ (100-1000 µM) were employed for calibration and values were represented as micromoles/gram Fe (II).

***ABTS radical decolorization assay*:** For the estimation of ABTS free radical scavenging ability of turmeric extracts, a method developed by Hossain *et al*. (2008) was followed with certain modifications. Freshly prepared ABTS radical contains 2.45 mM potassium persulfate (5 mL), 7 mM ABTS solution (5 mL) and volume was made to 10 mL. The mixture then transferred to amber bottle covered with aluminum foil and placed in dark for 16 hr until reaching a stable oxidized state. To get an absorbance of 0.700 at 734 nm, prepared mixture was diluted further with respective ethanol solution (1:89 v/v). Afterwards, ABTS solution (1

mL) was added to turmeric extract (10 µL) and mixed vigorously with vortex mixer for 10 sec and then after 30 min the absorbance was measured at 734 nm using a UV-visible spectrophotometer. Values obtained were compared with that of control ABTS solution and were reported in µmol Trolox/g sample extract.

***Ferrous ion chelating assay*:** The metal ion chelation was determined by method of Cousins *et al.* (2007) with modification. The chelating activity of the sample extract was quantified by computing the absorbance reduction of iron (II)-ferrozine complex at 560 nm. 100 µL solution of $FeSO_4$ (2 mM) was mixed with 100 µL of turmeric extract followed by addition of 100µL of ferrozine (5 mM). The mixture was stayed for 10 min and absorbance was calculated at 560 nm using spectrophotometer using EDTA (standard) as controls. The ability of turmeric extract to chelate ferrous ion relative to the control containing only iron ferrozine was calculated using the following equation:

$$\text{Chelating effect (\%)} = \frac{A_C - A_S}{A_C} \times 100$$

A_C = Absorbance of control; A_S = Absorbance of turmeric extract

***Supercritical fluid extraction (SFE)*:** Supercritical fluid extracts of dried turmeric rhizome were obtained through SFT-150system using 99.8% pure CO_2. After the placement of sample in 100 mL extraction vessel, CO_2 was liquefied by optimizing at three different time intervals *i.e.* 50, 100 and 150 min while keeping pressure and temperature conditions constant to accelerate the solvation & mass transfer of curcumin (Wakte *et al*., 2011).

***HPLC quantification of active ingredient*:** From the conventional solvent extracts (Table 1), three best treatments selected on the basis of *in vitro* studies and all SFE samples (Table 2) were tested for HPLC analysis. Accordingly, samples were prepared for analysis using 100 µL of three SFE and 500 µL of each CSE along with 900 µL & 500 µL of mobile phase, respectively. All the vials were subjected to vortex mixing using gyromixer and then filtered before subjected to HPLC analysis (PerkinElmer, Series 200, USA) equipped with shim-pack C_{18} column (15 cm x 4.6 mm, 5.0 µm particle size) along with autosampler of 10 µL sample at 25°C column temperature. Mobile phase comprised of solvent methanol with 1.2 mL/min flow rate using isocratic elution. Quantification of curcumin was carried out by UV detector at 245 nm, by comparing the retention time of sample peaks with standard (Himesh *et al*., 2011).

***Statistical analysis*:** The obtained results were analyzed through completely randomized design (CRD) using Cohort version 6.1 (Costat-2003). Furthermore, Analysis of variance (ANOVA) was performed to calculate significance level (Steel *et al*., 1997).

RESULTS

The observed values for effect of solvent elicited the highest TPC in ethanol turmeric extract 1106.54±12.17 mg GAE/100g trailed by methanol 736.29±8.83 mg GAE/100g and acetone 552.90±6.08 mg GAE/100g extracts. Considering time interval, the highest polyphenoles were detected at 65 min by 917.06±10.08 mg GAE/100g following 50 min by 803.52±9.64 mg GAE/100g. However, the initial time interval, 35 min showed the least TPC value (675.10±7.42 mg GAE/100g) (Fig. 1).

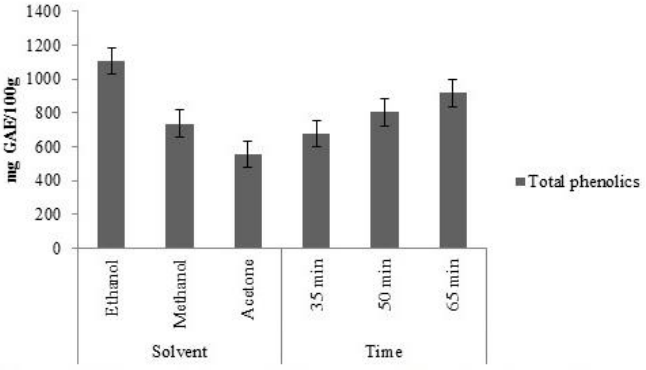

Figure 1. Effect of solvents and time on total phenolics of turmeric extracts.

Likewise, maximum DPPH value (65.71±3.40%) was exhibited by ethanol followed by methanol (59.82±2.51%) and minimum output (48.71±2.01%) in acetone extract. Time affected the DPPH with maximum value 65.12±2.87% at 65 min whereas the lowest value 50.49±1.72% at 35 min. (Table 1). The FRAP values in ethanol, methanol and acetone extract were 212.89±9.95, 190.41±7.04 and 149.63±4.94 µM Fe^{2+}/g, respectively. Similarly, means regarding effect of time showed highest FRAP (194.47±8.03µM Fe^{2+}/g) value in the resultant extracts at 65

Table 1. Effect of time and solvent on turmeric DPPH (%).

Treatments	Time			Means
	35 min	50 min	65 min	
Ethanol	54.93±1.86	66.37±2.45	75.85±2.57	65.71±3.40a
Methanol	53.85±2.20	60.39±2.12	65.24±2.84	59.82±2.51b
Acetone	42.71±1.79	49.16±2.23	54.27±1.78	48.71±2.01c
Means	50.49±1.72c	58.64±2.41b	65.12±2.87a	

Table 2. Effect of time and solvent on turmeric FRAP (µM Fe^{2+}/g).

Treatments	Time			Means
	35 min	50 min	65 min	
Ethanol	187.89±5.63	221.82±6.29	228.97±7.16	212.89±9.95a
Methanol	179.52±6.46	191.95±5.72	199.75±6.57	190.41±7.04b
Acetone	144.13±5.28	150.09±5.47	154.68±6.15	194.63±4.94c
Means	170.51±5.45c	187.95±7.33b	194.47±8.03a	

Table 3. Effect of time and solvent on turmeric ABTS (µM Trolox/g).

Treatments	Time			Means
	35 min	50 min	65 min	
Ethanol	170.87±5.46	175.14±6.28	178.55±6.07	174.85±7.86a
Methanol	152.51±6.13	159.05±5.79	165.68±6.24	159.08±6.71b
Acetone	141.30±5.27	143.58±5.42	145.17±5.76	142.32±5.14c
Means	154.90±6.35b	159.26±5.97ab	163.14±6.12a	

Table 4. Effect of time and solvent on turmeric metal chelation (%).

Treatments	Time			Means
	35 min	50 min	65 min	
Ethanol	69.51±2.23	73.34±2.78	77.23±3.45	73.36±3.32a
Methanol	61.52±2.46	67.12±1.82	69.94±2.58	66.19±2.48b
Acetone	48.25±1.49	51.66±1.65	53.59±2.14	51.16±2.17c
Means	59.76±3.32b	64.04±2.48ab	66.92±2.17a	

min. The extracts at 35 min reflected lowest value 170.51±5.45 µM Fe^{2+}/g for this trait (Table 2). The value for ABTS was also highest in ethanol and methanol as compared to acetone as 174.85±7.86, 159.08±6.71 and 142.32±5.14 µM Trolox/g, correspondingly. Moreover, extraction time of 65 min exposed maximum ABTS 163.14±6.12 µM Trolox/g while 35 min revealed minimum values as 154.90±6.35 µM Trolox/g, correspondingly (Table 3). Means for metal chelation illuminated the highest value (73.36±3.32%) in ethanolic extract followed by methanol (66.19±2.48%) whilst acetone exhibited the lowest value (51.16±2.17%). Furthermore, 65 min extraction time resulted in higher metal chelation activity 66.92±2.95% in comparison with 50 and 35 min by 64.04±2.48 and 59.76±2.04%, respectively (Table 4). However, interaction effect time and solvent on all these parameters is non-significant.

Inferences regarding HPLC quantification (Fig. 2) for turmeric bioactive moiety indicated momentous changes in curcumin content of Supercritical Fluid & Conventional Solvent Extracts (SFE & CSE) as function of treatments. Means for the effect of supercritical fluid extraction conditions on curcumin content elucidated highest yield (52.41±2.38 mg/g) in extract obtained at 150 min trailed by CO_2 (100) (46.03±2.15 mg/g) at 100 min. However, lowest curcumin content was measured in CO_2 (50) accounting 33.62±1.24 mg/g at 50 min. Amongst conventionally obtained extracts, the maximum curcumin yield was detected in ethanol as 31.48±1.35 mg/g followed by 28.75±1.09 and 23.19±1.12 mg/g dry matter in methanol and acetone, correspondingly.

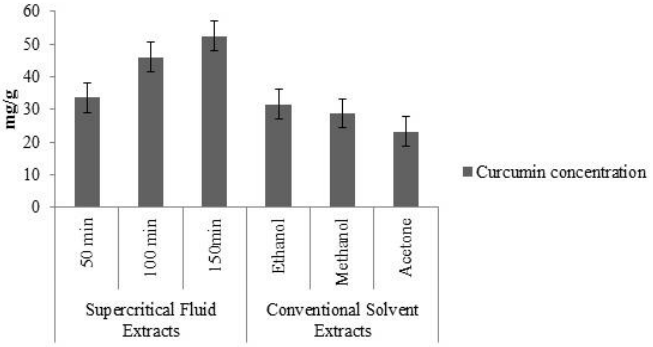

Figure 2. HPLC quantification for curcumin in different turmeric extracts.

DISCUSSION

In the current research, the comparative alterations in antioxidant profile of turmeric extract are elucidated using different organic solvents; ethanol, methanol & acetone and variation in extraction efficiencies of turmeric active ingredient *i.e.* curcumin using two different modes of extraction; Conventional Solvent Extraction (CSE) and Supercritical Fluid Extraction (SFE). In conventional solvent extraction system, choice of solvent is an important aspect that depends on its polarity, solubility of the desired compound, rate of mass transfer, cost and accessibility (Imran *et al*., 2016). For the extraction of lipophilic

ingredients like curcumin *i.e.* turmeric polyphenol, organic solvents such as ethanol, methanol, acetone and ethyl alcohol are normally practiced (Jansirani *et al*., 2014). We observed that antioxidant activity of turmeric extracts significantly ($p<0.05$) varies with solvents and extraction time. Resultantly, the antioxidant potential of all extracts increased with passage of time from 35 to 65 min. However, ethanol was regarded as a good extraction medium at 65 min due to its compatible polarity as that of turmeric polyphenols.

Innumerable evidences have confirmed ten times higher electron transferring ability of curcumin in contrast to α-tocopherol and considered it safer than other primary bodes. Additionally, turmeric polyphenols scavenge free radicals by deprotonation of phenolic group following the single electron transfer present in heptadienone structure of curcumin under polar medium (Aggarwal and Sung, 2009; Galano *et al*., 2009). The outcomes of present study are supported by the findings of Tiveron *et al.* (2012), observed TPC, ABTS and FRAP in turmeric powder as 1279.53 mg/100g GAE, 118.6 µmol Trolox/g and 169.1 µmol Fe^{2+}/g dry weight, respectively. Furthermore, effect of three solvents; ethanol, methanol and water on extraction efficiency of turmeric phenolics showed that ethanol extract exhibits more phenolics & antioxidant potential (745.76 mg GAE/100g & 52.19%) than methanol (682.43 mg GAE/100g & 49.83%) and water (496.76 mg GAE/100g & 31.33%), correspondingly. It is accredited to isolation competence of ethanol and chain breaking capability of extracted curcumin by the reason of capturing free radicals in conjugated structure (Nisar *et al*., 2015).

Extraction through conventional methods is very laborious and time consuming. Nowadays, fluid phase extraction above or near critical state *i.e.* temperature and pressure is among the novel methods for isolation of heat labile phytochemicals using pressurized liquids. It allows separation of nutraceuticals at low temperature, avoiding thermal degradation of desired components. Moreover, CO_2 is the most widely used fluid in supercritical fluid extraction as it liquefies at 300 bar pressure and temperature <31°C. It is a safer technique hence named as green extraction technology, inexpensive and facilitates mass transfer of active moieties without leaving any residues in resultant extract (Junior *et al*., 2010). Under supercritical conditions, extraction efficiency of curcumin can be modified by varying time and pressure. Moreover, accurate information about exact quantity of curcumin in turmeric is important to assess its effective dosage for higher biological activity. In this regard, HPLC analysis of ensuing extracts is a mandatory tool for the characterization and quantification of bioactive components.

In instant research, curcumin extraction was made at three different time intervals *i.e.* 50, 100 and 150 min. It was noticed that at higher extraction time (150 min), the duration for solid to solvent contact increased that allows maximum valorization of curcumin from dried turmeric powder. In this context, Pyo and Kim (2014) evaluated the effect of three independent variables *i.e*. pressure (200, 225, 250 and 275 atm), time (90, 120 and 150 min) & temperature (40, 50, 60 and 70°C) on extraction efficiency of curcumin under supercritical conditions. Maximum curcumin content was found at pressure of 250 atmosphere (atm) and 60°C temperature. But it decreased with rise in temperature due to very low density of supercritical fluid. On the other hand, increment in extraction time from 90 to 150 min enhanced curcumin recovery from 26.45 to 31.07 mg/g that significantly increased between 120 to 150 min. From current exploration, it is deduced that novel extraction methods like supercritical fluid extraction technique should be practiced to achieve maximal purity and yield of heat labile bioactive moieties.

***Conclusions*:** The isolation efficiency of curcumin varies as function of extraction techniques *i.e.* conventional and supercritical fluid separation tools. The instant findings explicated that extraction capacity of ethanol for curcumin is more than methanol and acetone as its polarity apt for its separation from raw material. Moreover, extraction using supercritical fluid as isolation medium allows rapid and higher yield of curcumin in contrast to conventional solvents.

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