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Possible mechanism and implications of phenolics-mediated reduction of XTT (sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene-sulfonic acid hydrate)

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Sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT)-based procedure is a simple method to estimate perhydroxyl/superoxide radical acid–base pair ($HO_2^{\cdot-}/O_2^{\cdot-}$) owing to the solubility of the reduced formazan. We show here that phenolic compounds, catechins and catechols, reduced XTT. Addition of superoxide dismutase in the medium enhanced the rate of oxygen consumption as well as XTT reduction. Based upon the data on XTT reduction and oxygen consumption, the mechanism of phenolics-mediated reduction has been proposed. Data on the limitation of adoption of the XTT-based procedure to estimate superoxide radicals in plant tissues is presented. Possible implications of the data have been discussed in wider perspectives.

ASSESSMENT of superoxide radical ($O_2^{\cdot-}$) in a living system is challenging due to rapid self-dismutation of the moiety and ubiquitous presence of an exceptionally proficient enzyme, superoxide dismutase, that catalyses dismutation of $O_2^{\cdot-}$ too efficiently to allow its accumulation and hence measurement (rate constant for the self-dismutation reaction = $< 2 \times 10^5\ M^{-1}\ s^{-1}$ as described by the reaction $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$; rate constant for the enzyme catalysed reaction is $2 \times 10^9\ M^{-1}\ s^{-1}$, a value near the diffusion limit[1,2]). The presence of an unpaired electron in the outer orbital of $O_2^{\cdot-}$, makes the moiety reactive, and this upon reacting with hydrogen peroxide gives rise to another highly reactive hydroxyl radical ($^{\cdot}OH$) through Haber–Weiss reaction[3]. These reactive species of oxygen react with proteins, nucleic acids, lipids and virtually with all the biomolecules to cause severe damage to the cell[4,5]. Under stressful environment, $O_2^{\cdot-}$ generation exceeds beyond the dismutation capability of the cell to force the system to undergo oxidative stress[6]. Therefore, it becomes essential to quantify $O_2^{\cdot-}$ in a living system.

Various available procedures for the purpose include chemiluminescence[7], spin trapping/electron paramagnetic resonance[8], electron spin resonance[9], and the reduction of

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tetrazolium dye such as nitroblue tetrazolium[10,11] or of the redox protein Cyt c[10,12], with some advantages and shortcomings associated with each of the above procedures[13]. With the synthesis of a new tetrazolium salt, sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) by Paull *et al.*[14], and its use for the analysis of cell growth and drug analysis[15], the dye was found to be useful for assaying perhydroxyl/superoxide radical acid–base pair ($HO_2^{\cdot-}/O_2^{\cdot-}$) generation[16]. XTT was recently used to monitor the production of $O_2^{\cdot-}$ by the tobacco cell cultures in response to pathogen attack[13,17]. It was shown that inclusion of either superoxide dismutase (SOD) or Mn (II) desferal significantly decreased XTT reduction, to make it possible to estimate $O_2^{\cdot-}$ using XTT.

A reagent to be adopted for assay should largely be free from interference of other cellular constituents. If, for example, the objective is to study the effect of a compound such as drug or plant extracts on $O_2^{\cdot-}$ generation, then the test compounds under study should not interfere with the reagent directly to the extent that the results obtained are misleading. Paull *et al.*[14] described a reduction reaction of XTT with ascorbic acid. This would mean that if the system under study has such compounds, the data obtained would be the additive result of $HO_2^{\cdot-}/O_2^{\cdot-}$ content and those reducing agents which would lead to false interpretation of the results.

Plant tissues contain an array of antioxidants/reducing compounds wherein phenolics (also used as antioxidants and as therapeutic agents[18]) represent the most obvious dominant group of compounds. The present study therefore, examines the phenolics-mediated reduction of XTT *vis-à-vis* studies on plant tissues with variable phenolic content.

All the experiments were performed at 25°C. Chemicals were purchased from M/s Sigma Chemical Co, St. Louis, USA. Two phenolic compounds namely, catechin and catechol were selected in the present study owing to their presence in plant systems. Stock solutions of catechin and catechol were prepared in acetone and ethanol, respectively. These were added in separate reactions to the reaction buffer consisting of phosphate buffer, 10 mM, pH, 7.5; XTT, 0.5 mM. XTT reduction was followed by measuring absorbance at 470 nm at frequent time intervals starting from 15 min onwards and up to 2 h. Unless and otherwise stated, the final concentration of phenolic compound used was 0.5 mM. A molar extinction coefficient of $2.16 \times 10^4\ M^{-1}\ cm^{-1}$ was used to quantify the amount of formazan produced[13]. Always, a control reaction was set up which had all the components except for phenolics.

Reduction of XTT by phenolic compounds was evaluated in the presence of 100 units of superoxide dismutase (SOD, EC 1.15.1.1, Sigma catalog no. S-4636) as well.

Oxygen consumption was monitored in all the reactions using a Clark type oxygen electrode attached to a control box unit (CB1D) and a chart recorder (M/s HansaTech, UK). Manufacturer's instructions were followed to set up the equipment and to record the data.

For experiments with leaf tissues, leaves of properly maintained plants of tea (*Camellia sinensis* (L.) O. Kuntze, comparatively a higher polyphenolic system[19]) and soybean (*Glycine max* var. bragg, leaf tissue of choice used in several investigations[20]) were selected. Since catechins are major polyphenols in the leaves such as that of tea, these were estimated as described by Singh *et al.*[21], to represent the level of polyphenols.

For the experiments of XTT reduction with plant tissue, the leaves of tea and soybean were harvested in the morning at 10 A.M. and the discs were prepared using a paper punch. These were thoroughly washed with distilled water and always a leaf area equivalent to 0.56 cm^2 was used in the experiments where required. All the experiments that were performed with leaf discs were also repeated with the whole leaf tissue (2nd leaf from the top) as well. The plant material was incubated in 1 ml of the reaction medium for different time intervals, followed by monitoring the XTT reduction starting from 15 min onwards and up to 2 h. Reduction of XTT by after dip solution (ADS) was also monitored. ADS was prepared by incubation of the discs in 1 ml of 10 mM phosphate buffer (pH, 7.5; XTT was not added) for 60 min followed by the removal of the tissue. Catechins were estimated in ADS also as described earlier. In ADS-mediated reactions, oxygen consumption was also monitored in the presence and absence of SOD.

Separate sets of experiments were conducted essentially as above except that 1% polyvinyl polypyrrolidone (PVPP) was included in the reaction medium and in the phosphate buffer to adsorb phenolic compounds leached out from the tissue.

XTT reduction leads to the synthesis of formazan. Therefore the expression, XTT reduction or the formazan production has been used interchangeably throughout the paper.

Incubation of XTT along with phenolic compounds showed a time-dependent reduction of XTT (Figure 1). Catechin was found to be more reactive as compared to catechol. Increasing the catechin content from 10 to 1000 μM, led to the increased formazan production by 51.30 times in 2 h, whereas these values were 45.36 for catechol (Figure 1 *a–c*).

While the data showed reduction of XTT by phenolic compounds, possible mechanism of the reduction was to be investigated. One possibility could be that phenolic compounds, in general, are reducing agents[18,22] and hence a nucleophilic attack on the positively-charged tetrazolium ring of XTT is possible to lead to the formazan production. This mode of reduction of XTT by phenolic compounds can be supported from the work of Paull *et al.*[14], who showed XTT reduction by reducing agent, ascorbic acid.

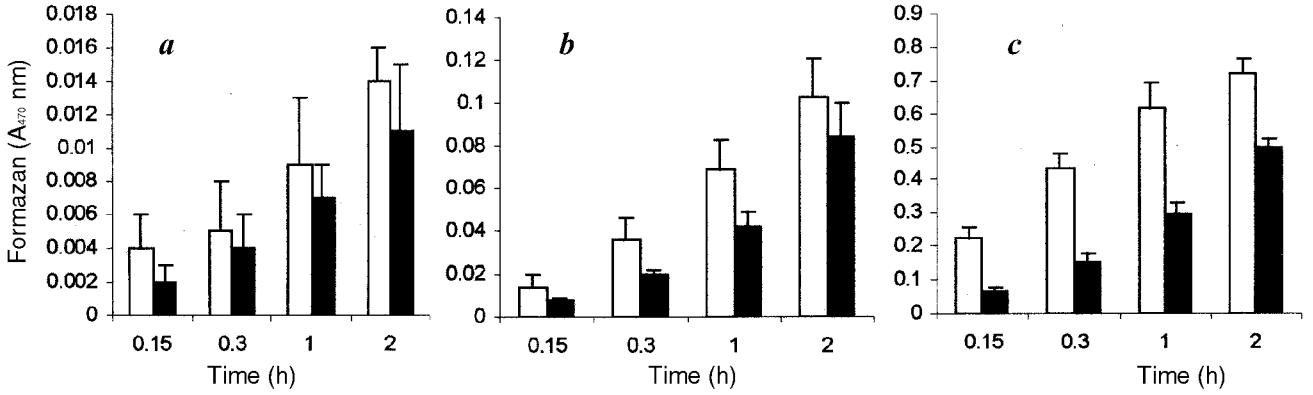

Figure 1. Effect of different concentrations of catechin () and cat echol () on XTT reduction. *a*, 10 μM; *b*, 100 μM; and *c*, 1000 μM. Error bars show standard deviation from the mean.

Also, the reactions involving auto-oxidation of phenolics in aqueous environment cannot be ruled out. Auto-oxidation leads to the production of phenoxy-free radical and hydronium ion as per the following reaction[23],

$$\text{Phenolic compound} + H_2O \rightarrow \text{phenoxy free radicalÿ} + H_3O^+.$$

The free radical species produced can in turn reduce XTT to its soluble formazan.

Alternatively, auto-oxidation of phenols[23–26] could also result in the formation of free radical species of semiquinones ($Q^{\cdot -}$) and $O_2^{\cdot -}$. These species can reduce XTT to form soluble formazan.

The above discussion suggested that the phenolics-mediated reduction of XTT could be either dependent or independent of $O_2^{\cdot -}$. If the reduction is $O_2^{\cdot -}$ dependent, O_2 uptake should take place during reduction and also, addition of SOD must exhibit reduced XTT reduction since the enzyme would compete with XTT for $O_2^{\cdot -}$.

O_2 measurement studies did exhibit O_2 uptake during XTT reduction by polyphenolics. For each mole of O_2 consumed per minute, 7.99 and 6.58 moles of XTT (Figure 2) were reduced on per minute basis in the presence of catechin and catechol, respectively. Interestingly, addition of SOD increased XTT reduction by 78 and 58% in the presence of catechin and catechol, respectively. Concomitantly, O_2 consumption increased by 91 and 80% by the addition of SOD and in the presence of catechin and catechol, respectively. Thus, the data clearly indicated the involvement of O_2 and hence $O_2^{\cdot -}$ in phenolics-mediated XTT reduction. Since, more than 6.5 moles of reduced XTT are obtained whereas only one mole of oxygen is consumed on per minute basis in the phenolics-mediated reduction, it is quite likely that other mechanisms independent of $O_2^{\cdot -}$, as mentioned earlier in the discussion, are also operative concomitantly or that $O_2^{\cdot -}$ initiates the chain reaction to account for higher moles of XTT reduced per mole of oxygen consumed.

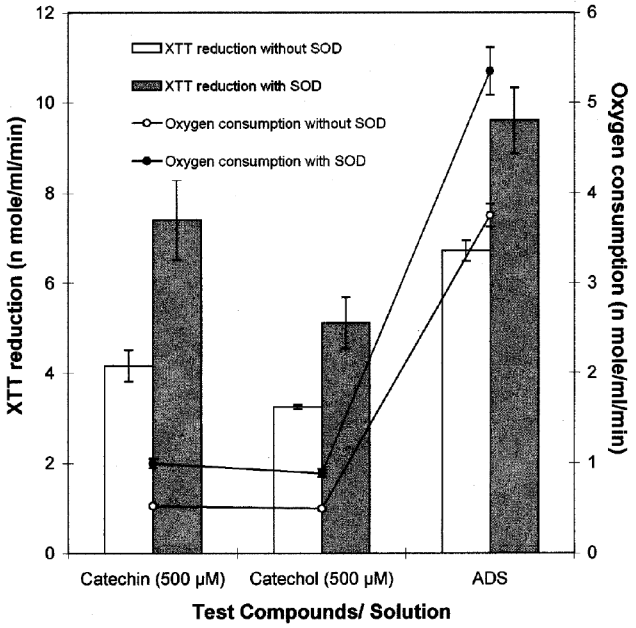

Figure 2. Effect of addition of superoxide dismutase (SOD) on phenolics-mediated reduction and oxygen consumption. Experiments were also performed with after dip solution (ADS). To prepare ADS, discs were incubated in 1 ml of 10 mM of phosphate buffer (pH 7.5) for 60 min, followed by removal of the tissue. XTT was then added and its reduction was monitored from 15 min onwards till 2 h. All the data presented is based upon the readings obtained at 2 h. Error bars show standard deviation from the mean value.

The result, that the addition of SOD led to increased rather than decreased reduction of XTT with concomitant increase in O_2 uptake (Figure 2) suggested the operation of the following mechanism,

$$2\ \text{Phenolics} + 2O_2 \rightarrow 2\ \text{Semiquinones}\ (Q^{\cdot -}) + 2O_2^{\cdot -}, \quad (1)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2, \quad (2)$$

$$2\ \text{Phenolics} + O_2 + 2H^+ \rightarrow 2\ \text{Semiquinones} + H_2O_2, \quad (3)$$

$$Q^{\cdot -} + \text{XTT} \rightarrow \text{QH} + \text{XTT}_{\text{reduced}}. \quad (4)$$

The mechanism implied that phenolics generated $Q^{\cdot-}$ and $O_2^{\cdot-}$ (eq. 1). In an aqueous medium $O_2^{\cdot-}$ undergoes spontaneous dismutation according to eq. (2). As is obvious from the sum up equation, there is net consumption of O_2 during oxidation of phenolics (eq. 3). Addition of SOD will greatly increase the dismutation by a factor of 10^4, thus driving the reaction (1) towards $O_2^{\cdot-}$ generation, leading to increased O_2 consumption and $Q^{\cdot-}$ generation. Greater $Q^{\cdot-}$ will reduce more XTT (eq. 4). Thus, the addition of SOD resulted in greater phenolics-mediated reduction of XTT as well as O_2 consumption.

Results thus showed that XTT reduction is possible by phenolics. If phenolic compounds could reduce XTT, an erroneous picture of the metabolic $O_2^{\cdot-}$ generation would be presented when these compounds are present in the system. Meaning thereby, if the metabolic $O_2^{\cdot-}$ status is same in two systems under consideration, the differential concentration of phenolic compound *per se* would render a differential status of estimated $O_2^{\cdot-}$. For example, metabolic $O_2^{\cdot-}$ generation and/or phenolics accumulation can be enhanced in plant tissues experiencing a variety of stresses[6,20]. While comparing $O_2^{\cdot-}$ in different plant systems under study, the phenolics content variation *per se* would lead to different values even if metabolic $O_2^{\cdot-}$ status has not changed. Since phenolics are important secondary metabolites in plant system, adoption of XTT-based procedures may pose logistic problems.

An attempt was made to estimate $O_2^{\cdot-}$ using XTT-based procedure in the leaves of tea and soybean along with the estimation of phenolics. Estimation of phenolics as catechins showed that tea leaves had as high as 20.57 mg/g fresh weight compared to 12.4 mg/g fresh weight for soybean. Tea leaves reduced significantly higher amount of XTT compared to that by soybean on per unit time basis (Figure 3 *a*). The interesting result was that upon removal of the leaf discs from the reaction medium after 60 min, formazan production continued. Secondly, even the 'after-dip-solution' (ADS) showed XTT reduction, which continued throughout the experimentation period of 2 h (Figure 3 *b*). ADS of tea produced 5.19 times higher formazan in 2 h than soybean ADS. When estimated catechins in ADS, tea ADS showed a value of 155 µM whereas soybean had only 27.5 µM (5.64 times higher in tea compared to soybean). Such a strong stoichiometric relationship between catechins content and XTT reductions, further strengthened our results on the involvement of phenolics in XTT reduction. The result that the XTT reduction continued even after removal of leaf discs and by ADS, indicates that XTT reduction obtained was probably mediated by phenolics and not by the metabolic $O_2^{\cdot-}$ status ($O_2^{\cdot-}$ has extremely short half life[1,27] of approximately $2 \times 10^5\ M^{-1} s^{-1}$ for the self-dismutation reaction at pH 7.4 as described by the reaction $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$). When the experiments were repeated with whole leaf tissue, the trend obtained was essentially the same except that the values were less compared to those obtained with leaf discs (data not shown). Further, the experiments wherein PVPP was included in the incubation medium to adsorb phenolics, XTT reduction was reduced substantially (up to 90%) compared to the experiments wherein PVPP was not included (Figure 3 *a*, *b*). These results further supported the data that phenolics are involved in XTT reduction. Slight reduction of XTT in the presence of PVPP could be because of incomplete adsorption of phenolics onto PVPP and also that available free radicals reacted with XTT.

The above results have further implications, for example while analysing a large number of plant samples, the question will be on how to stop the reaction? If the tissue

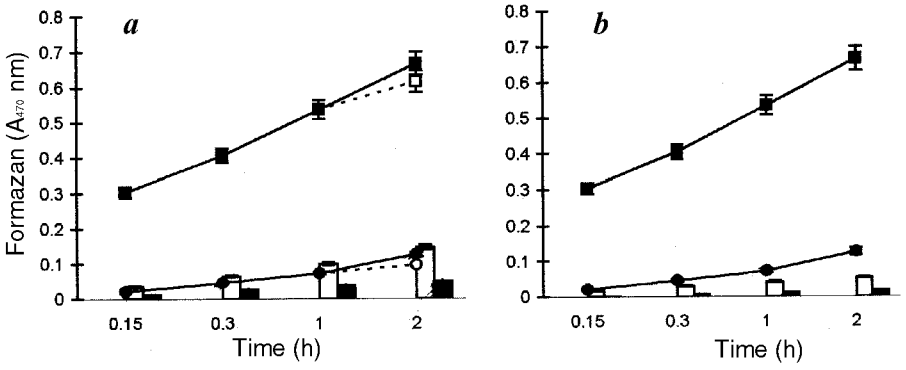

Figure 3. ***a***, Time course reduction of XTT by the leaf discs of tea (— —) and soybean (— —). Dotted lines show that the discs of tea (··· ···) and soybean (··· ···) were removed from the reaction mixture after one hour of incubation. XTT monitoring continued even after the removal of discs. ***b***, Experiments with after dip solution (ADS) of tea (— —) and soybean (— —). To prepare ADS, discs were incubated in 1 ml of 10 mM of phosphate buffer (pH 7.5) for 60 min, followed by removal of the tissue. XTT was then added and its reduction was monitored from 15 min onwards till 2 h. Error bars show standard deviation from the mean value. Experiments in (***a***) and (***b***) were performed in the presence of 1% PVPP as well; open bar () depicts experiments with tea whereas closed bar () shows results for soybean.

is removed to stop the reaction, XTT reduction would continue as shown earlier. This will incorporate enormous error in the absorbance between the first and the last sample. Even boiling the extract to stop the reaction was not successful, which in fact led to the development of much intense colour and diminished the differences, if any (data not shown).

Our conclusion that, phenolics mediate XTT reduction, becomes even more crucial for some of the experiments like those performed by Thabrew *et al.*[28] wherein the efficacy of catechin and other hepatoprotective plant components was tested by measuring the cell viability using tetrazolium salt (higher the cell viability higher the tetrazolium reduction). If tetrazolium salts are reduced by the compounds under test *per se*, as in the present example of XTT reduction by catechins, the data would lead to mistaken conclusions, i.e. catechin-treated cells would show more XTT reduction meaning thereby more cell viability, whereas, such an effect would, in fact, be a reflection of the reaction of catechin *per se* rather than representing cell viability. Earlier reports on XTT-mediated metabolic $O_2^{\cdot-}$ level measurements could not detect such a flaw because of the adoption of protoplast-based system which probably has much less of phenolics and there was no need to stop the reaction as it was a time span study for the superoxide anion burst[13,17].

Although some of the limitations of XTT-based procedure have been mentioned earlier as well[15], our results showed that XTT-based procedure to estimate $O_2^{\cdot-}$ in plant tissues is subjected to interference by phenolics, and hence the data obtained would lead to misinterpretation of the results in the presence of these compounds.

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