NPC Natural Product Communications

2011
Vol. 6
No. 9
1315 - 1319

Aqueous Extract from *Vitis vinifera* Tendrils is Able to Enrich Keratinocyte Antioxidant Defences

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Received: May 27th, 2011; Accepted: June 13th, 2011

An aqueous extract of *V. vinifera* L. tendrils was evaluated for its ability to enrich the antioxidant capacity of cultured cells. The long-time antioxidant capability of the extract was measured by *in vitro* chemical methods, and its influence on reduced glutathione levels and plasma membrane oxido reductase activity was determined in cultured human keratinocytes (NCTC 2544). Keratinocytes are cells normally exposed to oxidative stress, and for this reason adequately equipped with antioxidant defences. However, it has long been suggested that exogenous antioxidants may play an important role in minimizing the adverse effects of oxidative stress on skin.We demonstrated that *V. vinifera* tendril aqueous extract was able to increase, in a time- and dose-dependent manner, the reduced glutathione concentration and activity of trans plasma membrane oxido reductase as an indirect evaluation of the intracellular redox status of the cells demonstrating a relevant antioxidant activity of this phytocomplex.

Keywords: *Vitis vinifera* tendrils, antioxidant activity, NCTC 2544, GSH, PMOR.

The grapevine (*Vitis vinifera* L.) is native to southern Europe and western Asia and is cultivated today in all temperate regions of the world. The seeds and leaves of this species are used in herbal medicine and its fruits (grapes) are utilized as a dietary supplement and for wine production. Seeds, grape skin and leaves of grapevine contain several active polyphenols, including flavonoids such as kaempferol, quercetin, myricetin, catechins, epicatechins, procyanidins and derivatives [1]; proanthocyanidins [2,3]; resveratrol and other stilbene derivatives [4]. Extracts of various parts of grapevine have been reported to possess a broad spectrum of pharmacological and therapeutic effects, such as antioxidative, anti-inflammatory, and antimicrobial activities, as well as having cardioprotective, hepatoprotective, and neuroprotective properties [5]. Bouhamidi *et al*. [6] showed that polyunsaturated fatty acid peroxidation is inhibited by low concentrations of grape seed proanthocyanidins (2 mg/L), while other studies have confirmed that grape seed proanthocyanidin extract provides protection against free radicals in *in vitro* free radical scavenging assays and this effect was higher than that reported for vitamins C and E. Compared with other antioxidants, the same extract provided significant protection against 12-*O*-tetradecanoylphorbol-13-acetate induced oxidative damage [7,8]. The recent discovery of melatonin in grapes [9] opens new perspectives in the field of grape antioxidant activity research. In addition to its neurohormonal function, melatonin is a powerful antioxidant and acts as reducing and repairing electrophilic radicals. Procyanidin supplementation in rat and rabbit reduced myocardial ischemia/reperfusion damage and this was associated with an increase in plasma antioxidant activity [10]. Oral consumption of standardized grape extract (100 and 200 mg/kg) provided significant cardioprotection by improving post-ischemic ventricular recovery and reducing the amount of myocardial infarction in rats [11]. Another study demonstrated that oral administration of grape skin extract significantly reduced systolic, mean, and diastolic arterial pressure in a hypertensive rat model [12]. Regarding the hepatoprotective effect, a grape seed extract (50 mg/kg a day orally for 28 days) protected the liver from damage following bile duct ligation in rats [13] and reduced hepatic ischemia/reperfusion injury in the same animal model [14]. Grape seed extract reduced the incidence of free-radical-induced lipid peroxidation in the central nervous system of aged rats and reduced hypoxic ischemic brain injury in neonatal rat [15]. In some countries, raw young tendrils of *V. vinifera* are used in salads for their sour taste. To our knowledge, data concerning biological activity of the tendril extracts are lacking.

In the search for natural antioxidants able to counteract oxidative damage, we investigated the effects of the aqueous extract of *V. vinifera* tendrils on cultured human keratinocytes (NCTC 2544). It is widely accepted that oxidative stress conditions predispose cells to degenerative changes related to aging and cancer. Keratinocytes are particularly exposed to environmental stresses (UV light, electromagnetic field, relatively high oxygen tension, oxidizing chemicals and inflammatory conditions) [16-18] where reactive oxygen species (ROS) are often produced [19-20]. We decided to use the aqueous extract of young *V. vinifera* tendrils because this is very similar to that of tendrils used either as food in salads or in herbal tea.

Literature data have often highlighted that antioxidant ability probably derives from the additive and/or synergic effect of the various extract components [21-23]: for that reason, we decided to test the total *V. vinifera* tendril aqueous extract, as prepared in the Experimental section.

In many fruits and vegetables, the antioxidant activity can be attributed to the level of total polyphenols [24]. Therefore, the total polyphenol levels were measured in the aqueous extract of tendrils, and values of 31.5 ±1.8 mg/mL or 13.2 ± 0.9 mg/g, dry wt, were found. Flavonoids are known to be involved in antioxidative, antimicrobial, antimutagenic and anticarcinogenic processes. Considering these factors, studies on natural sources of flavonoids are relevant [25]. The total level of flavonoids in our extract was 8.7 ±0.7 μg/mL or 3.1 ± 0.2 μg/g, dry wt. Data concerning the total polyphenol and flavonoid content in young tendrils are lacking and consequently we cannot compare our data. Only Jeong *et al*. (2006) showed the presence of quercetin, myricetin and kaempferol, with a prevalence of quercetin in young tendrils of *V. vinifera* var Cabernet Sauvignon, but without quantifying the total content of flavonoids [26].

The total antioxidant activity of the tendril extract was determined using a spectrophotometric method based on the formation of a phosphomolybdenum complex [27]; the results demonstrated a significant dose-dependent antioxidant activity of the extract, when compared with that of ascorbic acid used in concentrations ranging from 25 to 200 μM (Figure 1). This activity was long lasting: repeating the Prieto test during three months showed that the aqueous extract retained significant antioxidant capacity when conserved at 4°C (data not shown).

We then examined the tendril extract for possible cytotoxic effects on human keratinocytes. Keratinocytes NCTC 2544, incubated in DMEM, were exposed to 25, 50, 62.5 and 100 mg/mL of tendril aqueous extract for 24 h at 37°C. The membrane integrity was evaluated through the release of lactate dehydrogenase [28]. As shown in Figure 2, the keratinocytes were not damaged by the aqueous extract used in concentrations from 25 to 62.5 mg/mL. After this last dose, a little decrease in viability was

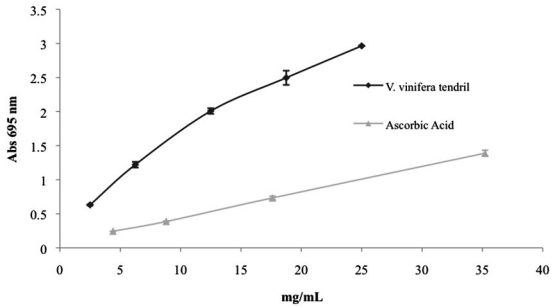

Figure 1: Antioxidant capacity of tendril extract. Ascorbic acid was used as the reference substance.

registered. For this reason all subsequent experiments were performed by incubating cells with a maximum of 62.5 mg/mL aqueous extract. The cytotoxic effect produced by high doses of tendril extract is not surprising; similar effects were described for phenolic compounds such as *p*-tyrosol and caffeic acid in normal fibroblasts [29]. Many antioxidants in high doses become pro-oxidants [30-32].

Because of the good antioxidant properties and lack of cytotoxicity of the *V. vinifera* tendril extract, we decided to verify further the antioxidant activity of this extract on an *in vitro* cellular system of human keratinocytes NCTC 2544, by incubation of the cells with increasing concentrations of the extract (from 0 to 62.5 mg/mL) in order to evaluate the effects on reduced glutathione (GSH) levels.

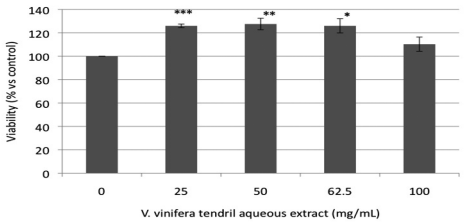

Figure 2: Tendril extract cytotoxicity. NCTC 2544 cells were exposed to 25, 50, 62.5 and 100 mg/mL of *V. vinifera* tendril aqueous extract in DMEM for 24 h at 37°C. The membrane integrity was evaluated through the release of lactate dehydrogenase in the medium. **P*<0.05.

NCTC 2544 cells were incubated with rising concentrations of *V. vinifera* tendril extracts (from 12.5 to 62.5 mg/mL) for 24 hours, then the medium was discarded and GSH levels were evaluated. Increases in the reduced glutathione cellular pool were noted, starting from the 12.5 mg/mL dose (Figure 3). These results indicate that the tendril extract is able to improve the cell antioxidant defences: the oxidation of glutathione is one of the first signs of damage caused by oxidative stress, despite it being continually recovered by GSSG-reductase activity in the presence of proper NADPH equivalents. The discarding of the cell medium, before GSH level evaluation, suggests that this response is triggered by an intracellular molecular mechanism. For these reasons and from the evidence presented that keratinocytes were reinforced in their antioxidant defenses after exposure to

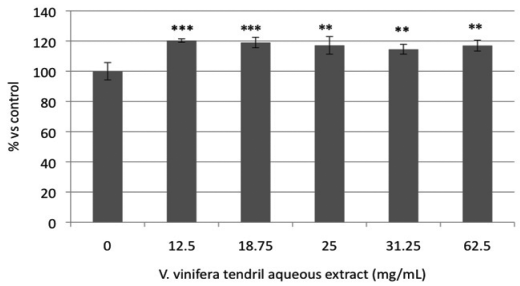

Figure 3: Effect of the tendril extract on GSH. NCTC 2544 cells were incubated with rising concentrations of the extract from 12.5 to 62.5 mg/mL. **P*<0.05.

tendril extract, we proceeded to measure the Plasma Membrane Oxido Reductase (PMOR) activity in NCTC 2544 cells incubated with 62.5 mg/mL of tendril aqueous extract after different incubation times (6, 14 and 24 hours) (Figure 4).

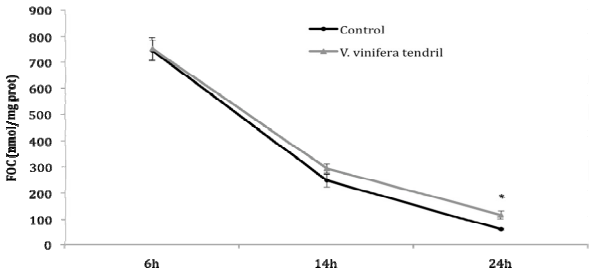

Figure 4: Tendril extract action on PMOR activity. NCTC 2544 cells were exposed to 62.5 mg/mL of the extract for 6, 14 and 24 h. **P*=0.0184

PMOR is a multi-enzyme complex contributing to cellular redox homeostasis regulation through the maintenance of the NAD(P)+/NAD(P)H ratio [33,34]. It is involved in functions such as cell growth control, iron uptake [34], ascorbate recycling [35], and signal transduction [33]. Therefore, it plays a role as a compensatory mechanism when energy is poor within cells, providing NAD+ for glycolytic metabolism [36]. From the obtained results, it is possible to declare that *V. vinifera* tendril aqueous extract is able to improve the activity of this enzyme in a time-dependent manner. Moreover, the maintenance of this capacity after 24 h of incubation is also relevant.

Since trans-plasma membrane electron transport is reported to be linked to intracellular redox status [34], we hypothesized that the positive influence of the extract may be useful in counteracting the oxidative damage.

In conclusion, we have shown that *V. vinifera* tendril extract exerts a significant, long lasting antioxidant activity, able to increase the reduced glutathione cellular levels in a dose-dependent manner. Furthermore, it enhances the plasma membrane oxido reductase activity in a time-dependent way. All the data obtained show that *V. vinifera* tendril extract possesses a reliable antioxidant activity that improves the intracellular defense system of keratinocytes, in a concentration- and time-dependent manner, increasing the GSH levels and activating the PMOR enzyme. These effects are very important and may contribute to the protection of cells from oxidative insults and for a possible future application of this extract on an *in vivo* system and in therapeutic fields.

Experimental

Chemicals: 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) and all reagents used for cell cultures and enzymatic assays were purchased from Sigma-Aldrich. Flasks and plates were from PBI International.

The composition of phosphate-buffered saline solution (PBS) was: 8 g/L NaCl, 1.44 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, and 0.2 g/L KCl, pH 7.4.

The Hank's solution was composed of 0.015 g/L $CaCl_2$, 8 g/L NaCl, 0.4 g/L KCl, 1.6 g/L glucose, 0.06 g/L KH_2PO_4, 0.048 g/L Na_2HPO_4, 0.35 g/L $NaHCO_3$, and 0.2 g/L $MgSO_4 \cdot 7H_2O$, pH 7.4.

Keratinocyte cell line NCTC 2544 was obtained by Interlab Cell Line Collection, Genova, Italy.

Plant material: Young tendrils of *Vitis vinifera* L. var. Sangiovese were collected in Urbino, Marche, central Italy, at 450 m above sea level, in June 2010 and identified by D. Fraternale. A voucher specimen is deposited in the herbarium of the Botanical Garden of the University of Urbino (V.v.T. 01).

Total polyphenol content: The total polyphenol content in the aqueous extract of fresh tendrils was determined by the Prussian Blue method described by Hagerman and Butler [37], with slight modifications. Aliquots of extract were made up to 1 mL with distilled water. After adding 60 µL of 0.1 M $FeNH_4(SO_4)_2$, they were incubated for 20 min at room temperature. Subsequently, 60 µL of 8 mM $K_3Fe(CN)_6$ was added to the sample, and after 20 min at room temperature the optical density of the mixture was determined at 720 nm (Jasco V-530 spectrophotometer, Tokyo, Japan). Quercetin was used as the standard to construct a calibration curve.

Flavonoid content: Flavonoid content was evaluated by the vanillin assay according to Monagas *et al*. [38]. 2.5 mL of H_2SO_4/methanol (25/75, v/v) solution and 2.5 mL of 1% (w/v) vanillin in methanol were added to 1 mL of the sample. A blank was prepared in the same way, but adding methanol instead of vanillin. Absorbance at 500 nm was read after a reaction time of 15 min at 30°C. Results were expressed as µg rutin/mL.

***Vitis vinifera* tendril aqueous extract:** An aqueous extract of fresh tendrils was prepared in a ratio of 1g/4 mL. To remove insoluble materials, the extract was centrifuged first at 7,000 rpm for 8 min, then at 15,000 rpm for 15 min,

twice. The supernatant was dried under vacuum; the powder was dissolved in H_2O to a concentration of 0.5 g/mL and maintained at -80° C in several batches. The *V. vinifera* aqueous extract was thawed, centrifuged at 14,000 rpm for 10 min and filtered (0.2 µm pore size) before use. Concentrations used in experiments are expressed as mg of fresh tendril weight per mL.

Antioxidant capacity of the *V. vinifera* tendril aqueous extract and time stability: Antioxidant capacity of the tendril aqueous extract was evaluated according to Prieto *et al*. [27]. This method depends on the ability of a compound to reduce Mo (VI) to Mo (V), which generates a green phosphate / Mo (V) at acidic pH that is measured at 695 nm. The values of absorbance were compared with the antioxidant capacity of ascorbic acid, used as a reference substance. The extract was maintained at 4°C and the test repeated every week for 1 month.

Cell culture: Cellular experiments were carried out on normal human keratinocytes (NCTC 2544). Cells, cultured at 37°C in an atmosphere of 95% air and 5% CO_2 in DMEM medium containing 1% streptomycin/penicillin, 2% glutamine, and 7% fetal bovine serum, were seeded 6 x 10^5 cells/well. *V. vinifera* tendril aqueous extract was added to the cells in either Hank's solution or DMEM medium without serum and incubated for different times at 37°C. Then, the medium was removed and the cells washed twice with PBS, harvested in PBS by gentle scraping, and then sonicated twice for 10 sec (50 W). The obtained lysate was analyzed by optical microscopy.

Cytotoxicity assay by LDH release: Keratinocytes were exposed to 25, 50, 62.5 and 100 mg/mL of *V. vinifera* tendril aqueous extract in DMEM for 24 h at 37°C. The membrane integrity was evaluated through the release of lactate dehydrogenase (LDH; EC 1.1.1.27) in the incubation medium, as previously reported [28].

Reduced glutathione determination: Keratinocytes were exposed to 12.5 to 62.5 mg/mL of *V. vinifera* aqueous extract in DMEM for 24 h at 37°C. Once the medium was discarded, the cellular lysate obtained as described above was centrifuged at 14.000 rpm for 5 min. The GSH content was performed in the supernatant with DTNB reagent, as described by Sedlak [39].

Protein concentration: The protein content was quantified using Bradford's method, with bovine serum albumin as a standard [40].

Measurement of ferricyanide reduction: Cells were incubated in a 6-well plate for 24 h in DMEM containing 62.5 mg/mL of *V. vinifera* tendril extract. After 6, 14 and 24 h of incubation, the medium was discarded, cells were washed twice with Hank's solution and re-incubated with 2 mL of 1 mM K ferricyanide in Hank's solution for 30 min at 37°C. After this time, ferricyanide reduction was estimated in the supernatants as reported by Avron and Shavit [41]. The ferrocyanide content was assayed by using 1,10-*o*-phenantroline as an indicator and measuring the absorbance at 510 nm ($e = 10.500\ M^{-1}cm^{-1}$).

Statistical analysis: All experiments were performed at least in quadruplicate. Results are expressed as mean ± SEM. Software GraphPad Prism was used for statistical analysis. Unpaired t test was applied for comparisons among the data obtained from controls and *V. vinifera* tendril aqueous extract treated groups.

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