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# GC-MS analysis and Mitochondrial Functionality Potential of the Fruits of *Tetrapleura tetraptera* By Cupric Reducing Antioxidant Capacity Assay

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## ABSTRACT

The use of antioxidants has emerged as a promising therapeutic strategy for the management of mitochondrial dysfunction and other oxidative stress-related degenerative pathologies. *Tetrapleura tetraptera* is a well-known tree and its fruit is applied traditionally as spice and in the preparation of remedies for different ailments. In this study, the gas chromatography-mass spectrometry (GC-MS) analysis and mitochondrial functionality potential of *T. tetraptera* fruits were investigated. GC-MS was used to detect compounds in the*n*-hexane and ethanol extracts of *T. tetraptera* fruits. Cupric reducing antioxidant capacity (CUPRAC) assay was used to evaluate the mitochondrial functionality potential of the ethanol extract. GC-MS analysis revealed the presence of six compounds in the*n*-hexane extract of *T. tetraptera* fruits. These compounds were detected in trace quantities, the most abundant being 2,3-dimethyl-3-buten-2-ol (0.04%). The compounds: cis-vaccenic acid (35.37%), *n*-hexadecanoic acid (28.09%), 6-octadecenoic acid (25.21%), and octadecanoic acid (11.33%) were identified in the ethanol extract of the fruits. Consequently, the ethanol extract was subjected to CUPRAC assay. The ethanol extract exhibited a concentration-dependent high cupric reducing capacity, returning CUPRAC values in the range of 0.090 to 0.403 at the concentration of 10 – 80 µg/ml. This activity was comparable to that of the positive control, naringenin, which showed CUPRAC values of 0.059 – 0.378 at the same concentrations. These results indicate that *T. tetraptera* fruits possess good antioxidant property as evaluated by other related antioxidant assays. This could be attributed to a synergistic effect of the phytochemical constituents. Hence consumption of *T. tetraptera* fruits could be beneficial for the prevention of mitochondrial dysfunction and other oxidative stress-related degenerative disorders.

**Keywords**: *Tetrapleura tetraptera*, mitochondrial dysfunction, degenerative disorders, CUPRAC, GC-MS, Nigeria

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## INTRODUCTION

Mitochondrial dysfunction is a major factor in ageing and the pathogenesis of a multitude of diseases, including neurodegenerative and metabolic disorders. Mitochondria play central role for neuronal function. Neurons possess low glycolytic capacity; thus they rely greatly on aerobic oxidative phosphorylation (OXPHOS) as their energy source [1-3].

The mitochondrial based OXPHOS is however a major source of reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\bullet}OH$). Cumulative oxidative stress may induce cellular damage, impairment of the DNA repair system and mitochondrial dysfunction, all of which have been known to be key factors in accelerating the aging process and the development of degenerative disorders [4].

Typically, the production of these ROS is managed by the body's antioxidative defenses; however, oxidative stress occurs when ROS generation overwhelms the body's anti-oxidative defenses and therefore is implicated in mitochondrial dysfunction and neuronal damage. In virtually all the diseases in which mitochondrial dysfunction is associated, a main source of damage is overproduction of ROS by mitochondria, either directly or as a secondary consequence of other disorders [2, 5]. It has been shown that antioxidants are promising therapeutic candidates for the treatment of neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), schizophrenia, tardive dyskinesia (TD) and spinocerebellar ataxia (SCA) [6-16].

Lipoic acid (LA), an antioxidant, was reported to promote stabilization of cognitive measures in AD patients [6, 7]. In another study, Andreassen *et al*. [8]reported that LA significantly increased survival in both R6/2 and N171-82Q transgenic mouse models of HD. Vitamin E, another antioxidant, has also been shown to prevent or slow the progression of PD [9].In a 4-years study, vitamin E and C supplements were shown to significantly improve cognitive function [10]. Huperzine A, a polyphenol antioxidant, exerted significant improvement in cognitive function in elderly people, patients with AD, patients with schizophrenia and patients with vascular dementia [11]. Resveratrol, another polyphenol antioxidant, has demonstrated remarkable neuroprotective activity. Resveratrol directly activated sirtuin 1 (SIRT1), a neuroprotective enzyme, and when administered prophylactically, it protected mice subjected to an optimized ischemic reperfusion stroke model and greatly reduced apoptotic neuronal PC12 death induced by 1-methyl-4-phenylpyridinium ion (MPP+), a neurotoxin [12-14]. Resveratrol and other polyphenol antioxidants, including, curcumin, alvidin, dihydroguaiaretic acid, ellagic acid, tannic acid, myricetin, exifone, piceid, oligonol, and salvianolic acid B are reported to possess protective effects against β-amyloidopathy and protein aggregation in different cell lines or animal models [15, 16]. Furthermore, Nigerian Propolis (NP) and its phenolic constituents also exhibited *in vitro* free radical scavenging activities [17].

*Tetrapleura tetraptera* (Schum &Thonn.) Taub. (Fabaceae) is a robust, deciduous, perennial tree widely distributed across tropical rainforests in Africa. *T. tetraptera* is highly sought for its economic and medicinal importance. The different parts of *T. tetraptera* have found various applications including, culinary; ethno-medicine; cosmetology and carpentry. For its several ethno-medicinal uses, *T. tetraptera* has been a subject of many scientific studies. Scientific evidences now abound to attest the medicinal value of this plant in malaria, diabetes mellitus, hypertension, convulsions, epilepsy, leprosy, mental illness, wound-healing, inflammation, arthritis and rheumatoid pain, schistosomiasis control, asthma, and as a general tonic. Comprehensive account of the documented

studies about the chemistry and various biological properties of the different parts of *T. tetraptera* has been given [18]. The fruit of *T. tetraptera* is the most important part for culinary, cosmetological and medicinal purposes. The fruit is used as spice and in the preparation of pomades and soaps. The fruit is also documented to have antimalarial, antidiabetic, antibacterial, anti-inflammatory, anticonvulsant, anxiolytic, hypotensive, hypolipidaemic, and molluscicidal properties [18].

There have been continuing efforts to find agents that can protect against cellular oxidative damage and potentially treat other associated diseases.

In this study, we investigated the constituents of the fruit of *T. tetraptera* using GC-MS analysis and its antioxidant activity as a mechanistic tool for mitochondrial functionality using cupric reducing antioxidant capacity (CUPRAC) assay.

## MATERIALS AND METHODS

### *Plant Material*

Fresh fruits of *T. tetraptera* were harvested in Ikwuano, Umuahia, Abia State, Nigeria. The plant material was authenticated and documented (with voucher specimen number LUH/2019/8385) by Dr. G.I. Nodza, a senior taxonomist at the Herbarium of the University of Lagos, Akoka. The fruits were dried under ambient conditions and milled into powder.

### *Extraction of Plant Material*

Fifty grams (50g) of the finely milled *T. tetraptera* fruits was macerated twice with 400 mL of *n*-hexane for 72h at room temperature. The extracts were filtered, combined and concentrated *in vacuo* on a rotary evaporator at 40 °C. The resulting concentrate was evaporated to dryness in an oven at 45 °C to give *T. tetraptera* fruit *n*-hexane extract (TtFHE). The extraction process was repeated with absolute ethanol to obtain *T. tetraptera* fruit ethanol extract (TtFEE).

### *Investigation of Chemical Constituents using GC-MS*

The *n*-hexane and ethanol extracts of *T. tetraptera* fruit, TtFHE and TtFEE, were investigated for their chemical constituents using an Agilent GC-MS system comprising of 7890A gas chromatograph (GC) equipped with a 30m × 0.25mm × 0.25µm SLB-5ms capillary column and a 5975C VL MSD mass spectrometer (MS) equipped with Triple-Axis Detector. The carrier gas was helium, injection was by split method (20:1), flow rate was 1.2 mL/min and column oven was programmed from 50°Cto 300°C (50 °C for 2 min, then 10 °C/min to 300 °C for 8 min) with total run time of 35 minutes.

### *Identification of Constituents*

The separated and detected constituents were identified by comparison of their mass spectra fragmentation pattern with those of reference compounds in National Institute of Standards and Technology NIST08 and Agilent RTLPEST3 mass spectral databases.

### *Cupric Reducing Antioxidant capacity (CUPRAC) Assay*

Cupric reducing antioxidant capacity assay was carried out on the ethanol extract of *T. tetraptera* fruit (TtFEE) as described by Apak *et al*. [19]. One mL of ammonium acetate solution (1.0 M; adjusted to pH 7.0 by dilute acetic acid/ or dilute ammonium hydroxide solution) was added to 1 mL of the extract solution (10, 20, 30, 50, 80 µg/ml prepared in ethanol) in a 25 mL volumetric flask. To this mixture, 1 mL of cupric chloride solution (10 mM) and 1mL of neocuproine (7.5 mM prepared in ethanol) were added. The resulting mixture was made up to 25 mL with distilled water and incubated at room temperature for 30 min. Thereafter, the absorbance was read at 450 nm against a reagent blank using an

Agilent 8543 UV spectrophotometer. Each test preparation was performed in triplicate. Naringenin was used as a positive control. The reagent blank was prepared by replacing 1 mL of the extract with 1 mL of ethanol in the test preparation. The cupric reducing antioxidant capacity was expressed in terms of absorbance, higher absorbance indicating a higher reducing power [19].

***Statistical Analysis***

Statistical analysis of the results was performed using GraphPad Prism Version 6.0 (GraphPad Software, San Diego, USA). The values were expressed as mean ± SEM and analysed for statistical significance using Student's t-test with $p \leq 0.05$ considered to indicate significant difference.

**RESULTS**

***Chemical Constituents of T. tetraptera fruit***

The chemical constituents of *n*-hexane and ethanol extracts of *T. tetraptera* fruit as determined by GC-MS analysis are presented in Tables 1 and 2. Six compounds were identified in TtFHE (Table 1) and their structures are presented in Figure 1. In TtFEE, four compounds were detected (Table 2) and their structures are presented in Figure 2.

**Table 1. The constituents identified in TtFHE using GC-MS**

| S/N | Compound name | Molecular formula | MW (g/mol) | RT (min) | Peak area (%) |
|---|---|---|---|---|---|
| 1 | 2-Methyl propyl octadecyl carbonate | $C_{23}H_{46}O_3$ | 370.6 | 2.554 | 0.03 |
| 2 | 2,3-Dimethyl-3-buten-2-ol | $C_6H_{12}O$ | 100.2 | 2.972 | 0.04 |
| 3 | 2-Chloroethyl caprate | $C_{12}H_{23}ClO_2$ | 234.8 | 3.189 | 0.03 |
| 4 | 2-Chloroethyl laurate | $C_{14}H_{27}ClO_2$ | 262.8 | 3.521 | 0.02 |
| 5 | 1-Ethylsulfanyl dodecane | $C_{14}H_{30}S$ | 230.4 | 4.866 | 0.02 |
| 6 | (4-Nitrophenyl) N-(4-chlorophenyl)carbamate | $C_{13}H_9ClN_2O_4$ | 292.7 | 5.243 | 0.01 |

MW: molecular weight, RT: retention time, TtFHE: *T. tetraptera* fruit *n*-hexane extract

**Table 2. The constituents identified in TtFEE using GC-MS**

| S/N | Compound name | Molecular formula | MW (g/mol) | RT (min) | Peak area (%) |
|---|---|---|---|---|---|
| 1 | n-Hexadecanoic acid | $C_{16}H_{22}O_2$ | 256.4 | 18.954 | 28.09 |
| 2 | cis-Vaccenic acid | $C_{18}H_{34}O_2$ | 282.4 | 20.648 | 35.37 |
| 3 | 6-Octadecenoic acid | $C_{18}H_{34}O_2$ | 282.4 | 20.688 | 25.21 |
| 4 | Octadecanoic acid | $C_{18}H_{36}O_2$ | 284.4 | 20.848 | 11.33 |

MW: molecular weight, RT: retention time, TtFEE: *T. tetraptera* fruit ethanol extract

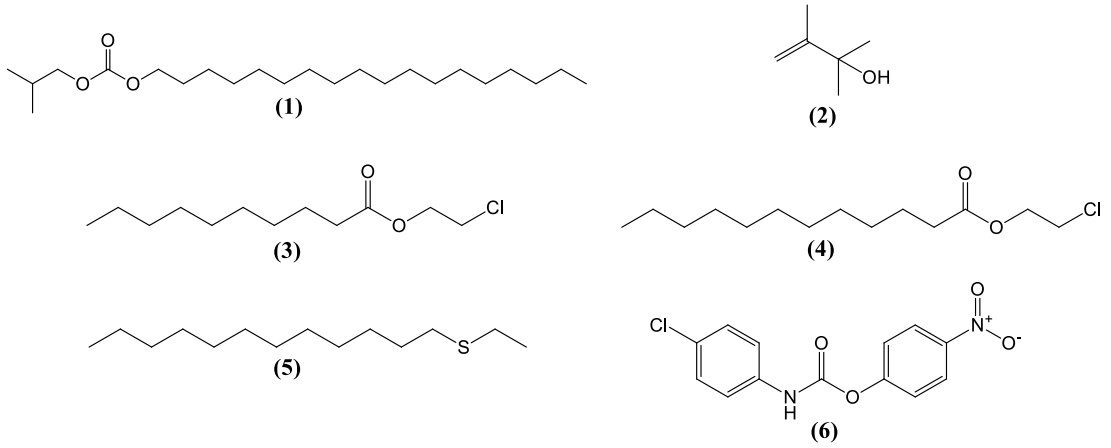


**Figure 1**. Structures of compounds identified in TtFHE: 2-Methyl propyl octadecyl carbonate (1), 2,3-Dimethyl-3-buten-2-ol (2), 2-Chloroethyl caprate (3), 2-Chloroethyl laurate (4), 1-Ethyl sulfanyl dodecane (5) and (4-Nitrophenyl) N-(4-chlorophenyl) carbamate (6)

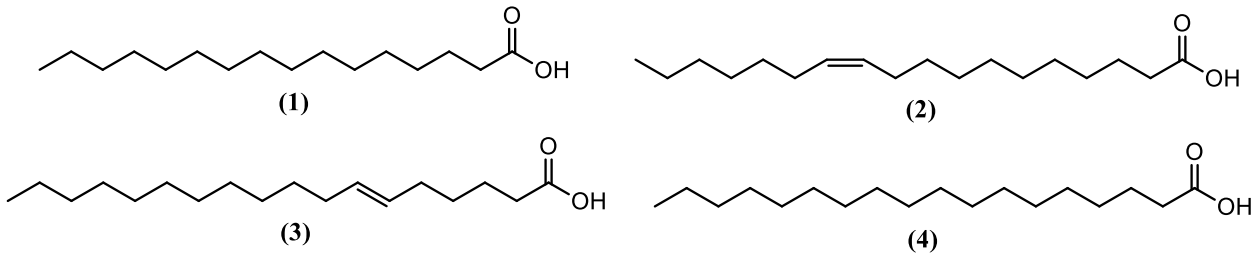


**Figure 2**. Structures of compounds identified in TtFEE: *n*-Hexadecanoic acid (1), cis-Vaccenic acid (2), 6-Octadecenoic acid (3), and Octadecanoic acid (4)

***Cupric Reducing Antioxidant Assay of T. tetraptera fruit***

The result of CUPRAC assay of TtFEE is given in Figure 3. The cupric reducing activity of TtFEE was found to be concentration-dependent and comparable to that of naringenin, used as positive control. TtFEE showed peak CUPRAC value of 0.403 at 80 µg/ml, while naringenin returned peak CUPRAC value of 0.378 at the same concentration.

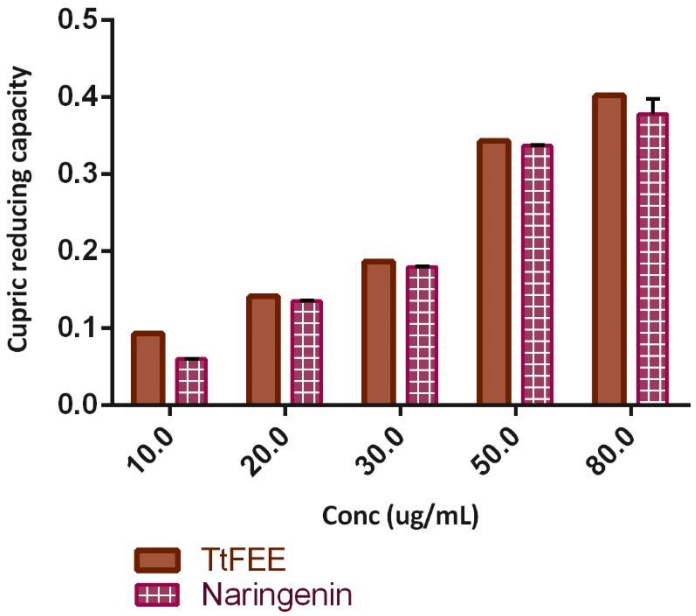


**Figure 3**. Cupric reducing antioxidant capacity (CUPRAC) of *T. tetraptera* fruit ethanol extract (TtFEE) and Naringenin. Values are given as mean ± SEM (n=3), No statistically significant difference ($p > 0.05$) between TtFEE and Naringenin groups.

## DISCUSSION

ROS-mediated mitochondrial dysfunction, excitotoxicity and apoptosis have been reported to be of pathological importance for ageing and age-related degenerative disorders such as AD, PD, HD and ALS. Scientific evidence indicates that antioxidants in form of diet and supplements may be of major importance for prevention of ROS-associated diseases. Scientists have further shown that a combination of antioxidants may be more beneficial than single entities for long term purpose [20]. In this study, the constituents and antioxidant activities of the fruit of *T. tetraptera* as a potential for mitochondrial functionality were investigated using GC-MS analysis and CUPRAC assay.

The six compounds identified in TtFHE were 2-methyl propyl octadecyl carbonate; 2,3-dimethyl-3-buten-2-ol; 2-chloroethyl caprate; 2-chloroethyl laurate; 1-ethyl sulfanyl dodecane and (4-nitrophenyl) N-(4-chlorophenyl) carbamate. These compounds however, were present in trace amounts, as they collectively made up less than 5% of the *n*-hexane extract; the remaining composition being undetected. The chemical nature and trace levels of these compounds suggest the unique nature of the *n*-hexane extract and/or that some of the compounds could be anthropogenic contaminants. Sulfur compounds abound in nature and are known for their odorous property. A sulfanyl compound, 1-(ethylsulfanyl) ethanethiol, was identified in durian, a tropic fruit, and was reported to contribute to its strong stinky aroma [21]. This suggests that 1-ethyl sulfanyl dodecane could contribute to the pungent odour of *T. tetraptera* fruits. Halogenated organic compounds were considered to be biosynthetic oddities of nature and mainly associated with marine

biota [22]. Although, evidences have now shown that organohalides could also be biosynthesized by some terrestrial organisms [22, 23], the organohalides detected in the *n*-hexane extract of *T. tetraptera* fruits: 2-chloroethyl caprate and 2-chloroethyl laurate, are documented contaminants of agricultural produce, arising as by-products of ethylene oxide fumigation [24, 25]. Carbamates are a class of agricultural pesticides. These compounds and their metabolites are of toxicological importance for their residual levels in agricultural produce. This suggest that (4-nitrophenyl) N-(4-chlorophenyl) carbamate detected in the *n*-hexane extract could be a contaminant from the use of carbamate pesticide on the fruits.

The four compounds detected in TtFEE were present at higher levels; these were *n*-hexadecanoic (palmitic) acid (28.09%), cis-vaccenic acid (35.37%), 6-octadecenoic acid (25.21%), and octadecanoic (stearic) acid (11.33%). In a similar study by Udourioh and Etokudoh [26] 15 fatty acids were identified in the fruits of *T. tetraptera*, and the major ones were palmitic acid (49.44%), linoleic acid (26.81%), oleic acid (19.72%) and stearic acid (3.20%); which is similar to the composition of the ethanol extract in this study. Studies have shown that the volatile components of *T. tetraptera* fruits are predominantly lower acids, their esters, ketones and alcohols. Ngassoum *et al*. [27] reported 2-methyl-2-buteic acid (67.3%), 2-methylbutanoic acid (31.0%) and limonene (1.7%) as the main components of the *n*-hexane extract of the fruits. Udourioh and Etokudoh [26] identified the major constituents of the essential oil of the fruits as acetic acid (34.59%), 2-hydroxy-3-butanone (18.25%), butanoic acid (8.35%), 2-methyl butanoic acid (7.58%), butanol (4.30%) and nerol (3.25%). Similar constituents were reported in the dichloromethane and solid-phase micro-extraction extracts of the fruits [27]. Most aromatic plants are known to contain predominantly terpenoids, fatty acids and their esters; the high levels of lower acids, their esters, ketones and alcohols in the fruits of *T. tetraptera* made it an unusual aromatic plant. Its characteristic aroma has been attributed mostly to 2-methyl butanol (7.45%), butanol (4.30%) and nerol (3.25%); its fruity flavor to 2-methyl butenoic acid ethyl ester (2.70%) and 2-methyl butanoic acid ethyl ester (2.09%) and its pungent odour mostly to 2-hydroxy-3-butanone (18.25%) [28].

The CUPRAC assay is an electron transfer antioxidant technique that involves the reduction of cupric ion ($Cu^{2+}$) to cuprous ion ($Cu^{+}$) by the antioxidative principles in the test substance. The cupric ion ($Cu^{2+}$) derived from cupric chloride ($CuCl_2$) reacts with neocuproine, a chelating agent, to form Cu(II)-neocuproine complex, an oxidizing agent that is reduced by the antioxidants to Cu(I)-neocuproine chelate, which shows maximum absorption at 450 nm. The reduction of Cu(II)-neocuproine to Cu(I)-neocuproine is reflected by a colour change from light blue to yellow-orange. The more intense this colour change, the higher the absorbance and the higher the cupric reducing capacity of the test substance [19]. Results showed that TtFEE had strong cupric reducing capability, giving CUPRAC values ranging from 0.090 to0.403 at 10 – 80 µg/ml. These values were comparable to or higher than those of naringenin, used as a positive control, showing CUPRAC values of 0.059 – 0.378 (Figure 3). However, statistical analysis showed that there was no significant difference ($p$> 0.05) in the cupric reducing capacity of TtFEE and naringenin. In a similar study, Famobuwa *et al*. [29] investigated the

antioxidant activity of ethanol extract of *T. tetraptera* fruits against 2,2-diphenyl-1-picryhydrazyl (DPPH) radical. The authors reported that the percentage DPPH radical scavenging activity of the fruits ranged from 10.56% to 66.01%, which was about 75% of the activity of ascorbic acid, used as positive control in the study. Although currently there is no single widely accepted method for evaluating 'total antioxidant capacity (TAC)' of a substance, the estimate of CUPRAC has been advocated as the most effective way to determine TAC in plants [30]. The CUPRAC method is applicable to both hydrophilic and lipophilic antioxidants (unlike DPPH and Folin assays) and requires physiological pH (7) in contrast to acidic pH used in FRAP (ferric reducing-antioxidant power) method or alkaline pH used in Folin method [31]. The CUPRAC assay also has a capability to assay thiol (–SH) bearing antioxidants (unlike FRAP method) [30]. The comparable CUPRAC values of TtFEE and naringenin observed in this study is an indication of the strong antioxidant potential of the former. This activity could be attributed to the chemical constituents of the extract. Palmitic acid, stearic acid and 6-octadecenoic acid, identified in this study, have been reported to possess antioxidant property [32, 33]. Some other undetected constituents, such as phenolics, might also have contributed to the observed antioxidant activity of *T. tetraptera* fruits. The antioxidant property of *T. tetraptera* fruit indicates that its consumption could be beneficial to human health. Its reported high level of acidic constituents however, portends toxicity. Toxicity studies have nevertheless indicated that *T. tetraptera* fruit is safe for human consumption at reasonable amounts [19].

**CONCLUSION**

*T. tetraptera* fruit is widely used traditionally for many purposes, including as spice and for the treatment of different ailments. In this study some chemical constituents of the *n*-hexane and ethanol extracts of the fruit were reported. This study further showed that the ethanol extract of the fruit possess antioxidant property comparable to that of naringenin, as indicated by CUPRAC assay. These findings suggest that *T. tetraptera* fruits could play preventive roles in mitochondrial dysfunctions and other ROS-associated disorders.

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