Effect of isoespintanol isolated from *Oxandra* cf. *xylopioides* against DNA damage of human lymphocytes

Maria Elena Marquez-Fernandez[1], Diana Carolina Munoz-Lasso[1], Juan Bautista Lopez[1], Karol Zapata[2], Miguel Angel Puertas Mejia[3], Camilo Lopez Alarcon[4] and Benjamin Alberto Rojano[2*]

[1]Grupo Biotecnología Animal, Escuela de Biociencias, Universidad Nacional de Colombia. Medellin, Colombia
[2]Grupo Quimica de los Productos Naturales y los Alimentos. Escuela de Quimica, Universidad Nacional de Colombia. Medellin, Colombia
[3]Facultad de Ciencias, Universidad de Antioquia, Medellin, Colombia
[4]Departamento de Farmacia. Facultad de Quimica, Pontificia Universidad Catolica de Chile. Santiago de Chile, Chile

Abstract: Synthetic antioxidants are used in the food and pharmaceutical industry, however, there is concern about their safety; this has prompted the search for new antioxidants that are effective, safe and act at low concentrations. The objective of this study is to evaluate the oxygen radical scavenging capacity and clastogenic effect of the Isoespintanol /2-isopropyl-3,6-dimethyl-5-methylphenol) in DNA of human lymphocyte compared with the BHA (Butylated hydroxyanisole). The oxygen radical scavenging ability was evaluated by methods $ORAC_{FL}$ and $ORAC_{PGR}$, genotoxicity was determined by comet assay and data analysis was performed using ANOVA and Duncan test. The results show that the oxygen radical scavenging capacity of the BHA is higher than Isoespintanol, however according to the reactivity concept proposed by Lopez-Alarcon and Lissi, the Isoespintanol it is more reactive than BHA. Furthermore, according to some studies, BHA presented adverse effects on the health of consumers. Comet assay results revealed that at concentrations between 3 and 1620 μM the Isoespintanol don't show clastogenic effects on DNA. In conclusion, the antioxidant capacity for the BHA is higher than Isoespintanol, but considering reactivity concepts proposed by Lopez-Alarcon and Lissi, the Isoespintanol is faster to neutralize radicals that the BHA, furthermore, according to the National Institute of Health "BHA" is a human carcinogen.

Keywords: DNA damage, antioxidants, BHA, biological products.

INTRODUCTION

Reactive Oxygen Species (ROS) involves free radicals like hydroxyl, alkoxyl, peroxyl, superoxide anion, the hydroperoxyl, and peroxynitrite and also certain non-radical species that are oxidants or easily become free radicals, in this group there are organic peroxides, singlet oxygen, hydrogen peroxide, nitrous acid, hypochlorous acid, and peroxynitrous acid. A free radical is an unstable chemical species and highly reactive for have an unpaired electron (Halliwell and Whiteman, 2004: Ebrahimzadeh *et al.,* 2010). This species has independent existence and short half-lives, the free radicals can be formed in living organisms (including the human body) derived from normal biological processes such as mitochondrial respiration and lipid peroxidation or as a result of daily exposure to solar energy or ionizing radiation, air pollution, smoke, fatty diets, and stress (Oliveira *et al.,* 2015).

To decrease the harmful effects of ROS, the body has its antioxidant defense mechanisms, integrated by enzymatic and non-enzymatic systems; the enzyme complex are glutathione peroxidase, superoxide dismutase and catalase, and as non-enzymatic systems are phenolic compounds, glutathione, ascorbic acid, tocopherols and carotenoids, among others. But when there is a disequilibrium among the generation of ROS and the natural antioxidant systems and this imbalance is in benefit of the oxidants, the oxidative stress is generated and the biomolecules are attacked by the ROS (Choksi *et al*., 2004), which has been related with cancer, cardiovascular diseases and aging (Lee *et al*., 2004).

Specifically nucleic acids are vulnerable to the oxidation, which includes alteration of nitrogenous bases, adduct formation between bases and sugars, strand breaks and cross links between DNA and other molecules (Beckman and Ames 1998). If oxidative stress is excessive and DNA repair systems are overwhelmed, these changes can lead to mutagenesis and carcinogenesis (Lee *et al.,* 2004).

The oxidative action caused by free radicals can be neutralized using natural or synthetic antioxidants. Currently, synthetic antioxidants are the most used in the food industry and pharmacy; however, there is concern about their safety. Butylhydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have shown adverse effects such as increased cholesterol, liver enlargement and induction of liver cancer, among others. Some antioxidants such as quercetin, α-tocopherol, β-carotene and ascorbic acid have fought diseases such as esophageal

**Corresponding author:* e-mail: brojano@unal.edu.co

cancer, stomach duodenal ulcer, male infertility and oxidative damage induced by endotoxins (Sierens *et al*., 2002; Da Silva *et al*., 2002; Wilms *et al*., 2005).

However, *in vitro* studies of antioxidants have shown pro-oxidant effects at high concentrations; such as increase in the sister chromatid exchange, chromosomal aberrations and mutations, so that their use in disease treatment related to biomolecule´s oxidation is regulated (Šamec *et al.,* 2015).

Therefore, the interest of consumers to ingest natural additives has prompted the search for new antioxidants with high activity at low concentrations, safe and harmless to human health (Cabral *et al.,* 2015; Dandu and Inamdar, 2009).

Isoespintanol (2-isopropyl-3,6-dimethyl-5-methylphenol) (fig. 1) extracted from the leaves *Oxandra cf xylopioides* (Annonaceae) showed a good antioxidant activity in assays such as ABTS $^{\bullet+}$, DPPH, lipid peroxidation TBARS, reducing ability of Fe^{3+}/TPTZ, which was outlined as a potential protector for genetic damage in biological systems (Rojano *et al.,* 2008a: Rojano *et al.,* 2008b).

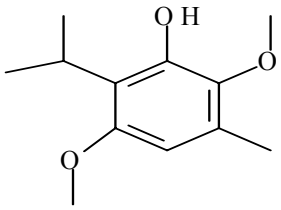

Fig. 1: Isoespintanol Structure

MATERIALS AND METHODS

Plant material

The vegetal material was harvested in Colombian caribbean region (Monteria), collected and stored at 4°C until its analysis.

Extraction

The dry leaves (0.5kg) of *Oxandra* cf. *xylopioides* were immersedin petroleum ether (7.5L) at 25°C and concentrated in vacuumto obtain an extract. The extract was passed through a column (5x80cm- Silica gel) eluting with gradient of hexane-CH2Cl2 (95:5, 80:20, 70:30, 100 each 0.5L), to give four fragments. Fragment II was mixed with CH2Cl2 and Fragment IV was recrystallized in hexane (1.5g) (1.5%). Finally, a crystalline solid was obtained with melting point (mp) 69-70°C and λ_{max} UV (MeOH) = 227, 273 nm.

Oxygen radical absorbance capacity (ORAC)

The ORAC methodologies are frequently used to determine the ability of a pure compound or complex mixtures to trap oxygen radicals, these methodologies are based in the protection that provides a sample to an objective compound that is being attacked by a radical. In this study the ability of Isoespintanol compared to the BHA of trap oxygen radicals generated by the precursor APPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) was evaluated.

$ORAC_{FL}$

This method is based in the evaluation of the capacity of a substance to capture peroxyl radicals that are the cause of the diminution of fluorescence of the probe (fluorescein). The protocol used was described by Prior (Prior *et al.,* 2003) and is now being carried out with some modifications as described: a volume of 30µL of the sample was added to 2,899µL of PBS (75mM), 21µL fluorescein $1x10^{-2}$M prepared in PBS and 50µL of AAPH 600mM, the temperature and pH, were controlled at 37°C and 7.4 respectively.

Readings were performed at λ/ slit excitation and λ/ slit emission of, 493/10nm and 515/15, respectively, the runs were made with attenuator 1%. The shielding effect of the sample was calculated by means of the differences in areas under the curve of diminution of fluoresce in with and without the sample, and compared against the standard Trolox. Results were reported as umol Trolox Equivalents Antioxidant Capacity (TEAC) per gram of sample using the following equation:

$$ORAC = \frac{(ABC - ABC^{0})}{(ABC_{Trolox-ABC^{0}})} f[Trolox]$$

Where ABC represents the Area Below of the Curve with the sample, ABC° is for the Area Below the Curve without the sample, ABC Trolox® for Area Below the Curve using Trolox and f is the dilution factor of the sample evaluated.

$ORAC_{RPG}$

The method described by Lopez-Alarco and Lissi with some modifications as described below was used. Pyrogallol Red (PGR) compound reacts with reactive oxygen species (ROS) generated by the azo compound APPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) in an aqueous medium and forms a colorless oxidized product. The compound tested traps free radicals and prevents the reduction of the absorbance due to decreased oxidized RPG formed (Lopez-Alarco and Lissi, 2006). To perform the reaction, 10µL of an aqueous solution of AAPH 0.3M were mixed with 50 µL of an aqueous solution of Pyrogallol Red (PGR) ($5x10^{-5}$M), 30µL of sample and 210µL buffer phosphate ([0.075M] and pH neutral, the absorbance of the mixture was monitored during 4000seconds at 540nm. For the spectrophotometric readings a Spectrophotometer Plate Reader UV-VIS was used. The shielding effect of the sample was calculated by

means of the percentage of inhibition of decay in absorbance when the reaction contains the sample; this value is compared against a standard Trolox®. Results were reported as umol Trolox Equivalents Antioxidant Capacity (TEAC) per gram of sample.

Isolation of human peripheral blood lymphocytes

Lymphocytes were isolated from heparinized human peripheral blood obtained from healthy patients. The blood was centrifuged at 1500rpm for 10minutes to remove the layer of white blood cells, which were resuspended in phosphate buffer (PBS) in the ratio 1:1, this mixture was added to 3ml of Hystopaque-1077 (SIGMA) and centrifuged at 2000rpm for 30minutes. Then, the cells were washed with saline solution for 30minutes at 2500rpm and finally resuspended in 2ml of phosphate buffer (PBS) (Sierens *et al*., 2001).

Cytotoxic testing

To evaluate the genotoxic effect on DNA, cell suspensions isolated (lymphocytes$1x10^6$ cells / ml) were treated with 10µl of Isoespintanol and BHA separately at different concentrations (1620, 1220, 630, 450, 206, 36, 5 and 3mM in dimethyl sulfoxide DMSO), subsequent it was added 10µl of H_2O_2 50mM (denaturing agent) to each treatment and stored for 180seconds at 4°C. As a Negative Control, cell suspensions were used only 10% DMSO without the denaturing agent and as Positive Control cell suspensions they were incubated with only a denaturing agent 50mM H_2O_2 (Çavaş and Könen, 2007).

Gel electrophoresis of individual cells

for this test the protocol proposed by Singh *et al* (Olive and Banáth, 2006) with slight modifications was used as described below: The cell suspension ($1x10^5$ células/ml) was preincubated at 4°C and then stirred in 90µl of Agarose (0.5% LMA in PBS Ca^{++}, Mg^{++}), the volume was placed on slides pretreated with 100µl agarose normal melting point (0.5% NMA in PBS Ca^{++}, Mg^{++}). The slides were incubated at 4°C for 12minutes to facilitate solidification of the agarose, and then were placed in lysis mix (2500mM NaCl, 100 mM EDTA, 0.01M Tris-HCL 10% DMSO, Triton X-100 at 1% and pH basic)at 4°C for 60minutes, they were then washed with cold PBS and incubated in dark for 30 minutes (4°C) in an electrophoresis chamber with scanning solution (300mM NaOH, EDTA 1mM and pH Basic) to 0.25cm on the slides. The electrophoretic sweep was performed at 25V and 300mA for 30minutes, and then the slides were covered with neutralizing mix (400mM Tris HCl pH neutral) at 25°C temperature for 900seconds and dehydrated with absolute methanol. Finally, the dry slides were colored with ethidium bromide (2g/L) and visualized on a Microscope Axiolab Reference Zeiss equipped by a fluorescence system with 20X objective. 25 photographs were taken, and then analyzed using the Comet Score software. The experimental steps were performed in the dark to prevent further DNA damage (Çavaş and Könen, 2007)

STATISTIC ANALYSIS

For testing, protective, cytotoxic and genotoxic, data was generated with an experimental design of randomized complete block, taking sample from each donor as blocking factor. The analysis of variance and the corresponding mean tests (Duncan) was performed using the statistical software Statgraphics Plus, version5.0.

RESULTS

Table 1 shows the $ORAC_{FL}$ and $ORAC_{PGR}$ values for Isoespintanol and BHA, the results show that the BHA has a higher antioxidant capacity than Isoespintanol, but considering the concepts of reactivity proposed by López-Alarcon and Lissi. We can conclude that the Isoespintanol molecules in solution are involved in a rapid neutralization of the free radical unlike the BHA.

One of the techniques used to evaluate breaks in DNA is gel electrophoresis of single cells or comet assay; this has been used to evaluate the genotoxicity of many physical, chemical and biological agents and to see the protective action of certain metabolites against denaturing the genetic material. In this study both antioxidants showed protective effect on the DNA of cells subjected to oxidative stress. Statistical analysis showed no statistically significant difference between the concentrations evaluated Isoespintanol (fig. 2). The concentrations of 3µM, 450µM and 630µM show remarkable protection against oxidative stress, suggesting that the compound can be used in a wide concentration range without losing its protective effect.

In the present study it was found that concentrations of 3.0µM, 8.0µM y 80µM Isoespintanol don't have genotoxic or cytotoxic effects on human lymphocytes peripheral blood compared to the positive control of both tests (figs. 2 and 3).

Table 1: Oxygen Radical scavenging capacity (ORAC) to Isoespintanol and BHA.

	$ORAC_{FL}$	$ORAC_{PGR}$	$ORAC_{PGR}/ORAC_{FL}$
BHA	8667,9 ± 632,6	1619 ± 23.25	0,19
Isoespintanol	3657,6 ± 148,6	1255 ± 16.28	0,34

* Values are means of each analysis for n = 3 ± SD

* The ORAC values are expressed as umol Trolox equivalent / g sample

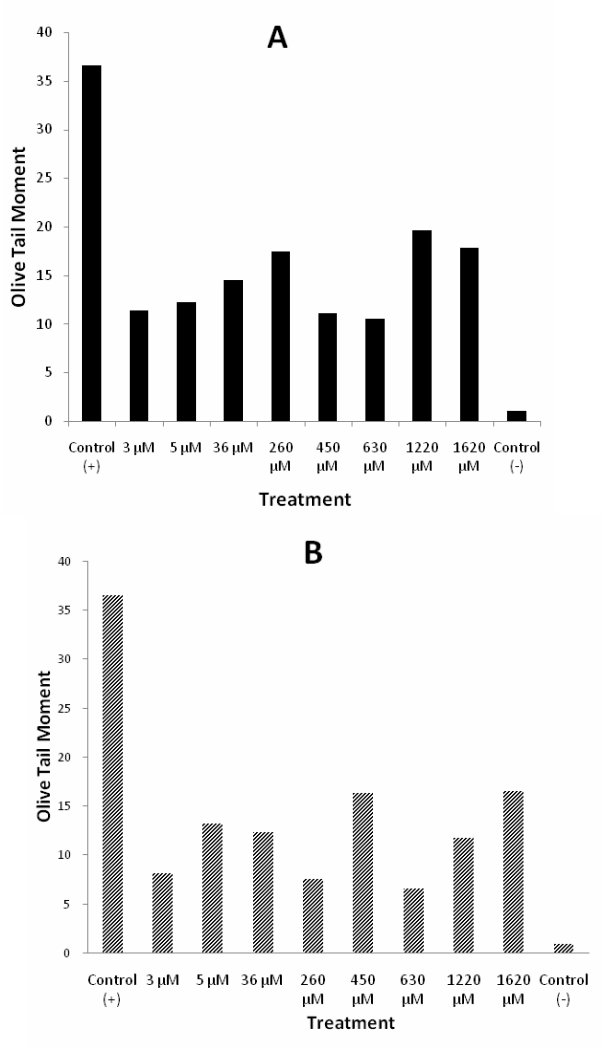

Fig. 2: Evaluation by the comet assay, protective effect of A) Isoespintanol and B) BHA on DNA. Negative control (DMSO 10%), positive control (50mM H_2O_2).

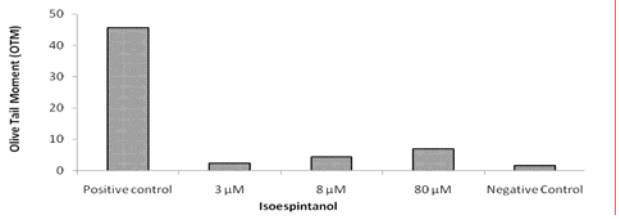

Fig. 3: Evaluation by comet assay genotoxic for three isoespintanol concentrations. The OTM is displayed. Negative control (10%DMSO) and positive control (50mM H_2O_2).

DISCUSSION

The ORAC method is often used to determine the ability of a pure compound or complex to trap oxygen radical mixtures. The ORAC methodologies varies with the source of radicals, the objective or target compound and the form to assess the level of protection. The procedure established by Cao *et al*., is the most used (Cao *et al.,* 1997), and it determines the ability of the tested antioxidant to prevent the fall in fluorescence of the fluorescein, using as source of radicals APPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) (Ou *et al*., 2001; Davalos *et al*., 2004; Perez *et al.,* 2000). Lopez-Alarcon and Lissi (Lopez-Alarcon and Lissi, 2006), established that the ORAC fluorescein methodology is deficient because it is influenced more by stoichiometric factors than by reagents factors.

There has been then proposed, an additional methodology to determine the ORAC value based on Pyrogallol Red (PGR) discoloration, which offers ORAC indices that are entirely determined by the reactivity of the evaluated compounds rather than the amount; thereof, this concept is important if the function of an antioxidant is considered. The PGR method offers information about of the reactivity of the compounds due to the high reactivity of PGR and the amounts of thisreagent used in the tests, compared to low levels of probes such as fluorescein. The way to verify the quantity and quality of a reactive compound is through the relationship between values $ORAC_{FL}$ and $ORAC_{PGR}$: so that ratios close to unity will have the perfect setting between quantity and quality.

Although Isoespintanol shows lower values of oxygen radical scavenging capacity compared to BHA, we must consider that the National Institutes of Health reported the BHA as a possible human carcinogen. BHA is a preservative that stores in body fat and can cause cancer and distorts the hormonal balance in some animals. Studies have shown that the BHA alters the reproductive performance in rats. These findings, along with the consumer interest in eating natural food additives has prompted the search for new antioxidants, which are safe, harmless and also act at low concentrations (Mesa-Vanegas *et al.,* 2010).

According to the results of the comet assay, both as Isoespintanol and BHA can be used in a wide concentration range as protective agents of the DNA damage of cells subjected to oxidative stress. Although there is not a clear mechanism described as mono- and polyphenols that acts on the protection of DNA, we can suggest that due to the high capacity of Isoespintanol to trap oxygen radicals, as evidenced by its ORAC values, the harmful effects of reactive species present on DNA are counteracted (Duthie *et al.,* 2003).

Previous studies found that thymol and carvacrol, structural analogues of Isoespintanol, showed protective effect on the DNA of human lymphocytes at low concentrations (0.5-50mM), however, at concentrations greater than 100µM prooxidant effects are presented (Aydin *et al.,* 2005). Numerous studies have shown that some polyphenolic antioxidants such as quercetin, rutin or

epicatechin gallate have genotoxic effects at high concentrations (Pérez, 2003; Johnson and Loo, 2000) contrary to the findings of this study with respect to Isoespintanol, which represents a great advantage.

CONCLUSION

Isoespintanol concentrations between 3-1620uM show protective effect on human lymphocyte DNA damage induced by H_2O_2 and do not show cytotoxic or genotoxic effects at concentrations of 3-80uM, suggesting that low concentrations can be used as an antioxidant without waiting for negative effects in the health.

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