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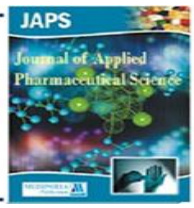

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Scavenging potential of reactive oxygen species by Tetra-hydrocurcumin

Radhika Prabhu P, Karunakar Hegde, A R Shabaraya and M N A Rao

Radhika Prabhu P, Karunakar Hegde, A R Shabaraya
Srinivas College of Pharmacy Valachil, Mangalore, India

M N A Rao
Manipal College of Pharmaceutical Sciences, Manipal, India

***For Correspondence**:
Radhika Prabhu P
Srinivas College of Pharmacy Valachil, Mangalore, India
Tel: +91-824-2274722,
Fax: +91-824-2274725

ABSTRACT

In this study, the scavenging response of tetrahydrocurcumin (THC) was evaluated by using series of *in vitro* models of chemicals compared with standard antioxidant Trolox and curcumin. The THC exhibited its radical scavenging effect in concentration dependent manner on super oxide, hydroxyl radicals, ferric ions, 1,1-diphenyl, 2-picryl hydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS). The THC was also evaluated for its inhibitory response on membrane lipid peroxidation by thiobarbituric acid reactive substances (TBARS) using rat liver homogenate. The present study shows that THC is a good antioxidant compared to the standard antioxidant, Trolox. Although, THC is less active than curcumin in many models, the increased superoxide scavenging and decreased ability to reduce ferric ions offers interesting advantages over curcumin. The THC was also effective in preventing membrane lipid peroxidation induced by $FeSO_4$/ascorbate in concentration dependent manner.

Key words: Antioxidant, Free radicals, Lipid peroxidation, Tetrahydrocurcumin.

INTRODUCTION

Free radicals and other oxygen derived species are constantly generated in vivo, both by accidents of chemistry and for specific metabolic purposes. The reactivity of different free radical varies, but some can cause severe damage to biological molecules especially to DNA, lipids and proteins. Antioxidant system of the body is generally able to combat the oxidative stress produced after normal physiological processes, but they are not completely effective (Yu, 1994). The increased production of free radicals leads to various diseases such as cancer, cardiovascular diseases, diabetes mellitus, liver disorders, brain dysfunction and in the process of aging (Joyce, 1987). Hence, externally given antioxidants may be particularly important in a diminishing cumulative oxidative damage. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in inhibiting lipid peroxidation and prevent chronic diseases.

Curcumin is a well known coloring principle in the rhizome of *Curcuma longa* (Turmeric) possessing potent antioxidant properties. Tetrahydrocurcumin (THC) is a saturated derivative of Curcumin. The antioxidant property of Curcumin is attributed to the presence of phenolic and diketone systems. In tetrahydrocurcumin, both structural features are retained. Further, THC is more stable and soluble than Curcumin. Hence, in the present study the tetrahydrocurcumin was investigated for its antioxidant properties.

MATERIALS AND METHODS

Materials

Tetrahydrocurcumin was procured from SAMI Chemicals and Extracts (P) Ltd, Bangalore (Batch No. RD.THC.01). 1,1-diphenyl, 2-picryl hydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) were obtained from Sigma Chemicals, USA.

The other chemicals used were EDTA, sodium nitroprusside, sulphanilamide, potassium superoxide, O-phosphoric acid, napthyl ethylene diamine dihydrochloride, potassium chloride, ferrous sulphate, thiobarbituric acid (TBA), trichloro acetic acid (TCA), butyrated hydroxyl toluene (BHT).

Experimental animals

Healthy Wistar albino rats of either sex, 8 -10 weeks old, weighing about 150-180 g were used for experiments. Animals were maintained under standard condition (12 h light / dark cycle; 25 ± 2^o C, 45-60 % humidity) and were fed standard rat feed (Hindustan Liver Ltd., India) and tap water *ad libitum*. All the animals were acclimatized to laboratory condition for a week before commencement of experiment. All experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the CPCSEA regulations.

Superoxide radical scavenging assay

The scavenging activity towards the superoxide radical ($O_2^{\cdot -}$) was measured in terms of inhibition of generation of $O_2^{\cdot -}$. The method was performed by using alkaline DMSO method. Potassium superoxide and dry DMSO were allowed to stand in contact for 24 h and the solution was filtered immediately before use. The filtrate (200 µl) was added to 2.8 ml of an aqueous solution containing NBT (56 µM), EDTA (10 µM) and potassium phosphate buffer (10 mM). THC 10, 100 and 500 µM concentrations were added and the absorbance was recorded at 560 nm against a control in which pure DMSO was added instead of alkaline DMSO (Henry et al, 1976). Ascorbic acid was used as standard. The experiment was repeated in triplicate and the percentage scavenging was calculated by using the formula:

$$\text{Inhibition (\%)} = [(\text{Control} - \text{Test}) / \text{Control}] \times 100$$

Inhibition of deoxyribose degradation induced by hydroxyl radicals

Hydroxyl radical was generated by Fenton reaction which degrades deoxyribose and the extent of degradation was measured as thiobarbituric acid reactive substances (TBARS). To the reaction mixture containing phosphate buffer (pH 7.4, 20 mM), deoxyribose (0.28 mM, 0.1 ml of 2.8 mM), ascorbic acid (0.05 mM, 0.05 ml of 1 mM), ferric chloride (0.05 mM, 0.05 ml of 1 mM), EDTA (0.0104 mM, 0.05 ml of 0.208 mM solution) and hydrogen peroxide (0.5 mM, 0.1 ml of 5 mM), various concentrations of THC and trolox was added in a final volume of 1 ml. The reaction was incubated for 60 min at 25 ± 1^{o}C. To the reaction mixture, 2 ml of TBA-TCA reagent was added. The tubes were kept in the boiling water for 20 min. After cooling, the absorbance was measured at 532 nm (Rajkumar and Rao, 1993). The percent inhibition was calculated.

Reduction of Ferric ions

Reduction of ferric ions to ferrous was estimated by O-phenanthroline method. To the reaction mixture containing THC (100 µM), O-phenanthroline (0.25 mg/ml) and phosphate buffer (pH 7.4, 20 mM) was added to ferric chloride (50 µM, 0.05 ml of 2 mM) in a final volume of 2 ml and incubated at ambient temperature for 10 min. Absorbance was measured at 510 nm (Rajkumar and Rao, 1993). In another tube ascorbic acid (0.2 mM) was added instead of THC to reduce all the ferric ions and the absorbance obtained was considered equivalent to 100 % reduction.

DPPH radical scavenging assay

To 1 ml of 1, 5 and 50 µM concentrations of THC, 1 ml solution of DPPH (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 min of incubation in the dark, absorbance was recorded at 517 nm and the experiment was performed in triplicate (Sreejayan and Rao, 1996). Ascorbic acid was used as standard. The percentage scavenging was calculated using the same formula as given above.

Preparation of rat liver homogenate

Wistar albino rats were fasted overnight and were sacrificed by cervical dislocation, dissected and the whole liver was excised and rinsed with 0.15 M Tris-HCl (pH 7.4). A 10% w/v of respective tissue homogenate was prepared in 0.15 M Tris-HCl buffer using Teflon homogenizer and filtered to get a clear homogenate. The tissue homogenate was used for the estimation of thiobarbituric acid reactive substances (TBARS).

Inhibition of lipid peroxidation

Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution to 3 ml of each tissue homogenate separately. Add 0.05 ml of different concentrations of THC. The mixture was incubated at 37^o C for 30 min. Then 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10 % TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67 % TBA in 50 % acetic acid. The mixtures were heated in a hot water bath at 95^o C for 30 min to complete the reaction. The intensity of pink colored complex formed was measured at 532 nm (Okhawa et al, 1979). The time dependent experiment was also done. The experiment was repeated in triplicate and the percentage scavenging was calculated using the same formula as given above.

ABTS radical scavenging assay

ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulfate and the mixture were allowed to stand in dark at room temperature for 12-16 h before use. For the study, different concentrations of the THC (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1 ml. The absorbance

was read at 745 nm and the experiment was performed in triplicate (Re et al, 1999). Ascorbic acid was used as standard.

RESULTS

Superoxide radical scavenging assay

Scavenging of superoxide by THC, Trolox and curcumin is given in Fig 1. All the compounds showed the concentration dependent scavenging of free radicals. THC was found to be most active compared to curcumin and trolox at all concentrations tested. At 100 µm, THC scavenged 48.88 % compared to Trolox (47.69 %) and curcumin (30.83 %).

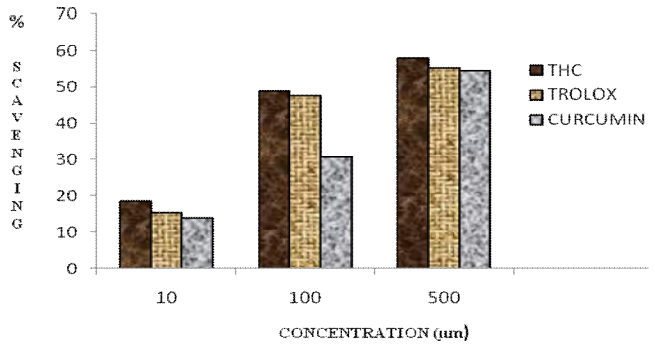

Fig 1. Scavenging of Superoxide anion by THC, Trolox and curcumin.

Inhibition of deoxyribose degradation induced by hydroxyl radicals

Hydroxyl radicals generated through Fenton reaction degrades deoxyribose to give TBARS which can be measured by absorbance at 532 nm. Scavenging of hydroxyl radicals decrease the amount of TBARS formed. The effect of THC and trolox on degradation of deoxyribose was studied (Fig 2).

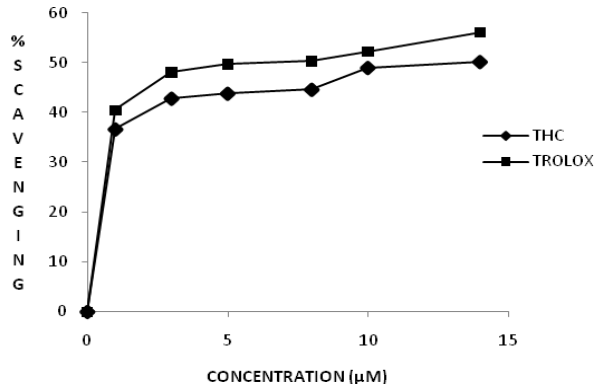

Fig 2. Hydroxyl radical scavenging by the THC and Trolox.

Curcumin could not be studied due to its poor solubility. Fig 2 clearly shows that THC has a good ability to scavenge hydroxyl radicals at all concentrations tested. However trolox was slightly more active than THC. During generation of hydroxyl radicals through Fenton reaction EDTA was added to keep the iron in solution by chelation and ascorbic acid to reduce ferric ions so that more hydroxyl radicals are generated. Hence the effect of THC was studied by deleting EDTA and ascorbic acid (Table 1).

Table 1. Effect of THC on the deoxyribose degradation in the presence and absence of EDTA and ascorbic acid

Reaction mixture (RM)	Absorbance at 532 nm
RM	0.464±0.006
RM+THC	0.220±0.007
RM-EDTA	0.193±0.003
RM-EDTA+THC	0.083±0.007
RM-Ascorbate	0.317±0.005
RM-Ascorbate+THC	0.062±.003

All values were expressed as mean ±SD

When EDTA was removed from the reaction mixture (RM) the amount of TBARS formed was decreased to appreciable extent. In presence of THC it was further decreased to a great extent. This indicates that THC has ability to chelate the iron and decrease its catalytic properties. Similarly when ascorbic acid was removed the amount of TBARS was decreased. This shows that THC does not have the ability to reduce ferric ions. By using O-phenanthroline method it was confirmed (Fig 3). Unlike THC curcumin was very active in reducing iron. At 200 µM curcumin reduced 59.67 % of ferric ions available compared to 1.61 % in case of THC (Fig 3).

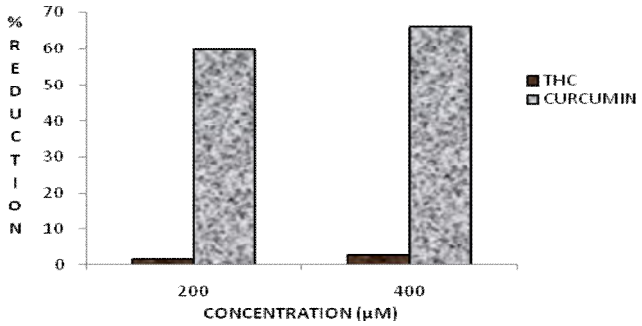

Fig 3. Reduction of Ferric ions by THC and Curcumin.

Scavenging of DPPH radicals

The effect of THC, curcumin and trolox on scavenging of the stable free radical DPPH is given in Table 2. Curcumin was most active followed by THC and trolox. At 50 µM curcumin showed 100 % scavenging compared to THC and trolox which showed 46.6% and 37.9 % respectively.

Table2. Interaction of THC, Curcumin and Trolox with DPPH

Drug	% Scavenging	
	At 10 µM	At 50 µM
THC	35.2±0.86	46.6±1.50
Curcumin	49.4±0.68	100
Trolox	27.4±0.97	37.9±0.82

All values were expressed as mean ±SD

Inhibition of lipid peroxidation

Effect of test compounds on lipid peroxidation is shown in Fig4. At 50 µM, curcumin was most active and inhibited lipid

peroxidation by 92.9 % compared to THC and trolox which showed 67.5 % and 12.3 % inhibition respectively. At lower concentration THC and curcumin were equally active. This trend was further confirmed by time dependent study (Fig 5). In control the TBARS formed increased with time. In presence of curcumin there was a little increase. THC was also very effective although less effective than curcumin.

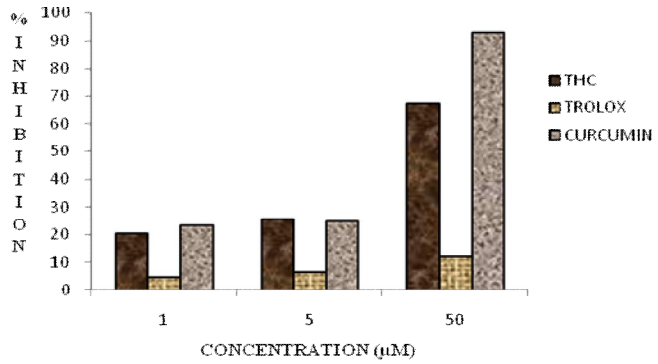

Fig 4. Inhibition of lipid peroxidation by THC,Trolox and curcumin.

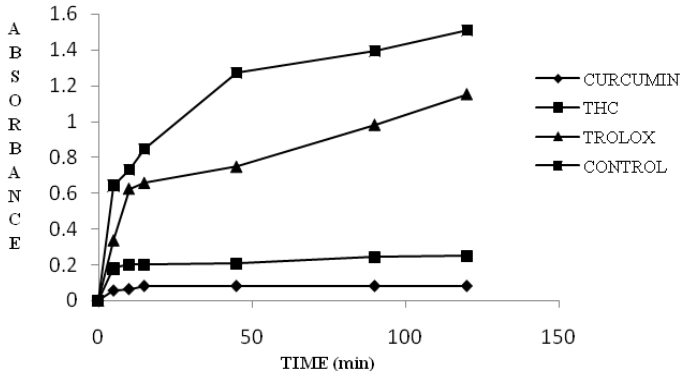

Fig 5. Time dependent lipid peroxidation inhibition.

Scavenging of ABTS radical

There is a inhibition of absorbance at 734 nm with the increase in concentration of THC, trolox and curcumin (Fig 6). THC was less active than curcumin but more active than trolox. This was further confirmed by time dependent study (Table 3).

DISCUSSION

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive tissue damage. Lipids, proteins and DNA are susceptible to attack by free radicals. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease (Youdim and Joseph, 2001).

The present study shows that tetrahydrocurcumin is a good antioxidant like curcumin. Although THC was less active

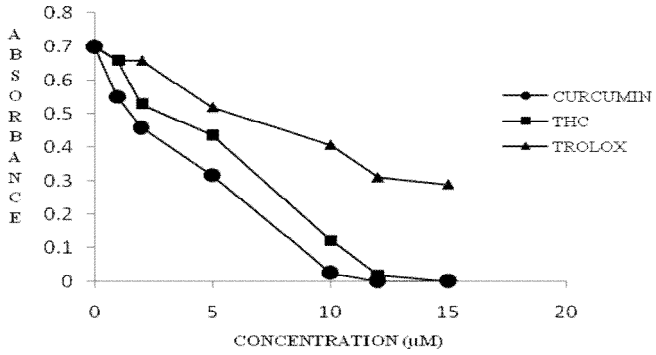

Fig 6. Effect of THC, Curcumin and Trolox on ABTS radical.

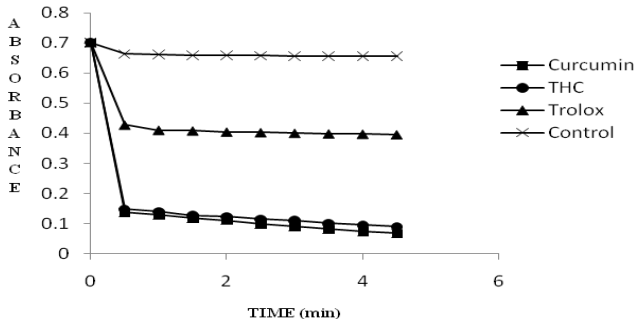

Fig 7. Time dependent effect of THC, Curcumin and Trolox on ABTS radical.

than curcumin in some models, it was more active than Trolox in all the models. Trolox was used as a standard antioxidant for comparison since it is a water soluble analog of Vitamin E (Rajkumar and Rao, 1995).

With reference to the superoxide anion scavenging activity, THC was found to be more active than curcumin and trolox, indicating that, presence of double bond is not essential for superoxide scavenging activity. Further saturation of two double bonds increased the scavenging activity. THC was also very effective in protecting deoxyribose against hydroxyl radical induced degradation. It was less active than trolox in scavenging hydroxyl radicals. Curcumin could not be tested because of its poor aqueous solubility. THC is also found to chelate iron ions. It was also interesting to note that THC is devoid of prooxidant potential because of its poor ability to reduce ferric ions. In comparison, curcumin ions very effective in reducing ferric ions. Most of the phenolic antioxidants are known to act as prooxidant mainly because of their ability to reduce ferric ions which result in recycling of ferrous ions for Fenton reaction and generating hydroxyl radicals (Kunchady and Rao, 1989). Thus saturation of double bond greatly decreases the ability of curcumin to reduce ferric ions.

DPPH and ABTS models are widely used to measure the antioxidant properties. In the present study using these models showed that THC was less active than curcumin in scavenging this radicals, however it was more active than trolox.

Similarly in case of lipid peroxidation induced by Ferric-ascorbate in soybean phosphatidyl liposomes, curcumin was more

active than THC in inhibiting the lipid peroxidation. But, THC was more active than Trolox. These results suggest that double bond contributes significantly to the antioxidant properties of curcumin in certain mechanisms. The double bond in conjugation with aromatic ring may help in electronic delocalization thus contributing to the increased stability of the free radical like phenoxalate radicals (Rajkumar and Rao, 1993).

CONCLUSION

In conclusion, the present study showed that THC is a good antioxidant compared to the standard antioxidant, Trolox. Although, THC is less active than curcumin in many models, the increased superoxide scavenging and decreased ability to reduce ferric ions offers interesting advantages over curcumin. Further, THC being colorless should be more stable than curcumin which is highly unstable at the physiological pH. In addition, THC is also more water soluble than curcumin. However, more studies are required to exploit these advantages of THC over curcumin.

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