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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and biological evaluation of gallic acid analogs

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Abstract A novel and efficient synthesis of pyrogallol moiety through a copper(I)-mediated C–O bond forming reaction is described. In particular, syntheses of 3,4,5-trihydroxyphenethyl alcohol and its methyl derivative are reported. Particular attention to dimethyl carbonate as an eco-friendly solvent/reactant has been paid, in order to improve the eco-compatibility of the whole synthetic pathway. Furthermore, the genotoxicity, cytotoxicity and the antioxidant activity of 3,4,5-trihydroxyphenethyl alcohol and its methyl derivative have been investigated.

Keywords Antioxidant · Phenols · Pyrogallol · Tyrosol

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Introduction

Polyphenols are the most abundant antioxidant in human diet (Manach *et al.*, 2004). They show a broad range of biological and pharmacological properties; in particular, current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases (Anthony *et al.*, 1996), cancers (Xiao *et al.*, 2007) and osteoporosis (Ishimi *et al.*, 2000) and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus (Scalbert *et al.*, 2005). Their biological activity is often correlated with the number and the position of hydroxy groups on the molecules, which can act as scavenger of free radicals by different mechanisms (Zhang, 2005). The most known molecules bearing a catechol moiety are hydroxytyrosol, flavonoids and epicatechins, while gallic acid owns a pyrogallol structure. The latter belong to plant kingdom, usually found as ester of chinic acid or bonded to glucose in galactomannans. Gallic acid is used in pharmacology as antiemorragic agent (Ow and Stupans, 2003). It is noteworthy that the most of naturally occurring compounds own a phenethyl substructure. In animal kingdom, they have fundamental neurotransmitter role (adrenaline, noradrenaline). A major topic in recent years is the high hydrophilicity of polyphenolics that can reduce their bioavailability. The lipophilicity can be enhanced protecting the side chain hydroxy group as ether or ester (de Pinedo *et al.*, 2007a, b; Pereira-Caro *et al.*, 2009). Our goal was to find out a general strategy to synthesize the pyrogallol moiety starting from a commercially available substrate. In recent years, a number of pyrogallol compounds were synthesized using available polyoxygenated starting materials, but in every case a pre-existent pyrogallol moiety was necessary (Fig. 1). All the above mentioned strategies lack of general interest since the exact

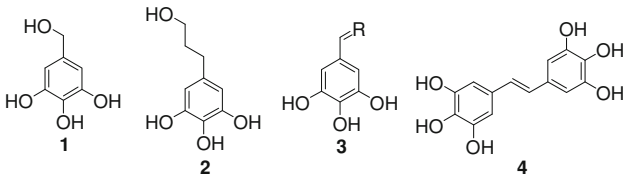


Fig. 1 Examples of synthetic molecules bearing a pyrogallol moiety

precursor should be chosen in order to obtain the target molecule (Morota *et al.*, 1990).

Pyrogallol molecules show a broad range of biological activities. In this paper we report a new, versatile and efficient synthesis of pyrogallol moiety starting from tyrosol. Above all, the first synthesis of 3,4,5-trihydroxyphenethyl alcohol (**11**) and its derivatives (**10**, **14**, **15**) was achieved exploiting a bromination/methanolysis protocol. Other methods for the insertion of hydroxyl groups on the aromatic ring are unable to form the pyrogallol structure. With the aim to reduce the environmental impact of our synthetic pathway, we turned our attention to dimethyl carbonate (DMC), a cheap commercially available and benign chemical having interesting solvating properties, low toxicity and high biodegradability (Tundo and Selva, 2002). To improve the eco-compatibility of the whole synthesis, the common solvent of every reaction step was replaced with DMC where possible. **11** and **15** have been tested to assess cytotoxicity and genotoxicity. Furthermore, the antioxidant activity has been evaluated, in order to better understand the role of the primary alcohol moiety on the base of preliminary structure–activity relationship studies.

Results and discussion—chemistry

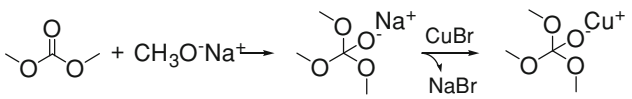
In this study, an efficient methodology to synthesize 3,4,5-trihydroxyphenethyl alcohol and its methylated derivate starting from tyrosol, a molecule found in olive oil production waste (Montedoro *et al.*, 1992; Mannino *et al.*, 1993; Angerosa *et al.*, 1995), is described. The pyrogallol moiety was achieved using a bromination/methanolysis protocol recently developed by our research group. The copper(I)-mediated C–O bond forming reaction easily achieved the desired galloyl moiety.

The bromination step was carried out modifying a well known procedure (Bovicelli, 2007); acetone was successfully replaced by eco-friendly DMC obtaining same results in conversion and yield (Scheme 1). The dibromo-derivative was then refluxed in DMC with CuBr and MeONa to obtain a mixture of dimethoxy-derivatives (**7** + **8**, Scheme 1).

The high efficiency of this procedure is probably due to the involvement of DMC in the formation of a reactive complex (Capdevielle and Maumy, 1993) (Scheme 2).

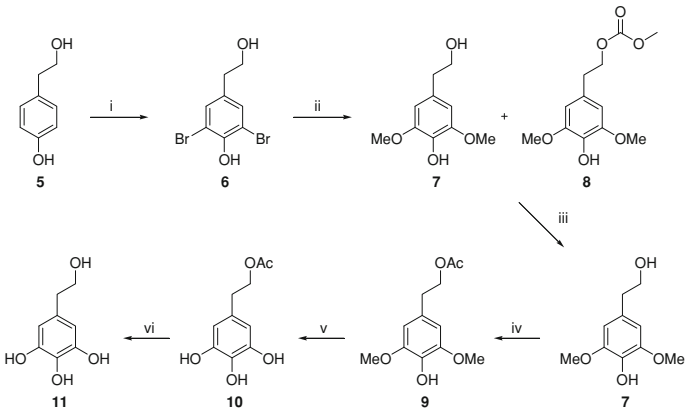
A basic hydrolysis in tetrahydrofuran (THF) followed by an acetylation reaction were needed to obtain the desired compound **9** (Scheme 1). Finally the methoxy groups were deprotected with BBr_3 at low temperature to avoid the competitive bromination of the side chain. The last step was the hydrolysis of the acetyl group in acidic media to obtain the target compound **11** (Scheme 1).

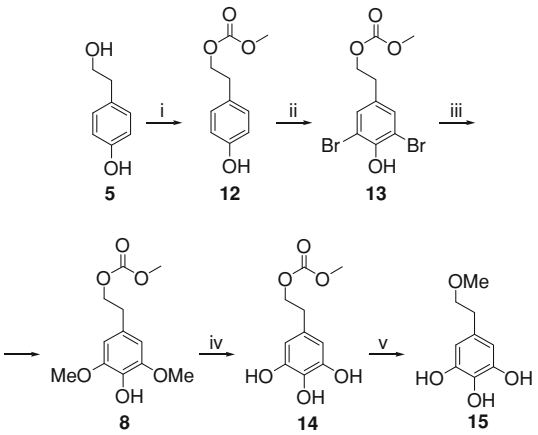
With the purpose of diminishing the polarity of **11** and enhancing its bioavailability, the methylether derivative **15** has been synthesized through a similar synthetic pathway. The choice of the new protecting group lies with convenient characteristics of DMC reactivity. Preliminary investigation on carboxymethylate phenethyl system showed the ability of carboxymethyl group to rearrange in acidic media into methyl ether. Moreover, the carboxymethyl group simplifies the synthetic pathway since it is consistent with methanolysis conditions. The first step was



Scheme 2 Plausible mechanism for the involvement of DMC in methanolysis step

Scheme 1 (*i*) NaBr, oxone, DMC/H_2O, rt; (*ii*) CuBr, MeONa, DMC, 90 °C; (*iii*) THF, NaOH 1 M, rt; (*iv*) DMC, AcCl, rt; (*v*) BBr_3, CH_2Cl_2, $T = -20$ °C; (*vi*) HCl 6 M, MeOH





Scheme 3 (*i*) DMC, Amberlyst 15, T = reflux; (*ii*) NaBr, oxone, DMC/H_2O, rt; (*iii*) CuBr, MeONa, DMC, T = 90 °C; (*iv*) BBr_3, CH_2Cl_2, T = –40 °C; (*v*) MeOH, Amberlyst 15, reflux

a selective protection of the primary hydroxyl group on the side chain, previously reported by us. DMC was used as solvent/reactant (Scheme 3). The pyrogallol moiety was then built as in the previous case, exploiting the bromination/methanolysis protocol followed by a demethylation step (Scheme 3). **15** was obtained refluxing **14** in methanol and amberlyst 15. The methylether formation is supposed to happen through a rearrangement in which the acidic media plays a key role (Scheme 3).

Results and discussion—biology

The pyrogallol compound **11** and the methylated derivative **15** were evaluated for their toxicity to better define the role of the pyrogallol moiety on structure–activity relationships. In particular, we assessed potential genotoxic effects in mammalian ovary hamster cells (CHO) in vitro by analyzing the induction of chromosomal aberrations and sister chromatid exchanges (SCEs), which are highly predictive of long term genetic and cancer risk (Ward, 1993; Rossner *et al.*, 2005; Bonassi *et al.*, 1982). Analyses of the mitotic index (MI), an indirect parameter used to evaluate cytotoxic effects, and proliferative replication index (PRI), used to assess cell division kinetics and interference with cellular check-points, were also determined. Chromosomal aberrations are highly predictive of long term genetic and cancer risk. SCE involves breakage of both DNA strands, followed by an exchange of whole DNA duplexes. This occurs during S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. The formation of SCEs has been correlated with recombinational repair and the induction of point mutations, gene amplification and cytotoxicity. MI and PRI values were only slightly lower (100 and 200 μM) in the

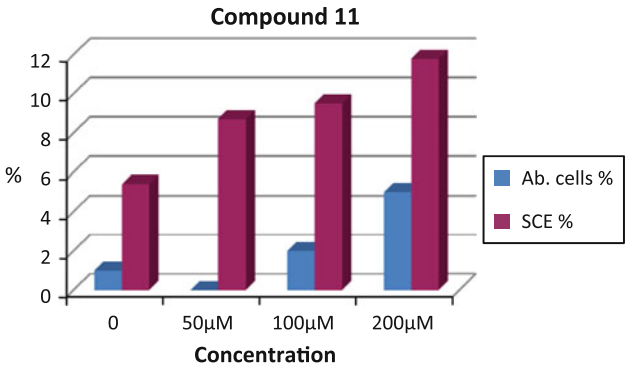


Fig. 2 Frequencies of SCEs and aberrations-bearing cells induced by compound **11** in CHO cells in vitro

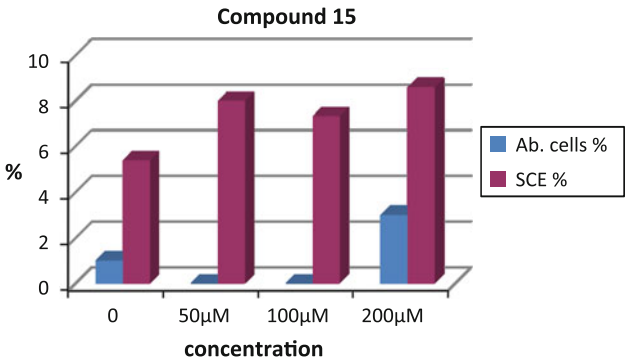


Fig. 3 Frequencies of SCEs and aberrations-bearing cells induced by compound **15** in CHO cells in vitro

cultures treated with both compound. No statistically significant increases in the incidence of chromosomal aberrations and SCEs were observed at all dose-level employed (Figs. 2, 3).

Compound **11** showed at 200 μM a slight increase of SCEs compared with same dose compound **15**, but no statistically significant. At higher dose levels (0.5, 1 and 2 mM), both compounds proved to be severely toxic, and no cytogenetic analysis was performed since no metaphases were recovered (Table 1). Summarizing, based on the data analysed, it can be affirmed that none of the two molecules show cytotoxicity or genotoxicity at doses lower than 200 μM. To investigate whether compound **11** and **15** confers protection against H_2O_2 induced chromosome aberration in CHO cells, we detected the cell viability after 200 μM H_2O_2 incubation for 2 h, with or without **11** and **15** pre-treatment for 15 min (Liu *et al.*, 2010). The results showed that pre-treatment prevented H_2O_2 damage in both cases and compound **15** has higher scavenger activity than compound **11** (Fig. 4). Since both of them bear a pyrogallol moiety, the higher scavenger activity of **15** could be due to its enhanced lipophilicity, resulting in an easier cell-membrane penetration.

Table 1 MI values observed in CHO cells for compounds **11** and **15**

Compound	Dose level (μM)	Mean MI (%)	Relative MI	Frequency of			PRI	Relative PRI
				M1	M2	M3		
None (control, DMSO)	1 %	11.5	100	10	90	0	1.90	100
11	50	11.1	96.5	8	92	0	1.92	101
11	100	11.4	99.1	25	75	0	1.75	92
11	200	6.7	58.3	50	50	0	1.50	79
11	500	0.5	4.3	/	/	/	/	/
11	1,000	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	50	9.1	79.1	6	94	0	1.94	102
15	100	9.3	80.8	18	82	0	1.82	96
15	200	10.1	87.7	34	66	0	1.66	87
15	500	1.8	15.6	/	/	/	/	/
15	1,000	0.0	0.0	0.0	0.0	0.0	0.0	0.0

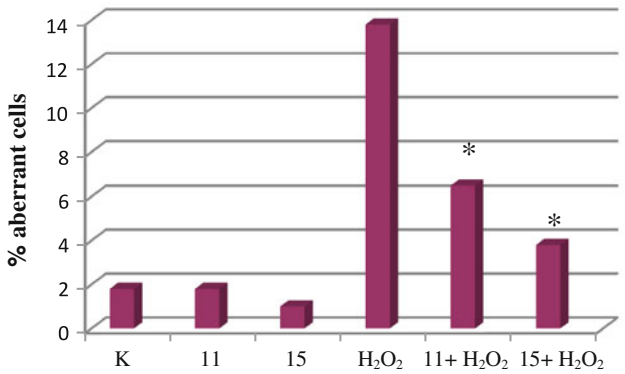


Fig. 4 Frequencies of cells bearing aberrations induced by a 2 h treatment 200 μM H_2O_2 in the CHO cells line. Statistical significance was evaluated by Fisher's exact test: $*p < 0.01$

Furthermore, in order to exclude the possible different interaction of the two compounds **15** and **11** with H_2O_2, they were kept in Hank's balanced salt solution (HBSS) (Gibco) and incubated at 37 °C in the dark for 1 h. Afterwards, the cells were treated for 1 h with these solutions and the protocol for detecting the induction of CA (chromosomal aberration) was followed.

We analyzed two different flasks containing respectively 200 μM of compound **15** and 200 μM of H_2O_2 (solution 1) and 200 μM of compound **11** with the same concentration of H_2O_2 (solution 2). Afterwards, the cells were treated for 1 h with these solutions and CA test was performed to study the frequency of CA. No difference in the frequency of CA was found in both solutions, in fact percentage of aberrant cells is around 5 % for the two samples. This result shows that compound **11** and **15** do not have different antioxidant activity. In fact the pre-treatment with equimolar concentrations of hydrogen peroxide, without cells, allows the two compounds to bind all of the H_2O_2 molecules. When we added the cells, there was no, in both cases, free H_2O_2 available to the cells and this is demonstrated with the same number aberrant cells. On the contrary, if compound **11** would had a lower antioxidant capacity the result would have been an increase of free H_2O_2 in the medium, that with addition of cells, would have caused increase of CA.

Experimental—chemistry

General procedures

*Synthesis of 4-hydroxy-3,5-dimethoxyphenethyl ethanoate (**9**)*

To a solution of **7** (1.0 mmol) in dimethyl carbonate (3 mL), under magnetic stirring at room temperature, acetyl chloride (1.2 mmol, 0.08 mL) was added. After 24 h, the complete disappearance of the substrate is observed. The solvent was removed by distillation under reduced pressure and the residue is solubilized with ethyl acetate (10 mL) and washed with brine (3 × 5 mL). The organic layer was dried over anhydrous Na_2SO_4 and the organic solvent was removed. 4-Hydroxy-3,5-dimethoxyphenethyl ethanoate **9** (0.93 mmol, 223 mg, 93 %) was obtained as colourless oil.

*Synthesis of 4-hydroxyphenethyl methyl carbonate (**12**)*

A mixture of 2-(4-hydrophenyl)ethanol (**5**) (1.0 mmol, 138 mg) amberlyst 15 (87 mg, 0.4 mmol) and DMC (8.0 mL) was heated to reflux. The reaction was monitored by thin layer chromatography (TLC). After the disappearance of the substrate the reaction mixture was cooled to room temperature and was filtrated through Celite. DMC

was evaporated under vacuum as an azeotropic mixture with methanol (DMC/CH_3OH = 1:3) boiling at 64 °C to afford 4-hydroxyphenethyl methyl carbonate **12** (0.98 mmol, 192 mg, 98 %) as colourless oil.

Bromination procedure

To a solution of the appropriate substrate (**5** or **12**, 1.00 mmol) and NaBr (2.00 mmol, 206 mg) in DMC (8 mL), a solution of Oxone® (1.00 mmol, 1,840 mg) in water (8 mL) was added at rt. The progress of the reaction was monitored by TLC and when the reaction was over (complete consumption of the substrate), AcOEt (15 mL) was added to the mixture. The organic layer was separated and the aqueous phase extracted with AcOEt (2 × 10 mL). The combined organic layer was washed with brine (2 × 15 mL), dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The crude product was purified over silica gel (20 % EtOAc/Hexane) to give 2,6-dibromo-4-(2-hydroxy-ethyl)-phenol **6** (0.87 mmol, 296 mg, 87 %) as yellow oil or 3,5-dibromo-4-hydroxyphenethyl methyl carbonate **13** (0.84 mmol, 301 mg, 84 %) as yellow oil.

Methanolysis procedure

A mixture of a 25 % w/v solution of sodium methoxide in methanol (32.00 mmol, 7.3 mL) and CuBr (0.80 mmol, 114 mg) in DMC (1.8 mL) was stirred at room temperature until the solution turned to a bright blue. The complex was added to a solution of the appropriate substrate (**6** or **13**, 1.00 mmol) in DMC (3.6 mL) at reflux in three portions. After 3 h all the substrate was consumed. The reaction mixture was then poured into 12 mL of 2 M HCl at 0 °C and the aqueous layer was extracted with AcOEt (3 × 10 mL). The combined organic extract was washed with brine (3 × 10 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuum. The crude product was purified over silica gel (33 % EtOAc/Hexane) to give 4-hydroxy-3,5-dimethoxyphenethyl methyl carbonate **8** (0.87 mmol, 223 mg, 87 % starting from **13**) as colourless oil or 4-hydroxy-3,5-dimethoxyphenethyl alcohol **7** as colourless oil (92 mg, 0.46 mmol, 46 %, starting from **6**) and 4-hydroxy-3,5-dimethoxyphenethyl methyl carbonate **8** as colourless oil (104 mg, 0.41 mmol, 41 %, starting from **6**).

Demethylation procedure

8 or **9** (1.00 mmol) was dissolved in dry CH_2Cl_2 (5 mL) at −40 °C. BBr_3 (3.00 mmol, 0.28 mL) was added dropwise to the solution and the reaction was monitored by TLC. After the total consumption of the substrate MeOH (1 mL) was added and the reaction mixture was poured into water (10 mL). The aqueous layer was extract with AcOEt (3 × 7 mL) and the combined organic layer was washed with brine (3 × 10 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuum to obtain 3,4,5-trihydroxyphenethyl ethanoate **14** (0.84 mmol, 178 mg, 84 %) as colourless oil or 3,4,5-trihydroxyphenethyl methyl carbonate **10** (0.90 mmol, 203 mg, 90 %) as colourless oil.

*Synthesis of 4-hydroxy-3,5-dimethoxyphenethyl alcohol (**7**)*

The mixture of **7** + **8** (217 mg) was dissolved in a mixture 1:1 THF:NaOH 2 M (10 mL). The reaction was left stirred overnight, then EtOAc (15 mL) was added and the aqueous layer was extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine (3 × 10 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to afford 4-hydroxy-3,5-dimethoxyphenethyl alcohol **7** (160 mg, 0.81 mmol, 93 %) as colourless oil.

*Synthesis of 3,4,5-trihydroxyphenethyl alcohol (**11**)*

A solution of **11** (0.43 mmol, 91 mg) in a mixture 1:1 MeOH:HCl 6 M (4 mL) was left stir at rt. After 1 h all the substrate was consumed. The solvent was evaporated under reduced pressure to give 3,4,5-trihydroxyphenethyl alcohol **11** (0.39 mmol, 66 mg, 91 %) as red-brown gummy oil.

*Synthesis of 3,4,5-trihydroxyphenethyl alcohol methylether (**15**)*

Amberlyst 15 (50 mg) was added to a solution of **14** (0.27 mmol, 62 mg) in MeOH (5.5 mL). The reaction mixture was stirred at reflux for 36 h then filtrate through Celite and washed with water (3 × 3 mL). The filtrate was evaporated under reduced pressure to obtain 3,4,5-trihydroxyphenethyl methyl ether **16** (0.20 mmol, 36 mg, 74 %) as brown oil.

Full characterization of all synthesized compounds is reported as supporting information.

Experimental—biology

Test compounds

3,4,5-Trihydroxyphenethyl alcohol (**11**) and 3,4,5-trihydroxyphenethyl methyl ether (**15**) were prepared immediately before treatment in dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich and added to the culture medium such that the final concentration of solvent did not exceed 1 %. 5-Bromo-2′-deoxyuridine (BrdU) used to evaluate the

frequency of cells in first (M1), second (M2) and third (M3) cell division, and colcemid to accumulate cells in metaphase, were also purchased from Sigma-Aldrich.

Test systems and culture conditions

The karyotype, generation time, plating efficiency, and absence of mycoplasmal contamination were checked at regular intervals. Permanent stocks of CHO cells were stored in liquid nitrogen (−170 °C) and subcultures are prepared from these stocks for experimental use. At the end of assay the cells were discarded and a new ampoule (containing cells from the stock under liquid nitrogen) was used. Cultures of the cells are grown in F-10 minimal medium supplemented with 10 % foetal bovine serum, 2 mM L-glutamine and antibiotics (1 % w/v penicillin and 86 mM streptomycin). All incubations were at 37 °C in a 5 % carbon dioxide atmosphere and 100 % nominal humidity. The doubling time was 12 h and the modal chromosome number was 21 ± 2. CHO cell line is particularly useful for this kind of studies because of its stable karyotype, short cell cycle and its high plating efficiency.

Chromosomal aberration assays

Test compound treatments of Chinese hamster CHO cells were performed in the absence of rat liver metabolism. The study was designed to comply with the experimental methods indicated in the OECD Guidelines for the testing of chemicals No. 487 (Draft June 2004). Following dose-range finding experiments, the assay was performed using dose levels of 200, 100 and 50 μM and a 3-h treatment time. Solvent-treated cells served as negative control. At the end of treatment (3 h), cultures were washed twice with a PBS solution and re-incubated at 37 °C in fresh complete culture medium for further 18 h (~1.5 cell cycle). Cultures set up for analysis of PRI received also BrdU at 9.8 mM to differentiate sister chromatids. To study antioxidant activity of compounds **11** and **15**, cells cultures were pretreated 15 min before H_2O_2 (200 μM) treatment. At the end of treatment (2 h), cultures were washed twice with a PBS solution and re-incubated at 37 °C in fresh complete culture medium for further 18 h (~1.5 cell cycle). Colcemid at 0.27 mM was added during the last 3 h of culture to accumulate cells in metaphase. Hypotonic shock was induced by 1 % trisodium citrate solution for 10 min. Cell suspension was fixed in a mixture of methanol and glacial acetic acid (v/v 3:1) followed by three washes. Cytogenetic preparations for analyses of chromosomal aberrations and mitotic indices were stained with an aqueous solution of Giemsa 1 %. The fluorescence-plus-Giemsa (FPG) technique (Perry and Wolff, 1974) was used for sister chromatid differentiation (SCD) staining. Slides were stained for 20 min with Hoechst 33258 (5 mg/ml), mounted in saline sodium citrate buffer (SSC) 2× powder (Anidra Company), and exposed to "black light" for 20 min at 50 °C. Finally, cells were stained with Giemsa and air-dried for evaluation of the frequency of cells in their first (M1), second (M2) and third (M3) cell division. Cell division kinetics was determined by the PRI according to the formula (Rojas *et al.*, 1992):

$$\mathrm{PRI} = \frac{(1 \times M1) + (2 \times M2) + (3 \times M3)}{100}$$

where M1, M2, and M3 are the proportions of first, second and third generations of mitotic cells respectively. To evaluate the MI, cytogenetic preparations stained by Giemsa were analyzed in a light microscope at 400× magnification. MI was expressed as number of metaphases per 1,000 nuclei analyzed. Scoring for chromosomal damage was undertaken blind with coded slides. A minimum of 100 metaphases per culture were scored for chromosomal aberrations. Chromosomal aberrations were classified as chromatid-type gaps, chromatid-type breaks, chromatid-type exchanges, chromosome-type gaps, chromosome-type breaks, chromosome-type exchanges and isolocus events (which include isochromatid and isolocus breaks when these cannot be distinguished), as described by Savage (Savage, 1976). For the chromosome aberration assay the number of aberration-bearing cells (excluding gaps) was utilized for statistical analyses.

Conclusions

In this work a new and efficient procedure for the formation of the pyrogallol moiety was described. In particular, 3,4,5-trihydroxyphenethyl alcohol (**11**) and its methylether derivative (**15**) were synthesized starting from an easily available starting material. The environmental impact of the whole process was reduced by the use of DMC as green reactant and solvent, where possible. Furthermore, 3,4,5-trihydroxyphenethyl alcohol and its methylated derivative were tested for their antioxidant activity and toxicity, showing interesting properties that could be applied in pharmaceutical and medicine. From the biological point of view, the presence of the methyl ether group on the side chain (compound **15**) does not modify cell viability compared with compound **11**. According to biological tests performed on compound **11** and **15** the presence of a methyl ether moiety improve the protection against H_2O_2 damage.

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