Dear Author,

Please, note that changes made to the HTML content will be added to the article before publication, but are not reflected in this PDF.

Note also that this file should not be used for submitting corrections.

Fitoterapia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

New findings on the in vivo antioxidant activity of *Curcuma longa* extract by an integrated ^{1}H NMR and HPLC–MS metabolomic approach

Stefano Dall'Acqua [a,*], Matteo Stocchero [b], Irene Boschiero [a], Mariano Schiavon [a], Samuel Golob [c], Jalal Uddin [d], Dario Voinovich [c], Stefano Mammi [d], Elisabetta Schievano [d]

[a] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via Marzolo 5, 35131 Padova, Italy
[b] S-IN Soluzioni Informatiche, Via Ferrari 14, 36100 Vicenza, Italy
[c] Department of Chemical and Pharmaceutical Sciences, University of Trieste, Via Giorgieri 1, 34127 Trieste, Italy
[d] Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy

ARTICLE INFO

Article history:
Received 16 November 2015
Received in revised form 15 December 2015
Accepted 17 December 2015
Available online xxxx

Keywords:
Antioxidants
In vivo
Curcuma longa
Metabolomics
NMR
HPLC–MS

ABSTRACT

Curcuminoids possess powerful antioxidant activity as demonstrated in many chemical *in vitro* tests and in several *in vivo* trials. Nevertheless, the mechanism of this activity is not completely elucidated and studies on the *in vivo* antioxidant effects are still needed. Metabolomics may be used as an attractive approach for such studies and in this paper, we describe the effects of oral administration of a *Curcuma longa* L. extract (150 mg/kg of total curcuminoids) to 12 healthy rats with particular attention to urinary markers of oxidative stress. The experiment was carried out over 33 days and changes in the 24-h urine samples metabolome were evaluated by ^{1}H NMR and HPLC–MS. Both techniques produced similar representations for the collected samples confirming our previous study. Modifications of the urinary metabolome lead to the observation of different variables proving the complementarity of ^{1}H NMR and HPLC–MS for metabolomic purposes. The urinary levels of allantoin, *m*-tyrosine, 8-hydroxy-2′-deoxyguanosine, and nitrotyrosine were decreased in the treated group thus supporting an *in vivo* antioxidant effect of the oral administration of *Curcuma* extract to healthy rats. On the other hand, urinary TMAO levels were higher in the treated compared to the control group suggesting a role of curcumin supplementation on microbiota or on TMAO urinary excretion. Furthermore, the urinary levels of the sulphur containing compounds taurine and cystine were also changed suggesting a role for such constituents in the biochemical pathways involved in *Curcuma* extract bioactivity and indicating the need for further investigation on the complex role of antioxidant curcumin effects.

1. Introduction

Food supplements and nutraceuticals are largely used for health-promoting purposes mainly ascribed to the antioxidant properties of the phytochemicals contained in these products. Nevertheless, it is well accepted that the antioxidant activity is poorly related to the radical scavenging properties that can be demonstrated with *in vitro* chemical assays. Furthermore, studies related to the real *in vivo* antioxidant activities of these chemicals as well as to their effects on healthy subjects are still missing. In general, the study of *in vivo* antioxidant activity is difficult due to the complex multiple targets of purified natural products or extracts possessing this effect [1,2]. Current studies of antioxidant phytochemicals are generally focused on specific compounds and their effects are evaluated on a limited number of markers [1].

Curcuma longa L. is extensively used in Ayurveda, Unani, Siddha, and Chinese medicine for the management of various diseases. This spice is highly regarded for its numerous biological activities especially related to antioxidant, anti-inflammatory and cancer preventive properties [3–11]. The effects of *C. longa* are ascribed to the presence of diarylheptanoid compounds known as curcuminoids (namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin), which are considered the main active principles of the plant, although their bioavailability is poor because of scarce absorption, rapid metabolism and systemic elimination [12,13]. Extremely low serum levels of curcumin after oral administration were observed [12], making it difficult to explain its antioxidant properties on the basis of simple radical scavenging action. Nevertheless, extensive scientific research over the past decade [6,14–17] has shown that this compound is able to modulate multiple cellular targets and hence that it possesses preventive and therapeutic value against a wide variety of diseases thus showing the need for new approaches in the study of this natural product. Previous studies in rats have shown the ability of curcumin to upregulate the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which is responsible for phase 2 antioxidant and detoxification genes expression, indicating that this compound increases the total superoxide dismutase and glutathione peroxidase activities [18,19].

* Corresponding author.
E-mail address: stefano.dallacqua@unipd.it (S. Dall'Acqua).

http://dx.doi.org/10.1016/j.fitote.2015.12.013
0367-326X/

Metabolomics can offer new opportunities in this research area since it allows the observation of changes in particular bio-fluids caused by the overall effect of a natural product on different biochemical pathways. Urinary biomarkers of oxidative status present a great opportunity to study redox balance because specimen collection is non-invasive [20] and long-term observation experiments are possible. Therefore, studies using urinary metabolome analysis are attractive especially for the evaluation of antioxidants in healthy subjects or in healthy *in vivo* models.

In a previous study, we used a metabolomic approach to study the changes of the urinary metabolic profile after the administration of *C. longa* extract in rats. Compared to the control group, the treated animals were characterized by decreased levels of allantoin, a urinary biomarker of oxidative stress [21].

As a continuation of our previous study, we evaluated the effect of oral daily administration of standardized *Curcuma longa* Linn extract (corresponding to 150 mg/kg of total curcuminoids) to 12 healthy rats by untargeted metabolomics. Treatment was carried out over 33 days and changes in the urinary metabolome were evaluated by monitoring the 24-h urine composition by ^{1}H NMR and HPLC–MS. Urinary collections at 42 days (after stopping the treatment at day 33) were also analysed. We attempted to use the combined potential of NMR and MS in a unified metabolomic approach as a powerful tool to assess the modification of urine composition caused by curcumin supplementation in a healthy animal model. Both techniques produced similar representations for the collected samples confirming our previous study using similar methodology. The two different approaches were able to detect variations in the urinary metabolome, leading to the observation of different variables thus proving the complementarity of these two analytical techniques for metabolomic purposes.

2. Experimental

2.1. Materials

Curcumin standard, methanol, acetonitrile, formic acid, hydrochloric acid, deuterated water, methanol, glutathione (GSH), sulphosalicylic acid (SSA), γ-glutamyl-glutamic acid (γ-Glu-Glu), ethylenediaminetetracetic acid (EDTA), and N-ethylmaleimide (NEM) were obtained from Sigma-Aldrich (Milan, Italy). Curcumin glucuronide was synthesized in our laboratory using a previously published protocol [22]. *C. longa* Linn. dried extract was purchased from a local market; the total curcuminoid content was measured as previously described [21,23] as 94%; specifically, 71.0%, curcumin, 20.5% demethoxycurcumin, and 2.5% bisdemethoxycurcumin were determined using HPLC–MS and HPLC-DAD measurements [21].

2.2. Animals and urine collection

All experimental protocols involving animals were reviewed and approved by the Ethical Committee for animal experiments of the University of Padua (CEASA, protocol number 49571). The study involved 12 Sprague–Dawley rats: 6 males and 6 females, 8 ± 1 weeks of age, at the beginning of the experiments; male animals weighted 78.0 ± 2.3 g and female animals 79.5 ± 4.0 g. They were caged in a temperature- and photoperiod-controlled (12-h light/dark cycle) room with rodent maintenance diet and water *ad libitum*. Rats were randomly divided into a control (three males and three females) and a curcumin-treated group (three males and three females). No differences were observed between the two groups at the beginning of the experiment, based on HPLC–MS and NMR preliminary data (not shown). Six hundred milligrammes of *C. longa* extract were suspended in 12 mL of water. The treated group received a daily dose of 160 mg/kg of *C. longa* extract (corresponding to 150 mg/kg total curcuminoids or 112 mg/kg of curcumin) orally by gavage for 33 days. An equal dose of water was given to the control group. At day 0, 6, 15, 22, 28, 33 and 42 (10 days after the end of the treatment), the animals were housed individually in metabolic cages for the collection of the 24-h urine outputs. The collected samples were stored at −80 °C until ^{1}H NMR and HPLC–MS analysis.

2.3. HPLC–MS urine analysis

To obtain a metabolic profiling of urine, an HPLC–MS full scan method was used. A Varian MS 500 equipped with a prostar 430 autosampler and binary chromatograph 212 series (Varian, Palo Alto, USA), was used as HPLC–MS system. An Agilent (Milan, Italy) Eclipse XDB C-8 column (2.1 × 150 mm 3.5 μm) was used as stationary phase. The mobile phase was composed of solvent A (acetonitrile with 0.5% acetic acid) and solvent B (water with 2% formic acid). Linear gradients of A and B were used, as follows: 0 min, 10% A; 20 min, 85% A; 21 min, 100% A, 21.30 min, 10% A; 27 min, 10% A. The flow rate was 200 μL/min and the injection volume was 10 μL. The mass range explored was 50–1000 m/z. MS were recorded both in positive standard mode and in turbo depending data scanning (tdds) mode that allows the elucidation of the fragmentation patterns of the detected ions. Collected urine samples were centrifuged (13,000 g for 10 min) and directly injected in the HPLC. Each HPLC–MS data set was processed with MZmine 2.9 software [24]; from the raw data files, we obtained a data set composed of 102 variables. Median Fold Change normalization was applied to take into account the effects of sample dilution. Data were log-transformed and mean centred.

2.4. ^{1}H–NMR urine analysis

Aliquots of 700 μL of urine at pH 2.50 ± 0.05 were centrifuged at 13,000 rpm for 10 min and mixed with 70 μL of 2 mM 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 (TSP) in D_2O solution. ^{1}H NMR spectra were recorded at room temperature using a Bruker (Rheinstetten, Germany) Avance DMX 600 spectrometer. One-dimensional spectra were acquired using the NOESYGPPR1D pulse sequence. Parameters used were: 64 scans, 32 k data points, spectral width of 8389.26 Hz, 2 s relaxation delay, 50 ms mixing time, 1.95 s acquisition time. Prior to Fourier transformation, the FIDs were zero-filled to 64 k points and an exponential line broadening factor of 0.3 Hz was applied. All spectra were manually corrected for phase and baseline distortions using ACD/NMR Workbook software (Advanced Chemistry Development, Inc. Toronto, Ontario, Canada) and were referenced to the CH_3 resonance of creatinine at 3.13 ppm. Spectra were aligned using the CluPA algorithm (VU T.N., Laukens K., Valkenborg D. (2012) speaq: an R-package for NMR spectrum alignment and quantitation. R package version 1.1.). The spectral region between 4.7 and 5.0 ppm was removed prior to statistical data analysis to avoid variability due to the residual water signal. Data were reduced to 470 bins by intelligent bucketing; the obtained data set was normalized by Total Sum Normalization and mean centring and Pareto scaling were applied.

2.5. Blood sample, glutathione and curcumin quantification

Whole blood samples were collected at day 34 and stored in heparinised tubes at −20 °C until analysis. GSH was measured using a previously described method [25]. Briefly, a 20 mM GSH stock solution in water was used to prepare calibration curves. The precipitating solution was prepared by mixing 150 μL of a solution containing NEM, EDTA and γ-Glu-Glu (in water/methanol, 85/15 (*v*/*v*)) with 50 μL of SSA; the final concentrations in the precipitating solution were 20 mM, 2 mM, 250 μM and 2% (*w*/*v*) for NEM, EDTA, γ-Glu-Glu and SSA, respectively. Curcumin was measured using a previously published method using SPE extraction [23].

Please cite this article as: S. Dall'Acqua, et al., New findings on the in vivo antioxidant activity of Curcuma longa extract by an integrated 1H NMR and HPLC–MS metabolomic appro, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.12.013

2.6. Statistical data analysis

Multivariate data analysis based on projection methods was applied for statistical data analysis. Specifically, exploratory data analysis was performed by principal component analysis (PCA) while a new projection to latent structures (PLS)-based method was applied to study the changes in the urinary metabolome during the experiment. While PCA is a well-known technique used in multivariate data analysis [26], the PLS-based approach applied to model the data collected during our longitudinal study was recently published by our group [21] and will be summarized in the following. A more detailed description of our proposed method is reported in the supplementary materials. Projection to latent structures by partial least squares regression (PLS) [27] is an effective and robust regression technique used to investigate the relationships existing between two blocks of data, usually called X- and Y-block. In metabolomics applications, PLS often produces a large number of latent components with the result to compromise a clear interpretation of the model. For this reason, we elaborated a post-transformation method, called post-transformation of PLS2, able to decompose the structured variation of the X-block discovered by PLS into two main blocks corresponding to the variations correlated (the so called parallel or predictive block) and orthogonal to the Y-block by a suitable rotation of the weights of the PLS model. Post-transformation of PLS2 is a three step approach. In the first step, a PLS regression model is built on the data; in the second step, the weight matrix of the model is rotated while in the third step a regression model is rebuilt by using the same framework of the PLS algorithm but the new weight matrix to project the data. The relationships between the X-block and the Y-block can be investigated by exploring only the parallel part of the model by using suitable correlation loading plots. As a result, the model obtained by post-transformation of PLS2 maintains the same power in prediction and regression coefficients of the unrotated PLS model but can be easily interpreted because the number of components useful to interpret the model is usually reduced. Post-transformation of PLS2 can be applied to model longitudinal studies by considering the experimental data and the design matrix as X- and Y-block, respectively. In our study, we supposed an interaction model to define the design matrix. The significance of the terms time, treatment and time × treatment included in the model was evaluated by permutation tests.

To avoid over-fitting and prove the robustness of the obtained models, we performed *N*-fold full cross-validation with different values of *N* ($N = 6, 7, 8$) and a permutation test on the response (500 random permutations) according to good practise for model validation. Data set comparison was performed by Bidirectional Orthogonal Projections to Latent Structures (O2PLS) [28].

PCA and PLS models were built using SIMCA 13 (Umetrics, Umea, Sweden) while the platform R 3.0.2 (R Foundation for Statistical Computing) was used to perform t-test and Mann–Whitney test, to post-transform the PLS model (user-written R function) and to build the O2PLS model (user-written R function).

3. Results

3.1. Animal weight and urinary output

No differences in the treated *vs* control group were observed in animal weight and 24-h urinary output during the experiment. Data are summarized in Table 1.

Table 1
Variations of body weight and 24-h urine output of the rats during the course of the experiment. The differences between the two groups were not statistically significant according to t-test and Mann–Whitney test (both p-values were >0.10).

	Body weight (g)		Urine volume (mL)	
Day	Control	Treated	Control	Treated
0	78.8 ± 3.7	78.8 ± 3.1	6.3 ± 4.0	7.0 ± 2.4
6	110.0 ± 5.0	107.6 ± 7.3	10.7 ± 3.0	15.3 ± 6.5
15	190.3 ± 20.4	180.8 ± 20.8	12.7 ± 2.2	15.7 ± 5.3
22	230.8 ± 38.1	217.0 ± 39.6	13.8 ± 2.0	16.5 ± 2.7
28	254.8 ± 44.8	236.8 ± 45.1	11.2 ± 1.8	11.8 ± 2.1
33	278.5 ± 60.8	262.8 ± 55.0	11.5 ± 1.5	10.6 ± 1.2
42	300.3 ± 68.7	290.3 ± 64.0	10.0 ± 2.9	10.3 ± 3.1

3.2. Data analysis of the ^{1}H NMR and HPLC–MS data sets

Exploratory data analysis on the two data sets did not show the presence of outliers in the data. PCA models of the urines collected on day 0 did not show differences between rats belonging to the control or the treated group.

As a first step of our data modelling strategy, the two data sets were compared to investigate the common information shared by NMR and HPLC–MS. To this end, we scaled both data sets to unit variance and applied O2PLS (Fig. 1). The obtained model showed a joint overlapping variation described by 3 latent components ($R^2 = 0.59$ for NMR and $R^2 = 0.45$ for HPLC–MS), a unique systematic variation for NMR having 4 latent components ($R^2 = 0.19$) and a unique systematic variation of 4 latent components for HPLC–MS ($R^2 = 0.29$). As a consequence, we can conclude that a large part of the systematic variation of the two data sets contains the same information while only a small part is unique and non-overlapping in the two data sets. Fig. 1 shows the score scatter plot for the first and the second latent components describing the joint co-variation of the two data sets: the common information can be qualitatively interpreted in terms of the effects of time evolution and curcumin supplementation. This first model considers only the correlation structure existing between the NMR and the HPLC–MS data sets, and ignores the chemical identity of the variables used. For this reason, we cannot consider the two data sets as equivalent as will be proven with the following models.

In the second part of our data modelling strategy, post-transformed PLS2 models of the data related to urine samples collected from day 6 to day 33 proved that the interaction term time × treatment was not significant at the level of 95% and that a simple linear model can be used for the design matrix. Both the model obtained for the ^{1}H NMR data set and that obtained for the HPLC–MS data set clearly showed the effects of time and treatment on the metabolic profile of the urine as it can be observed in the score scatter plots of Figs. 2 and 3. The model for the ^{1}H NMR data set had A = 2 + 4 components, $R^2 = 0.74$ and $Q^2 = 0.52$ for treatment and $R^2 = 0.77$ and $Q^2 = 0.70$ for time while

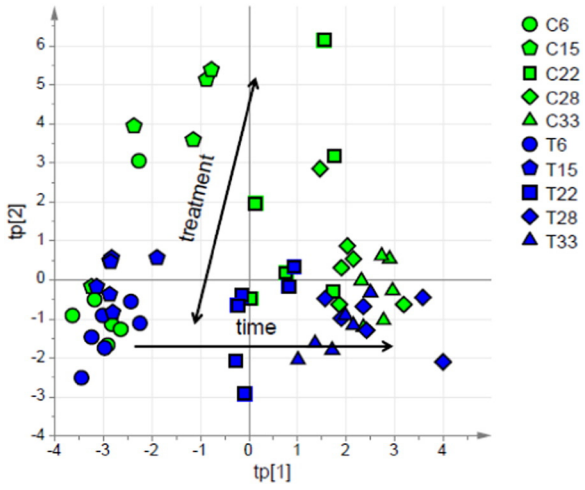

Fig. 1. Score scatter plot describing the joint systematic variation explained by the first and the second latent component of the O2PLS model; different symbols and colours were used to allow the interpretation of the observed patterns in terms of time evolution and treatment effect. C = control; T = treated; the numbers refer to the day of urine collection.

Please cite this article as: S. Dall'Acqua, et al., New findings on the in vivo antioxidant activity of Curcuma longa extract by an integrated 1H NMR and HPLC–MS metabolomic appro, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.12.013

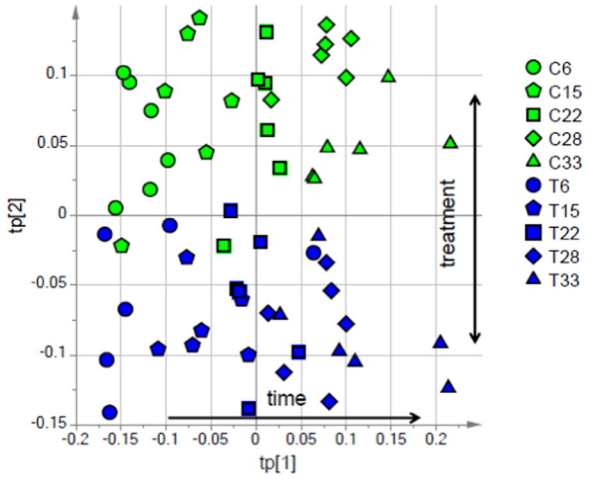

Fig. 2. ^{1}H NMR data set: score scatter plot for the post-transformed PLS2 model; the time evolution of the composition of the collected urines is described by the horizontal axis (tp [1]) while the vertical axis (tp [2]) represents the effects of the different treatment. C = control; T = treated; the numbers refer to the day of urine collection.

the model for the HPLC–MS data set had A = 2 + 2 components, R^2 = 0.73 and Q^2 = 0.44 for treatment and R^2 = 0.85 and Q^2 = 0.64 for time. (See Fig. 4.)

By analysing the correlation loading plots of the obtained models, it was possible to find variables characterizing the time evolution of the samples and the effects of curcumin administration on the rat urine metabolome.

Days 15, 28 and 33 were selected as key points of the experiment to check the presence of significant modifications of control *vs*. treated groups in the metabolites highlighted by our analysis.

Considering the ^{1}H NMR data, resonances associated with the discrimination between control and treated groups were observed in the range of deshielded protons (8.17–8.50 ppm), but it was not possible to assign any known metabolite to those signals. Tentative assignments were deduced on the basis of spectral data for bins as reported in Table 2. Hippuric acid, 2-oxoglutarate and trimethylamine N-oxide (TMAO) levels were higher in the treated group compared to the control. On the other hand, the urinary marker of oxidative stress, 8-hydroxy-2′-deoxyguanosine (8-*OHdG*), was higher in the control group. The correlation loading plot obtained for the model of the HPLC–MS data set highlighted significant differences in a set of variables

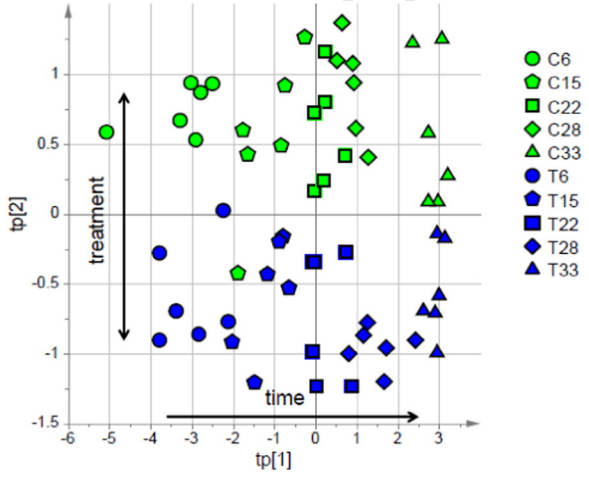

Fig. 3. HPLC–MS data set: score scatter plot for the post-transformed PLS2 model; the time evolution of the urinary metabolome can be observed along the horizontal axis (tp [1]) while the effects of the treatment are included in tp [2] (vertical axis). C = control; T = treated; the numbers refer to the day of urine collection.

that were chemically identified and are related to oxidative stress. Specifically, our untargeted approach confirmed the metabolites found in our previous targeted approach [21]. Indeed, four urinary markers of the oxidative status of the animals, i.e., allantoin, *m*-tyrosine, 3-nitrotyrosine, and 8-*OHdG*, were identified in the urine on the basis of their *m/z* value and fragmentation patterns compared to those registered in the Human Metabolome Database and Mass Bank Database. Furthermore, urinary levels of two sulphur containing compounds, namely taurine and cystine were modified during the experiment. In Table 3, the calculated reduction of these metabolites as average measured data of treated *vs*. control animals on days 15, 28 and 33 are reported. Allantoin, 3-nitrotyrosine, *m*-tyrosine, and 8-*OHdG* levels were significantly decreased in the treated compared to the control group starting from day 6 of urine collection, and also at the end of the treatment (Table 3). Surprisingly, on day 28 the average *m*-tyrosine levels were higher (+13%) in the treated group.

On day 33, allantoin, *m*-tyrosine, and 8-*OHdG* reductions in the treated group compared to controls were significant (p-value < 0.10 for both t-test and Mann–Whitney test). Significant (p-value < 0.10 for both t-test and Mann–Whitney test) changes were also observed for taurine (−24%) and cystine (−40%) in the treated group compared to controls (Table 3). The levels of these metabolites were measured also 10 days after the administration of *Curcuma* extract stopped (day 42). At this point (day 42) no significant differences in urinary composition were observed between treated and control groups showing that the observed changes were reversible with the interruption of curcuma extract administration.

3.3. Whole blood glutathione (GSH) and curcumin levels

Blood samples were collected at the end of the treatment (day 34) and GSH and curcumin levels were measured. We did not find any difference between the average concentration of whole blood GSH in the control and the treated group on day 34. Curcumin, curcumin glucuronide and curcumin sulphate were not detectable in either the treated or control groups.

4. Discussion

Food supplements and nutraceuticals with claimed antioxidant properties are enjoying a growing diffusion because of their health-promoting effects. Polyphenols are a large group of phytochemicals that present strong chemical antioxidant properties. Their health benefits are often claimed based on their antioxidant properties *in vitro*, but evidence for *in vivo* antioxidant effects is still limited since no validated *in vivo* biomarkers have been identified and no long-term studies are available [1,29–31]. Metabolomics offers new opportunities for the evaluation of *in vivo* antioxidant properties of complex mixtures such as natural products [32–34]. The study of urinary metabolome and urinary biomarkers of oxidative stress is attractive because sample collection is simple and non-invasive [20] and may lead to the observation of modified levels of metabolites that can be considered as a starting points for depicting new mechanisms of *in vivo* antioxidant activity. In this paper, we report significant changes in the urinary metabolome of healthy rats, orally treated with curcumin, compared to controls in data sets obtained both by NMR and HPLC–MS. These results confirm our previous findings obtained using a different experiment design: a lower dose of curcumin extract (80 mg/kg) was administered, and a targeted HPLC–MS approach was used, by selecting 25 metabolites in the urine chromatogram [21]. In the present work, we identified a larger number of metabolites that are related to curcumin supplementation in healthy rats.

The metabolic changes revealed by NMR data are related to phenolic compound metabolism. Previously published papers reported increase in urinary hippuric acid levels after administration of fruit and polyphenol rich foods [35]. Other authors reported that the oral administration

Please cite this article as: S. Dall'Acqua, et al., New findings on the in vivo antioxidant activity of Curcuma longa extract by an integrated 1H NMR and HPLC–MS metabolomic appro, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.12.013

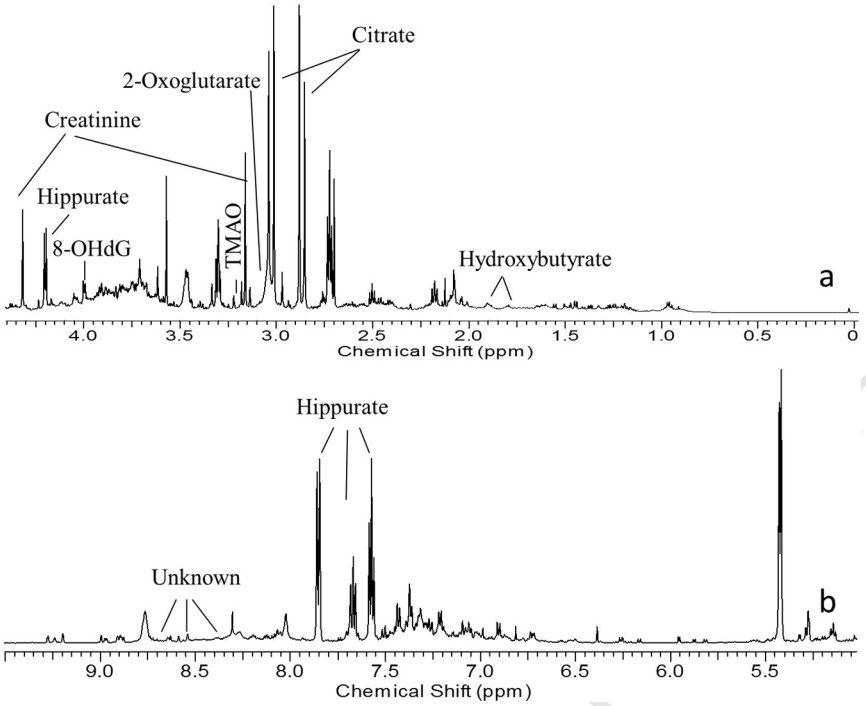

Fig. 4. Representative 600 MHz ^{1}H NMR spectra of urine after oral administration of curcumin in rats. (a) Aliphatic region (0.0–4.5 ppm). (b) Aromatic region (5.0–9.5 ppm). The vertical scale in the aromatic region was magnified four times compared with that in the aliphatic region.

of the flavonoid quercetin to rats, in an NMR-based metabolomic study, resulted in an increase in choline, creatinine, dimethylglycine, hippurate, taurine, and TMAO and in the reduction in acetate, alanine, and lactate [36]. These authors considered the general changes of such compounds as modification in osmolyte levels thus suggesting that these data may indicate improved glomerular or general renal function and correlated their observation in kidney osmolyte activity to the potential beneficial effects of quercetin on kidney function and hypertension [36]. Our data showed a higher level of creatinine and taurine in the control group. It is difficult to correlate the decreased urinary creatinine levels in the treated group with any biological meaning. On the contrary, the reduced urinary taurine levels, confirmed our previous observations [21] and other published papers that reported lowered taurine brain concentrations in curcumin-treated rats [37]. Taurine has been previously reported to decline in a number of tissues with advancing age and also in rats, the urinary levels were significantly reduced with ageing [38]. On the other hand, increased urinary taurine levels have been indicated as a specific marker of liver toxicity [37,38]. Cystine levels were also significantly reduced on day 33, but this metabolite can also derive from cysteine modification during 24-h urine collection so that it is difficult to assess its meaning in this bio-fluid.

Trimethylamine N-oxide (TMAO) is an oxidation product of trimethylamine (TMA), and both these compounds are products of choline metabolism. The methylamine pathway is a typical example of microbial–mammalian co-metabolism and is well known that intestinal microbiota plays a role in the catabolism of choline in humans and rodents [39]. Dietary choline is converted in TMA by gut microbiota and TMA is mainly oxidized to TMAO [39–41]; thus, an increase of such metabolite may be related to the influence of the oral curcumin treatment on intestinal microbiota. There is strong interest in the evaluation of TMAO plasmatic or urinary levels due to various physio-pathological functions that have been proposed for this compound. In fact, recent animal studies have shown a link between intestinal microbial metabolism of the choline moiety in dietary phosphatidylcholine and coronary diseases through the production of TMAO, considered as a proatherogenic compound. In humans, the production of TMAO from dietary phosphatidylcholine is dependent on metabolism by the intestinal microbiota. Furthermore, ingestion of different types of foods, such as eggs or fish, may influence TMAO plasma levels [41–43]. Other authors reported the ability of oral broad-spectrum antibiotics to temporarily suppress the production of TMAO suggesting that intestinal microorganisms participate in phosphatidylcholine metabolism to form circulating and urinary TMAO [41,44]. Increased plasma TMAO levels are associated with an increased risk of incidence of major adverse cardiovascular events [41,44]. The role of TMAO appears to be complex,

Table 2
Comparison of selected bins observed in the NMR data set; differences between treated and control group are reported in terms of increase or decrease of the NMR integral. *p-value < 0.10 for both t-test and Mann–Whitney test; #difference in the mean values, but not significant at the level of 90% for both t-test and Mann–Whitney test.

ppm	Assignment	Day 15	Day 28	Day 33
8.50–8.17	Not Assigned	>C*	>C#	>C*
7.57–7.55	Hippuric acid	>T#	>T*	>T#
4.30	Creatinine	>C#	>C*	>C*
3.95	8-*OHdG*	>C#	>C*	>C*
3.32	Not assigned	>T*	>T#	>T#
3.23–3.22	TMAO	>T*	>T*	>T#
3.19	2-Oxoglutarate	>T*	>T#	>T*
2.81	Citrate	>C#	>T*	>T*
1.81–1.79	Hydroxybutyrate	>C*	>C*	>C#

Table 3
Oxidative stress urinary markers reduction in the treated group as a percentage of the value of the control group; *p-value < 0.10 for both t-test and Mann–Whitney test, #difference in the mean values, but not significant at the level of 90% for both t-test and Mann–Whitney test.

	Day 15	Day 28	Day 33
Allantoin	−31.0*	−16.0#	−34.0*
3-Nitrotyrosine	−23.0#	−18.0#	−27.0#
m-Tyrosine	−0.2#	+13.0#	−20.0*
8-*OHdG*	−0.7#	−19.0#	−28.0*
Taurine	*−4.4#*	*−26.0**	*−24.0**
Cystine	*−24.0#*	*−29.0**	*−40.0**

but our data indicate that curcumin oral administration modifies the microbiota or influences the urinary excretion of this compound thus leading to changed urinary levels in the treated group. This result suggests a role of the intestinal microbial population in the mechanism of action of curcuminoids. Recently, the ability of curcumin supplementation to modulate colonic microbiota during colitis and colon cancer prevention was studied showing an increase in microbial diversity and restoration of colonic microbial composition to that observed in healthy WT animals compared to mouse model of IBD-associated colon cancer [45].

Urinary levels of some markers of oxidative stress were significantly reduced because of the treatment, as demonstrated by HPLC–MS results. Allantoin is considered a urinary marker of oxidative stress, because it is the predominant product of non-enzymatic oxidation of uric acid by many types of free radicals, and it is considered a valid biomarker of oxidative state especially in humans [20].

m-Tyrosine is considered a promising biomarker for oxidative damage to proteins [46]. The highly reactive hydroxyl radical oxidizes phenylalanine residues to *o*-tyrosine and *m*-tyrosine and increased levels of these metabolites are correlated to an increased ROS production from normal metabolic processes or from exposure to exogenous factors. Also reactive nitrogen species react readily with tyrosine and protein-associated tyrosine to form free 3-nitrotyrosine and protein-associated 3-nitrotyrosine, respectively [47]. Urinary 3-nitrotyrosine is a potential biomarker that may reflect the enhanced generation of reactive nitrogen species and it has been proposed as a biomarker to detect changes in oxidative stress and to evaluate the efficacy of therapeutic interventions aimed at reducing oxidative stress [48].

Urinary 8-*OHdG* is considered a biomarker of generalized cellular oxidative stress because it is one of the predominant products of oxidized DNA repair [49]. Because it is fairly water-soluble, it will be excreted into the urine without being further metabolized and it is considered a stable end product of non-enzymatic DNA oxidation [20]. Therefore, increased urinary levels of 8-*OHdG* could be correlated to an increase of oxidative DNA damage [20]. Our experiment showed significant reduction of some of these urinary markers of oxidative stress after 33 days of treatment (see Table 3), suggesting an *in vivo* antioxidant effect of curcumin supplementation in the healthy rat.

To have a further parameter related to the oxidative state of the animals at the end of the treatment, blood samples were collected on day 34 and GSH levels were measured. The average blood GSH levels showed no difference (t-test p-value < 0.01) between the control and the treated group. Other authors reported similar results in a study that evaluated the effect of curcumin and one analogue [bis-1,7-(2-hydroxy-phenyl)-hepta-1,6-diene-3,5-dione] (BDMC-A) on carbon tetrachloride-induced hepatotoxicity in rats. Control and curcumin-treated groups presented the same GSH values, while significant increase in plasma GSH levels was observed in the animals group treated with CCl_4 and curcumin compared with the CCl_4 treated ones [50].

However, a previous study reported a role for curcumin against GSH depletion-mediated mitochondrial dysfunction *in vitro* and *in vivo* [51]. Another study reported a significant effect of curcumin on GSH biosynthesis in alveolar epithelial cells [52]. Other researchers have considered that some of the most important effects of curcumin, such as the anticarcinogenic, antimutagenic, antioxidant and cytoprotective activities can be explained by its inhibitory effect on glutathione S-transferase (GST) [15]. The capacity of curcumin to protect rats from adriamicin (ADR) nephrotoxicity was demonstrated [53]. Curcumin protected against ADR-induced renal injury by suppressing oxidative stress and increasing kidney glutathione content and glutathione peroxidase activity; nevertheless, in the same paper, kidney GSH levels of animals treated either with saline or curcumin were similar showing that curcumin treatment does not increase kidney GSH levels but can restore ADR-induced GSH depletion.

Our results indicate that curcumin supplementation does not increase blood GSH levels in healthy subject. Considering this result the low plasma concentration of curcumin due to its poor absorption, must be underlined. Previous studies have reported that, due to poor bio-availability, only traces of curcumin are detected in plasma after oral administration [12]. Also our data showed no detectable curcumin and curcumin conjugated metabolites in blood 24 h after the last administration (day 34) indicating rapid elimination of the compound from the bloodstream, in agreement with previously published results that reported the curcumin plasma peak 40 min after oral administration of 500 mg/kg in rats [54]. A large amount of orally administered curcumin, due to poor absorption, is present at the intestinal level so that the intestinal mucosa is exposed to higher concentrations of curcumin and for this reason, interactions of curcumin with GSH and GST are likely to be more significant in intestinal epithelial cells rather than in plasma [55]. In our healthy animal model, curcumin supplementation is not likely to play a prominent role to change GSH levels in plasma.

5. Conclusions

In this work we used both ^{1}H NMR and HPLC–MS techniques to study the modification of urinary composition in rats treated with *C. longa* extract correlated with *in vivo* antioxidant activity. Multivariate analysis on ^{1}H NMR and HPLC–MS data produced similar representations for the collected samples. The two different approaches were able to detect variations in the urinary metabolome, leading to the observation of different components, showing the complementarity of these two analytical techniques for metabolomic purposes. The results of the present study are in agreement with our previously published data obtained with a lower curcumin dose and using a targeted ^{1}H NMR and HPLC–MS approach [21]. The evaluation of the effects of *Curcuma* extract on urinary composition in healthy rats by a metabolomic approach led us to observe evidence for an *in vivo* antioxidant effect caused by a significant reduction in the amount of urinary biomarkers of oxidative stress such as allantoin, *m*-tyrosine, 8-*OHdG*. A tendency to the reduction of 3-nitrotyrosine was also observed. Our metabolomics-based study supports an *in vivo* antioxidant effect of the oral administration of *Curcuma* extract to healthy rats. The observation that urinary TMAO levels are increased in the treated compared to the control group may be related to the influence of curcumin supplementation on microbiota, as recently indicated by other research groups, or on the urinary excretion of this metabolite. Urinary levels of taurine and cystine, sulphur containing compounds, were also changed suggesting a role for such constituents in the biochemical pathways involved in *Curcuma* extract bioactivity and indicating the need for further investigation on curcumin effects. The undetectable plasmatic levels of curcumin and its conjugates confirmed its rapid elimination from the bloodstream after oral administration indicating that the contribution to the whole antioxidant activity by a direct radical scavenging action is negligible. The unchanged plasmatic GSH amount in treated and control group indicates that curcumin supplementation in the health subject does not increase this endogenous antioxidant's levels.

Acknowledgments

The authors gratefully acknowledge funding from the University of Padova (PRAT CPDA118080).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.12.013.

References

[1] C. Manach, J. Hubert, R. Llorach, A. Scalbert, The complex links between dietary phytochemicals and human health deciphered by metabolomics, Mol. Nutr. Food Res. 53 (2009) 1303–1315.

Please cite this article as: S. Dall'Acqua, et al., New findings on the in vivo antioxidant activity of Curcuma longa extract by an integrated 1H NMR and HPLC–MS metabolomic appro, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.12.013

[2] A.R. Ndhlala, M. Moyo, J. Van Staden, Natural antioxidants: fascinating or mythical biomolecules? Molecules 15 (2010) 6905–6930.
[3] M. Teiten, S. Eifes, M. Dicato, M. Diederich, Curcumin-the paradigm of a multi-target natural compound with applications in cancer prevention and treatment, Toxins 2 (2010) 128–162.
[4] A. Goel, A.B. Kunnumakkara, B.B. Aggarwal, Curcumin as "Curecumin": from kitchen to clinic, Biochem. Pharmacol. 75 (2008) 787–809.
[5] H. Hatcher, R. Planalp, J. Cho, F.M. Torti, S.V. Torti, Curcumin: from ancient medicine to current clinical trials, Cell. Mol. Life Sci. 65 (2008) 1631–1652.
[6] B.B. Aggarwal, C. Sundaram, N. Malani, H. Ichikawa, Curcumin: the Indian solid gold, Adv. Exp. Med. Biol. 595 (2007) 1–75.
[7] R.K. Maheshwari, A.K. Singh, J. Gaddipati, R.C. Srimal, Multiple biological activities of curcumin: a short review, Life Sci. 78 (2006) 2081–2087.
[8] R.A. Matchanickal, M.M. Rafi, Curcumin: potential health benefits, molecular mechanism of action, and its anticancer properties in vitro and in vivo, ACS Symp. Ser. 925 (2006) 92–107.
[9] A. Duvoix, R. Blasius, S. Delhalle, M. Schnekenburger, F. Morceau, E. Henry, M. Dicato, M. Diederich, Chemopreventive and therapeutic effects of curcumin, Cancer Lett. 223 (2005) 181–190.
[10] R.A. Sharma, A.J. Gescher, W.P. Steward, Curcumin: the story so far, Eur. J. Cancer 41 (2005) 1955–1968.
[11] I. Chattopadhyay, K. Biswas, U. Bandyopadhyay, R.K. Banerjee, Turmeric and curcumin: biological actions and medicinal applications, Curr. Sci. 87 (2004) 44–53.
[12] P. Anand, A.B. Kunnumakkara, R.A. Newman, B.B. Aggarwal, Bioavailability of curcumin: problems and promises, Mol. Pharm. 4 (2007) 807–818.
[13] C. Ireson, S. Orr, D.J.L. Jones, R. Verschoyle, C. Lim, J. Luo, L. Howells, S. Plummer, R. Jukes, M. Williams, W.P. Steward, A. Gescher, Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production, Cancer Res. 61 (2001) 1058–1064.
[14] B.B. Aggarwal, K.B. Harikumar, Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases, Int. J. Biochem. Cell Biol. 41 (2009) 40–59.
[15] B.B. Aggarwal, A. Kumar, A.C. Bharti, Anticancer potential of curcumin: preclinical and clinical studies, Anticancer Res. 23 (2003) 363–398.
[16] P. Anand, C. Sundaram, S. Jhurani, A.B. Kunnumakkara, B.B. Aggarwal, Curcumin and cancer: an "old-age" disease with an "age-old" solution, Cancer Lett. 267 (2008) 133–164.
[17] A.C. Bharti, N. Donato, S. Singh, B.B. Aggarwal, Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-κB and IκBα kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis, Blood 101 (2003) 1053–1062.
[18] C. Yang, X. Zhang, H. Fan, Y. Liu, Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia, Brain Res. 1282 (2009) 133–141.
[19] I. Carmona-Ramírez, A. Santamaría, J.C. Tobón-Velasco, M. Orozco-Ibarra, I.G. González-Herrera, J. Pedraza-Chaverrí, P.D. Maldonado, Curcumin restores Nrf2 levels and prevents quinolinic acid-induced neurotoxicity, J. Nutr. Biochem. 24 (2013) 14–24.
[20] D. Il'yasova, P. Scarbrough, I. Spasojevic, Urinary biomarkers of oxidative status, Clin. Chim. Acta 413 (2012) 1446–1453.
[21] S. Dall'Acqua, M. Stocchero, M. Clauser, I. Boschiero, E. Ndoum, M. Schiavon, S. Mammi, E. Schievano, Changes in urinary metabolic profile after oral administration of curcuma extract in rats, J. Pharm. Biomed. Anal. 100 (2014) 348–356.
[22] E. Pfeiffer, S.I. Hoehle, S.G. Walch, A. Riess, A.M. Sólyom, M. Metzler, Curcuminoids form reactive glucuronides in vitro, J. Agric. Food Chem. 55 (2007) 538–544.
[23] T.H. Marczylo, W.P. Steward, A.J. Gescher, Rapid analysis of curcumin and curcumin metabolites in rat biomatrices using a novel ultraperformance liquid chromatography (UPLC) method, J. Agric. Food Chem. 57 (2009) 797–803.
[24] M. Katajamaa, J. Miettinen, M. Orešic, MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data, Bioinformatics 22 (2006) 634–636.
[25] J. Steghens, F. Flourié, K. Arab, C. Collombel, Fast liquid chromatography-mass spectrometry glutathione measurement in whole blood: Micromolar GSSG is a sample preparation artifact, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 798 (2003) 343–349.
[26] J.E. Jackson, A Users Guide to Principal Components, John Wiley, New York, 1991.
[27] S. Wold, M. Sjöström, L. Eriksson, PLS-regression: a basic tool of chemometrics, Chemom. Intell. Lab. Syst. 58 (2001) 109–130.
[28] J. Trygg, S. Wold, O2-PLS, a two-block (X-Y) latent variable regression (LVR) method with an integral OSC filter, J. Chemom. 17 (2003) 53–64.
[29] C. Manach, G. Williamson, C. Morand, A. Scalbert, C. Rémésy, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, Am. J. Clin. Nutr. 81 (2005) 230S–242S.
[30] B. Halliwell, Are polyphenols antioxidants or pro-oxidants? what do we learn from cell culture and in vivo studies? Arch. Biochem. Biophys. 476 (2008) 107–112.
[31] B. Halliwell, J. Rafter, A. Jenner, Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am. J. Clin. Nutr. 81 (2005) 268S–276S.
[32] J. Godzien, M. Ciborowski, S. Angulo, F.J. Ruperez, M.P. Martínez, F.J. Señorans, A. Cifuentes, E. Ibañez, C. Barbas, Metabolomic approach with LC-QTOF to study the effect of a nutraceutical treatment on urine of diabetic rats, J. Proteome Res. 10 (2011) 837–844.
[33] A. Krastanov, Metabolomics — the state of art, Biotechnol. Biotechnol. Equip. 24 (2010) 1537–1543.
[34] R. Llorach, M. Garcia-Aloy, S. Tulipani, R. Vazquez-Fresno, C. Andres-Lacueva, Nutrimetabolomic strategies to develop new biomarkers of intake and health effects, J. Agric. Food Chem. 60 (2012) 8797–8808.
[35] T.P. Mulder, A.G. Rietveld, J.M. Van Amelsvoort, Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine, Am. J. Clin. Nutr. 81 (2005) 2565–2605.
[36] D. An, Q. Zhang, S. Wu, J. Wei, J. Yang, F. Dong, X. Yan, C. Guo, Changes of metabolic profiles in urine after oral administration of quercetin in rats, Food Chem. Toxicol. 48 (2010) 1521–1527.
[37] J. Pyrzanowska, A. Piechal, K. Blecharz-Klin, M. Lehner, A. Skórzewska, D. Turzynska, A. Sobolewska, A. Plaznik, E. Widy-Tyszkiewicz, The influence of the long-term administration of *Curcuma longa* extract on learning and spatial memory as well as the concentration of brain neurotransmitters and level of plasma corticosterone in aged rats, Pharmacol. Biochem. Behav. 95 (2010) 351–358.
[38] R. Dawson Jr., S. Liu, B. Eppler, T. Patterson, Effects of dietary taurine supplementation or deprivation in aged male Fischer 344 rats, Mech. Ageing Dev. 107 (1999) 73–91.
[39] W.R. Russell, L. Hoyles, H.J. Flint, M. Dumas, Colonic bacterial metabolites and human health, Curr. Opin. Microbiol. 16 (2013) 246–254.
[40] S.H. Zeisel, K.A. DaCosta, M. Youssef, S. Hensey, Conversion of dietary choline to trimethylamine and dimethylamine in rats: dose–response relationship, J. Nutr. 119 (1989) 800–804.
[41] W.H.W. Tang, S.L. Hazen, The contributory role of gut microbiota in cardiovascular disease, J. Clin. Invest. 124 (2014) 4204–4211.
[42] C.A. Miller, K.D. Corbin, K. Da Costa, S. Zhang, X. Zhao, J.A. Galanko, T. Blevins, B.J. Bennett, A. O'Connor, S.H. Zeisel, Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose–response study, Am. J. Clin. Nutr. 100 (2014) 778–786.
[43] A. O'Gorman, H. Gibbons, L. Brennan, Metabolomics in the identification of biomarkers of dietary intake, Comput. Struct. Biotechnol. J. 4 (2013).
[44] W.H.W. Tang, Z. Wang, B.S. Levison, R.A. Koeth, E.B. Britt, X. Fu, Y. Wu, S.L. Hazen, Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk, N. Engl. J. Med. 368 (2013) 1575–1584.
[45] R.T. McFadden, C.B. Larmonier, M.T. Midura-Kiela, R. Ramalingam, C.A. Harrison, D.G. Besselsen, J. Chase, G. Caporaso, F.K. Ghishan, P.R. Kiela, 275 The Role of Curcumin in Modulating Colonic Microbiota During Colitis and Colon Cancer Prevention, Gastroenterology 146 (2014) S-66.
[46] H. Orhan, N.P.E. Vermeulen, C. Tump, H. Zappey, J.H.N. Meerman, Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 799 (2004) 245–254.
[47] D. Tsikas, A. Mitschke, M. Suchy, F. Gutzki, D.O. Stichtenoth, Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography-tandem mass spectrometry and evaluation of the excretion after oral intake, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 827 (2005) 146–156.
[48] M. Schwemmer, B. Fink, R. Köckerbauer, E. Bassenge, How urine analysis reflects oxidative stress — nitrotyrosine as a potential marker, Clin. Chim. Acta 297 (2000) 207–216.
[49] L.L. Wu, C. Chiou, P. Chang, J.T. Wu, Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics, Clin. Chim. Acta 339 (2004) 1–9.
[50] N. Kamalakkannan, R. Rukkumani, P.S. Varma, P. Viswanathan, K.N. Rajasekharan, V.P. Menon, Comparative effects of curcumin and an analogue of curcumin in carbon tetrachloride-induced hepatotoxicity in rats, Basic Clin. Pharmacol. Toxicol. 97 (2005) 15–21.
[51] B. Jagatha, R.B. Mythri, S. Vali, M.M.S. Bharath, Curcumin treatment alleviates the effects of glutathione depletion in vitro and in vivo: therapeutic implications for Parkinson's disease explained via in silico studies, Free Radic. Biol. Med. 44 (2008) 907–917.
[52] S.K. Biswas, D. McClure, L.A. Jimenez, I.L. Megson, I. Rahman, Curcumin induces glutathione biosynthesis and inhibits NF-κB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity, Antioxid. Redox Signal. 7 (2005) 32.41.
[53] N. Venkatesan, D. Punithavathi, V. Arumugam, Curcumin prevents adriamycin nephrotoxicity in rats, Br. J. Pharmacol. 129 (2000) 231–234.
[54] K. Yang, L. Lin, T. Tseng, S. Wang, T. Tsai, Oral bioavailability of curcumin in rat and the herbal analysis from *Curcuma longa* by LC-MS/MS, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 853 (2007) 183–189.
[55] S. Awasthi, U. Pandya, S.S. Singhal, J.T. Lin, V. Thiviyanathan, W.E. Seifert Jr., Y.C. Awasthi, G.A.S. Ansari, Curcumin–glutathione interactions and the role of human glutathione S-transferase P1-1, Chem. Biol. Interact. 128 (2000) 19–38.

Please cite this article as: S. Dall'Acqua, et al., New findings on the in vivo antioxidant activity of Curcuma longa extract by an integrated 1H NMR and HPLC–MS metabolomic appro, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.12.013