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Antioxidant and Anti-inflammatory Activities of *Odontella aurita* Aqueous Extract on Human Activated Neutrophil Granulocytes or in Stimulated Human Whole Blood

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Abstract

The present study is aimed at investigating the effect of an aqueous extract of *Odontella aurita* (*O. aurita*) on oxidative-stress and inflammation. The antioxidant effects of the extract were determined throughout the inhibition of human neutrophil granulocytes generating oxygen free radicals and their by-products superoxide anion ($O_2^{\cdot -}$) and hypochlorous acid (HOCl). The extract was assessed for its effects on inflammation as a potential reducer of cyclo-oxygenase and lipoxygenase activities. It was found that *Odontella aurita* is antioxidative as inhibitor of $O_2^{\cdot -}$ and HOCl productions. Moreover, the extract showed an Oxygen Radical Absorbance Capacity (ORAC) with a Trolox Equivalent Antioxidant Capacity of 3.55 nM comparable to that of Chlorella (3.16 nM) and Spirulina (3.60 nM). As far as anti-inflammatory activity is concerned, *O. aurita* inhibits the releasing of 12 HHTr marker of cyclo-oxygenase (COX 1 and COX 2) and 13 HODE marker of lipoxygenase (15 LOX 1). Based on above, *Odontella aurita* could be a potential candidate for healthy nutraceutical.

Keywords: *Odontella aurita*; Antioxidative; Anti-inflammatory; Nutrition; Chlorella; Spirulina; Blue algae

Introduction

Algae are a safe component of human foods and animal feeds. They are used as foods, food additives or nutraceuticals [1,2]. Microalgae-derived ingredients are expected to have growing applications not only in nutrition and health, but also in cosmetics and other high value products [3]. Although there are some 300,000 microalgae around the world, 100 of which are studied, only about 10 are used for nutritional purposes. Among the most studied microalgae are *Spirulina*, *Chlorella* and *O. aurita*. In Europe, only those three micro algae have been authorized for human foods under *Regulation* (*EC*) *No 258/97* of the European Parliament. *Odontella aurita* is a marine microalga which belongs to the family of seaweed known as diatom algae. This micro alga has 28-37 per cent mineral, 18-30 per cent protein, 17-28 per cent glucide and 6.5-11.8 per cent lipid content. 29% of the lipids in the algae are made up of omega-3 fatty acids; *Odontella aurita* is a source of omega-3 like EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). *Odontella aurita* was the first alga in France to be validated as food fit for humans. The alga was approved for sale on a European level in 2002. The beneficial effects of *O. aurita* on human health maybe derive from the presence of three categories of constituents: fibers, proteins and minerals as well as metabolites with antioxidant properties including carotenoids and vitamins [4].

On the one hand, an excessive increase in reactive oxygen species (ROS) production has been implicated in the pathogenesis of atherosclerosis, cardiovascular diseases and immuno-inflammatory diseases. Within the cells, ROS can act as secondary messengers and induce the oncogenic phenotype of cancer cells, cellular senescence or apoptosis [5]. Moreover oxidative stress (OS), a physiological abnormal phenomenon, occurs inside our cells or tissues when production of oxygen radicals exceeds their antioxidant capacity. OS has been pathologically linked with the systemic inflammatory response and antioxidant supplementation could have a clinical benefit [6].

On the other hand, many beneficial health properties of microalgae water extracts are attributed to their antioxidant or anti-inflammatory activities [7]. For example, *Chlorella* has been reported to show antioxidant activity in exhibiting attenuating effects on oxidative stress and suppressing inflammatory mediator [8,9]. In addition, experimental studies have demonstrated the antioxidant activity of *Spirulina* water extracts in decreasing lead-induced lipid peroxidation [10] or *Spirulina* anti-inflammatory action by selectively inhibited COX 2 [11]. All these reports exhibited the potential prospects of *Chlorella* and *Spirulina* water extracts as functional ingredient to prevent the ROS or pro-inflammatory agents related to diseases. While for *Odontella aurita* water extract scientific investigations are needed.

Therefore, we have performed a preliminary screening in ongoing research on *Odontella aurita* used as beneficial in the trial of oxidative-stress and inflammation as it contains various components with antioxidant activities [12,3], such as pigments (chlorophyll, fucoxanthine and β-carotene) and vitamins (B6, C and E). The purpose was, firstly to evaluate the interaction of *Odontella aurita* water extract with anion superoxide and hypochlorous acid generated by activated human neutrophil granulocytes and determinate its capacity through ORAC test. Secondly, anti-inflammatory activities of the micro alga extract were evaluated throughout the whole blood by monitoring inflammatory mediators of arachidonic acid catabolism and their by-products 12 rHHT and 13 HODE.

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Materials and Methods

Microalgae and chemicals products

Milli-Q (Millipore, Billerica, MA, USA) water was used throughout the study. *Chlorella* and *Odontella* were purchased from Sword-Age Vert while *Spirulina* was obtained from ELFERDY SARL. Buffer compounds and chemical reagents were purchased from Sigma Chemical Compagny (St Louis MO, USA).

Preparation of microalgae aqueous extracts

One gram per alga was crushed in 20 ml of Milli-Q water for 25 min in an ice bath. After centrifugation (400 g, 5 min at 4°C) the supernatant was collected and filtered through a 20 µm membrane to form the aqueous extracts (45 mg/mL) prior to analysis.

Isolation of human neutrophil granulocytes

Neutrophils were purified from fresh heparinized venous blood of healthy human subjects as previously described [13]. Briefly, 15 mL of blood was diluted with 15 mL of phosphate-buffered saline 0.1 M pH 7.4 (PBS). The diluted samples were then laid over 10 ml Histopaque® 1077. After centrifugation, granulocyte fraction and erythrocytes were retrieved at the bottom of the tube. The pellet was resuspended in 10 mL plasma and haemolysis was performed with ammonium chloride. After 10 min centrifugation (400 g), the neutrophils pellet was washed with PBS. Neutrophils were resuspended in Hanks Buffer Saline Solution pH 7.4 (HBSS). The concentration of the suspension was established using a Thoma counting chamber. Cell purity checked by May-Grünwald-Giemsa staining was at least 95%.

Assessment of Reactive Oxygen Species

Measurement of Superoxide anion ($O_2^{\circ-}$)

Neutrophil production of superoxide anion: $O_2^{\circ-}$ production by human Neutrophils stimulated by PMA was measured by reducing ferricytochrome C [14]. First, 400 µL of neutrophils (2.5×10^6 cells/mL) was pre-incubated with various concentrations of aqueous extract of *Odontella aurita* in HBSS for 30 min at 37°C. Secondly, 100 µL of stimulus Phorbol-12-Myristate-13Acetate (PMA 160 nM), 100 µL of ferricytochrome C (2 mg/mL) and 100 µL of Superoxide dismutase (2500 U/mL) in reference tube only, were added to a final volume in each vial of 1 ml and incubated for 15 min at 37°C. After 5 min centrifugation at 1125 g, absorbance of the supernatants was measured with a Kontron Uvikon 923 spectrophotometer against a reference cuvette. The slope of the absorbance curve at 550 nm was converted to nanomoles of $O2^{\circ-}$ using the extinction coefficient E550=21.1 $mM.Cm^{-1}$. The results were expressed as the percentage of O2°- inhibited by *Odontella aurita* water extract.

Acellular model: The potential scavenging effect of *Odontella aurita* water extract on $O_2^{\circ-}$was studied as previously described [15]. Briefly, an assay system was set up containing the extract at the desired concentration, with 0.15 mM hypoxanthine, 1.5 mM EDTA, 0.025 mM cytochrome C. The reaction was started by adding 0.1 U/mLxanthine oxidase. The $O_2^{\circ-}$mediated increases in absorbance values at 550 nm was spectrophotometrically recorded with and without the extract after 5 min at 25°C, using appropriate controls.

Measurement of hypochlorous acid (HOCl)

Neutrophil production of hypochlorous acid (HOCl): The generation of hypochlorous acid (HOCl), from humain neutrophils stimulated by PMA, was measured by the chlorination of taurine [16], as previously reported [17]. Briefly, a reaction mixture of 1.5×10^6 neutrophils were pre-incubated with *Odontella aurita* water extract for 30 min. Afterwards neutrophils were stimulated by 16 nM PMA for 30 min at 37°C in the presence of 15 mM taurine. After addition of 10 µl of KI 2M to cell-free supernatants absorbance was measured against a reference cuvette at 350 nm.

Acellular model: The effect of *Odontella aurita* water extract on hypochlorous acid (HOCl) concentration was investigated via a taurine chlorination system [17]. Briefly, a desired concentration of *Odontella aurita* water extract was mixed with 60 µM NaOCl and 15 mM taurine in a PBS buffer. After 30 min at 37°C, 10 µl of KI 2M was added to the system and absorbance was measured against a reference cuvette at 350 nm.

Oxygen Radical Absorbance Capacity (ORAC) essay

Algae water extract ORAC were measured using the Spectrofluorometer (Fluoromax-3, JOBIN YVON-HORIBA) at 37°C in an adapted Huang and coll procedure [18]. Briefly, 400 µl of 2, 2'-azobis (2-amidinopropane) dihydrochloride (or AAPH) 0.5M was added to a reaction mixture containing 2000 µl of Fluorescein (Fl) 1.18 µM and 10 µl of Trolox (concentration range 0-15 nM: for construction of a standard curve) or alga water extract. The Fl fluorescence decay was monitored at 1 second intervals for 2500 seconds (excitation, 440 nm; emission, 512 nm). All fluorescent measurement is expressed relative to the initial reading. The final results were achieved by first calculating of the area under the kinetic curve (AUC) and net AUC ($AUC_{sample} - AUC_{blank}$). Secondly, a standard curve was obtained by plotting the concentration of trolox (water-soluble analogue of vitamine-E) and the AUC. Finally, the trolox equivalents of a sample were calculated using the standard curve on an Excel program. AUC were calculated with SigmaPlot SPW10 (systat software).

HPLC analysis of 12 rHHT and 13 HODE

Fresh blood was collected in heparinized tubes from normal volunteers. Then, 1 ml aliquots were transferred to heparinized tubes preloaded with either 5 µL of the vehicle(DMSO) or 5 µLof test compounds and incubated for 15 min at 37°C. For 13 HODE and 12 rHHT analysis, 100 µl of LPS 5 mg/L was added before 20 h incubation at 37°C. Finally, 12 rHHT and 13 HODE were extracted by ethyl acetate. HPLC analysis was performed on a hypersil ODS 5 µm column (150 mm x 4.6 mm) using a methanol/water/acetic acid: 70:30:0.08, PH 6.0, mobile phase flow rate of 1 ml/min and UV detection at 245 nm and 270 nm as previously described by Pommery N and colleagues [19].

Statistical analysis: Statistical analysis of the results obtained was performed using statistical software. All data were expressed as mean ± standard error to standard mean (M ± ESM). The data are representative of six distinct observations. The difference is significant for $p<0.05$.

Results and Discussion

We had produced an aqueous extract of *Odontella aurita with a mass* concentration of 45 mg/mL. Our extract has 7.29 ± 1.61 mg/mL of proteins and its polyphenol rate was 4.00 ± 0.067% compare to pyrogallol. *Odontella aurita* is known as a marine micro-algae diatom source of omega-3 like EPA and DHA. It is also rich in silicium, vitamins (B2, B6, C, E, PP), provitamin A and amino acids. All these components maybe the source of antioxidant and anti-inflammation whose play on: fighting against cellular aging and in the nutrition of connective tissue (cartilage, skin, skeletal mineralization), manufacturing cells and the

nervous system's smooth function [20,21]. Bearing this in mind, we decided to explore the activities of our *O. aurita* aqueous extract in the field of oxidative stress and inflammation.

Evaluation of the aqueous extract of *Odontella aurita* antioxidant activity and determination of its antioxidant capacity

Effects of ***Odontella aurita (O. aurita) aqueous***extracts on **Reactive Oxygen Species (ROS: $O_2^{\cdot-}$ and HOCl):** In the cellular experimentation, *O. aurita* was able to inhibit Reactive Oxygen Species (ROS: $O_2^{\cdot-}$and HOCl) production by stimulated human neutrophils in a dose-dependent manner (Figures 1 and 2). The 50% inhibitory concentrations (IC_{50}) of *O. aurita* for PMA-induced $O_2^{\cdot-}$production was 200 +/- 0.2 µg/mL. As far as hypochlorous acid rate is concerned, the corresponding IC_{50} was 80 +/- 0.5 µg/mL. In the free cellular experiment, no significant effect was observed for $O_2^{\cdot-}$ or HOCl and it wasn't possible to evaluatean IC_{50}. *O. aurita* acellular effect in $O_2^{\cdot-}$and HOCl level was very shorter than the one observed in the cellular system (Figure 3 and Figure 4). Thus, *O. aurita* might react via a biochemical interaction in neutrophil oxidative metabolism. This cellular effects was not due to cytotoxicity as in each vial, LDH activity was lower than 20%.

Oxygen radical absorbance capacity of algaeaqueousextract: The time-dependent fluorescence decay of Fl oxidated by AAPH was monitored in the presence of antioxidant Trolox or by succesively each of our micro algae aqueous extracts (chlorella, *Odontella aurita* and spirulina). The ORAC of tested sampleswas evaluated as expression of their net Area Under the Curve (AUC): (Table 1). We haveobserved that AAPH reduced Fl fluorescence at 84.40%. By adding trolox or aqueous extracts of algae, this reduction of Fl AUC was less important and had reached the percentage of 47%, 59.3%, 63% and 67% respectively for trolox, *Odontelle aurita, spirulina and chlorella*.

The net AUC was calculated from the Trolox kinetic curves and plotted against concentration, results in a linear relationship as shown in Figure 5. Linear regression analysis resulted in a correlation coefficient (R^2) of 0.998. The resultant standard curve can then be interpolated for determination of antioxidant capacity of algae aqueous extract samples and reported as Trolox equivalents: (Table 2).

On the one hand, our *O. aurita* aqueous extract at 180 µg/mL have a Trolox equivalent in ORAC of 3.55 nM. On the other hand, its final concentration in ORAC assay (180 µg/mL) was between the IC_{50} of inhibitory concentrations of *O. aurita* for PMA-induced $O_2^{\cdot-}$ production (200 +/- 0.2 µg/mL) and hypochlorous acid (80+/- 0.5 µg/mL). Thus, at 180 µg/ml *O. aurita* will be effective to inhibit as well as ROS ($O_2^{\cdot-}$ and HOCl) production by stimulated human neutrophils in a state of oxidative stress or maintaining an antioxidant capacity with a Trolox equivalent of 3.55 nM. Our results could be compare to those of Xia S. and colleagues using purified fucoxanthin [22]. This team showed interesting antioxidant properties with an effectiveIC_{50} of 1,1-dihpenyl-2-picrylhydrazyl(DPPH) radicaland 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid(ABTS) radical at 140 and 30 µg/mL respectively.

Effects of aqueous extract of *Odontella aurita* on the catabolism of arachidonic acid: The arachidonic acid pathway overproduces proinflammatory eicosanoids (Figure 6). The purpose of this study was to examine the effect of our aqueous extract of *O. aurita* on the production of two proinflammatory eicosanoids: 12 HHTr and 13 HODE marker of lipoxygenase (15 LOX 1). Anti-inflammatory activities of the micro alga extract were evaluated throughout the whole blood by the monitoring inflammatory of those two mediators of arachidonic acid catabolism by-products.

In Figure 6, O. *aurita* extract at 450 µg/mLshowed an effective inhibition of cyclo-oxygenase and 15 LOX 1: 30% for 13 HODE and 10% for 12 HHTr. While at 45 µg/ml *O. aurita* inhibiting seems to be at the same level. Between those two values of concentration 45-450 µg/ml we have the corresponding IC_{50} 80 µg/ml and 200 µg/ml respectively for HOCl and $O_2^{\cdot-}$. Moreover the mass concentration of *O. aurita* in ORAC test was 180 µg/ml. All those results could suggest synergistic effectsof our extract regarding antioxidant and anti-inflammatories activities.

Conclusion

We have prepared an aqueous extract of *O. aurita* with a mass concentration of 45 mg/mL. This extract is rich in proteins and polyphenols. Our *O. aurita* aqueous extract has shown potential synergistic effects of antioxidant and anti-inflammatories activities

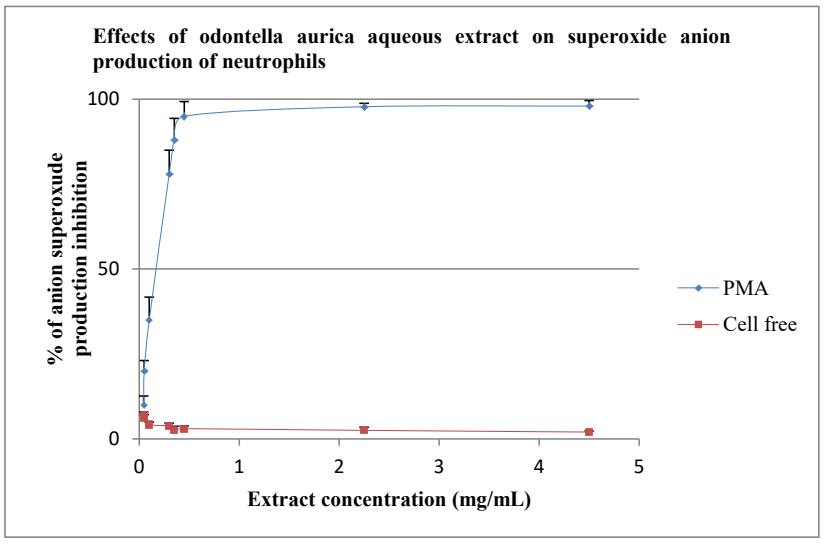

Figure 1: Reduction of superoxide anion production by various concentrations of *Odontella aurica* aqueous extract.

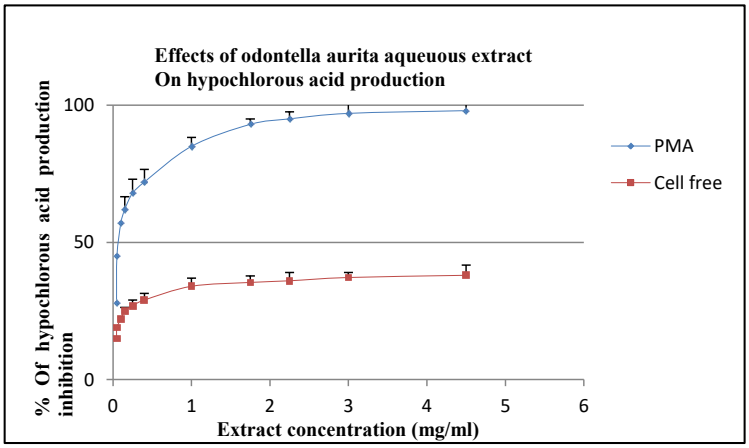

Figure 2: Reduction of hypochlorous acid production by various concentrations of *Odontella aurita* aqueous extract.

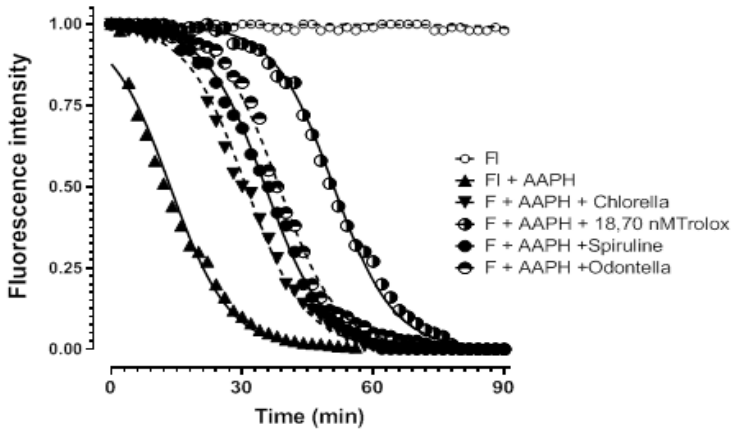

Figure 3: Representative curves from ORAC assay. 2, 2'-azobis (2-amidinopropane) dihydrochloride (or AAPH) oxydatingfluorescein (Fl) in the presence or absence of water-soluble antioxidant Trolox or micro algae aqueous extracts (*chlorella*, *odontella aurita* or *spirulina*: at final concentration of 180 µg/ml).

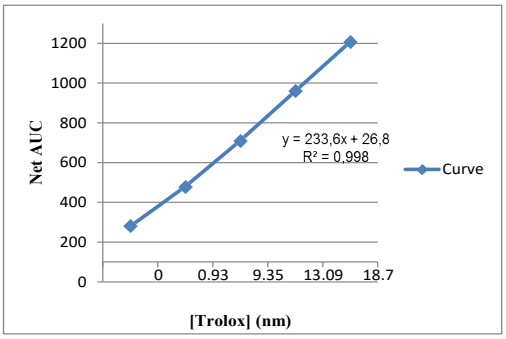

Figure 4: Trolox standard curve. The net AUC of varying concentration of Trolox antioxidant standards ranging from 0 to 18.7 nm are plotted *vs.* Concentration. The subsequent calibration curve was used to interpolate the antioxidant capacity of the three algae aqueous extracts (*Odontelle aurita, spirulina and chlorella)*.

Table 1: The net Areas Under the fluorescence intensity Cuve (AUC) corresponding for the sample used in Figure 3.

Samples / Parameters	F	F + AAPH	F + AAPH + *Chlorella*	F + AAPH + 18,70 nMTrolox	F + AAPH + *Spiruline*	F + AAPH + *Odontella*
Baseline	0,0	0,0	0,0	0,0	0,0	0,0
Total Area	95,09	14,87	31,33	50,58	35,20	38,69
Total Peak Area	95,09	14,87	31,33	50,58	35,20	38,69
Number of Peaks	1,000	1,000	1,000	1,000	1,000	1,000

Table 2: Antioxidant capacity of algae aqueous extract samples reported as Trolox equivalents.

Micro-algae	Troloxequivalent (nM)
Chlorella	3.16
Odontellaaurita	3.55
Spirulina	3.60

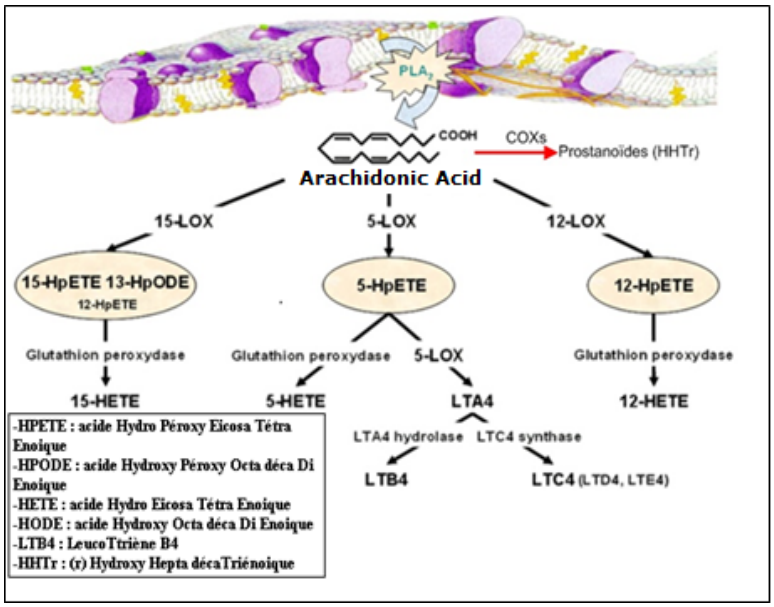

Figure 5: Diagram of the catabolism of arachidonic acid and their inflammatory markers.

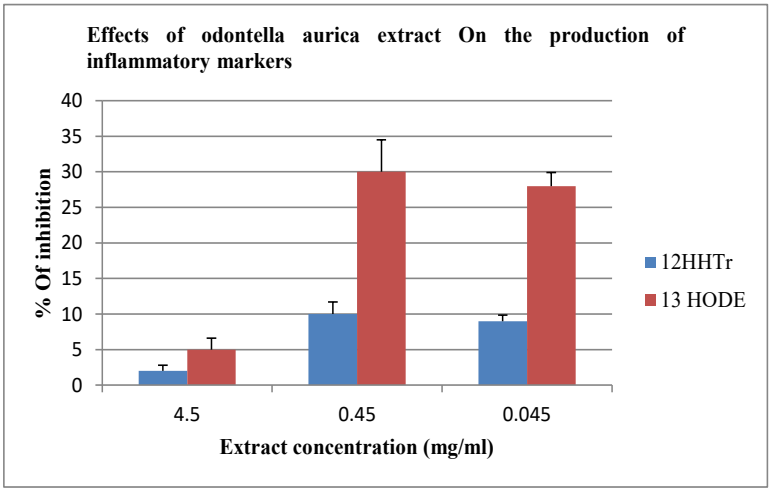

Figure 6: Inhibition action of O. aurita aqueous extract on inflammatory markers production rate.

with Trolox equivalent of 3.55 nM and inhibiting activities at 45 µg/ml throughout pro-inflammatory markers 12 HHTr and 13 HODE. Also, *O. aurita* aqueous extract has shown inhibition of human neutrophil granulocytes generating oxygen free radicals and their by-products superoxide anion (O_2°-) and hypochlorous acid (HOCl) in a dose-dependent manner with respectively IC_{50} of 200 +/- 0.2 µg/mL and 80 +/- 0.5 µg/mL. All those results suggested that *O. aurita* can be a natural source of antioxidant and anti-Inflammation for human health and nutrition.

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