Effects of Grape Seed Proanthocyanidin on 5-Hydroxytryptamine3 Receptors in NCB-20 Neuroblastoma Cells

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Proanthocyanidin is a phenolic compound present in plants, that has antioxidant, antinociceptive, antiemetic, and neuroprotective properties. We investigated the actions of proanthocyanidin from grape seeds on 5-hydroxytryptamine (5-HT)3 receptors in NCB-20 neuroblastoma cells using a whole-cell voltage clamp technique. Co-treatment of proanthocyanidin (0.3—100 μg/ml) and 3 μM 5-HT (near EC50) produced a slight inhibition of 5-HT-induced inward peak current (I5-HT) in NCB-20 cells, but pretreatment with proanthocyanidin for 30 s before application of 5-HT induced a much larger inhibition of I5-HT in an irreversible, concentration- and time-dependent manner (IC50 = 6.5 ± 0.4 μg/ml, Hill coefficient = 2.5 ± 0.1). Proanthocyanidin also produced a concentration-dependent inhibition of currents induced by 30 μM 5-HT, near-maximal concentration (IC50 = 22.1 ± 0.4 μg/ml, Hill coefficient = 2.4 ± 0.3). High concentrations (≥30 μg/ml) of proanthocyanidin caused a concentration-dependent inhibition of the activation and desensitization of currents induced by 30 μM 5-HT. Further studies showed that pretreatment of 20 μg/ml proanthocyanidin caused not only a rightward shift of the dose-response curve for 5-HT (EC50 shift from 2.7 ± 0.4 to 6.2 ± 0.5 μM), but also a decreased Emax (inhibition by 37.5 ± 1.3%). The proanthocyanidin-induced inhibition of 5-HT3 receptors did not show a significant difference within the testing holding potential ranges (−50 to +30 mV). These results suggest that proanthocyanidin inhibits 5-HT3 receptor function in NCB-20 cells in a noncompetitive mode, and that this inhibitory effect of proanthocyanidin probably contributes to the pharmacological actions of proanthocyanidin.

Key words proanthocyanidin; 5-hydroxytryptamine, receptor; patch clamp; NCB-20 cell

5-Hydroxytryptamin (5-HT)3 receptors belong to the ‘Cys-loop receptor’ superfamily, which also includes nicotinic acetylcholine (nACh) receptors, γ-aminobutyric acid type A (GABA_A) receptors and glycine receptors.1–4) The 5-HT3 receptors are distributed throughout the central nervous system (CNS) and the peripheral nervous system (PNS), with strongest expression found in the nucleus tractus solitarius, area postrema and dorsal motor nucleus of the vagus nerve, where they play an important role in the initiation and coordination of the vomiting reflex.5,6) In the CNS, postsynaptic 5-HT3 receptors mediate the fast synaptic response to serotonin, and presynaptic receptors regulate the synaptic release of serotonin and several other neurotransmitters.7,8) Also, the 5-HT3 receptors are established drug targets. For examples, competitive antagonists targeting central and gastrointestinal 5-HT3 receptors are used in the clinical treatment of the nausea and/or emesis associated with radio- and chemotherapy treatment of cancer, postoperative nausea and vomiting, and irritable bowel syndrome.9,10) Furthermore, central 5-HT3 receptors have been proposed as potential targets for the treatment of various psychiatric disorders, cognitive dysfunction, drug abuse and withdrawal, and certain forms of pain.10)

Proanthocyanidin is a kind of phenolic compound (oligons are catechin-type monomers, dimmers and trimers, as well as oligomeric proanthocyanidins) present in plants, especially highly present in the grape seed, that has received attention recently.11,12) Experimental and clinical studies have revealed that proanthocyanidin has antioxidant, antinociceptive, anti-emetic, and cardioprotective properties, without inducing significant toxicological effects.12–16)

In view of the above reports, we try to investigate whether proanthocyanidin has any effects on the activity of 5-HT3 receptors. Accordingly, we tested the effects of proanthocyanidin on 5-HT3 receptor function using patch-clamp recording combined with a fast drug application technique in order to explore a new pharmacological mechanism for the effect of proanthocyanidin.

MATERIALS AND METHODS

Materials NCB-20 neuroblastoma cells were kindly provided by Dr. Lovinger (National Institute on Alcohol Abuse and Alcoholism, U.S.A.). Proanthocyanidin was purchased from InterHealth Nutraceuticals Inc. (Benicia, CA, U.S.A.), as a grape seed proanthocyanidin extract (GSPE) containing approximately 76% oligomeric proanthocyanidins and 3% monomeric bioflavonoids. Cell culture reagents were obtained from Gibco BRL (Rockville, MD, U.S.A.). Serotonin and all other chemicals were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.).

Cell Culture NCB-20 cells were maintained under conditions previously described.17) Frozen cell stocks were maintained in liquid nitrogen, and thawed as needed. Cells were routinely grown in medium containing 90% Dulbecco’s modified Eagle’s medium, which contained 3.7 g/l NaHCO3 (pH adjusted to 7.4 with NaOH), 10% fetal bovine serum, and 1% hypoxanthine aminopterin thymidine supplement (HAT, which gave a final concentration of 13.6 mg/l hypoxanthine, 0.178 mg/l aminopterin, and 0.4 mg/l thymidine) and were cultured in an incubator containing 5% CO2 at 37 °C. Cells were seeded onto 35-mm culture dishes at least 2 d prior to electrophysiological experiments.

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Electrophysiological Recordings  Whole-cell patch-clamp recordings were performed in 35-mm culture dishes at room temperature (21—24°C) on the stage of an inverted microscope (TS 100; Nikon, Tokyo, Japan), as described previously.15 Cells were continuously superfused with an extracellular solution containing 150 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl$_2$, 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), and 10 mM D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm/kg with sucrose) at a rate of 2—3 ml/min. Patch pipettes were pulled from thin-walled borosilicate glass tubing (Molecular Devices, Inc. Sunnyvale, CA, U.S.A.) with a filament using a puller (P-97; Sutter instrument Co., Novato, CA, U.S.A.). Pipette tips had resistances of 2.0—3.0 MΩ when filled with an internal solution containing 140 mM CsCl, 2 mM MgCl$_2$, 5 mM HEPES (pH adjusted to 7.4 with CsOH, osmolality adjusted to 310 mOsm/kg with sucrose). Whole-cell currents were amplified using an EPC-7 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and saved on a PC using DigiData1322 and pClamp 9.0 software (Molecular Devices, Inc.). All experiments were carried out at a holding potential of −50 mV unless otherwise stated. Currents were filtered at 10 kHz using a three-pole Bessel filter and digitized at 10 kHz. Capacitive currents were compensated with analog compensation, but leak subtraction was not used in this study.

Drug Preparation and Application  After establishing a whole-cell configuration, the cell was lifted and placed in front of one side of a θ-tubing pipette, which had been pulled to an inner diameter of ca. 300 μm. The extracellular solution, which contained the agonist, flowed through a different side of the θ-tubing. Solutions were rapidly switched around the cell using a piezo-electric translator (P-287.70; Physik Instrument, Waldbronn, Germany), which displaced the θ-tubing laterally so that the cell could be exposed for a defined period of time to the drug-containing solution, and then rapidly returned to the drug-free solution. The piezo-electric translator was controlled by a high-voltage amplifier (E-507; Physik Instrumente) and triggered by pClamp 9.0 software (Molecular Devices, Inc.). The rate of solution exchange was typically 24.4 ± 5.3 ms (n = 11) in the open pipette tip configuration.17 Flow solution was driven by gravity from wells placed above the preparation, and the application of the solutions was controlled by a valve placed upstream of the drug-containing tubing. Immediately after achieving a whole-cell configuration, a variable amount of current run-down was typically observed. However, the currents stabilized 20 min after achieving whole-cell mode. During the experiment, drug applications and current recordings were carried out at 2 min intervals to minimize any effects of desensitization. Because the effect of proanthocyanidin was irreversible, the cell had to be changed after a one time application of proanthocyanidin to exclude the possibility of a carry-over effect.

RESULTS

Effect of Proanthocyanidin on 5-HT-Induced Currents  Lambert et al.18 reported that application of 5-HT to NCB-20 cells produced ion current exclusively via 5-HT$_3$ receptor activation. This conclusion was based on the potent blockade of the response observed with GR 38032F and ICS 205-930, the 5-HT$_3$ receptor antagonists.18 In our study, we firstly determined the concentration–response relationship for the effects of 5-HT on 5-HT$_3$ receptor–mediated currents. The application of 0.1—300 μM 5-HT for 5 s at 2 min intervals to NCB-20 cells caused a concentration-dependent inward current at −50 mV, and 30 μM 5-HT induced a near-maximal response. The EC$_{50}$ value was 2.7 ± 0.4 μM, with a Hill coefficient of 2.1 ± 0.1 (Fig. 4A). The characteristics of 5-HT-induced ion currents were similar to those in our previous reports.17,19

We tested the concentration–dependence effects of proanthocyanidin on the currents evoked by 3 μM 5-HT (near EC$_{50}$). Application of 0.3—100 μg/ml proanthocyanidin in the absence of 5-HT did not induce any currents (the effect of treatment of 100 μg/ml proanthocyanidin alone shown in Fig. 1A). Co-treatment with proanthocyanidin and 5-HT produced a small inhibition, although statistically significant at high concentrations (≥30 μg/ml) of proanthocyanidin, on 5-
HT-induced inward peak current ($I_{5\text{HT}}$) with the maximal inhibition of 9.89±1.2% in 100 μg/ml proanthocyanidin ($n=6$, $p<0.01$, when compared to control) (Fig. 1B). However, pretreatment of cells with proanthocyanidin for 30 s before application of 5-HT greatly inhibited $I_{5\text{HT}}$ in a concentration-dependent manner. Representative traces are shown in Fig. 1A and Fig. 2A. Figure 1B illustrates concentration–response curves for proanthocyanidin-induced inhibition when it was used to pre-treat cells for 30 s or when it was co-applied with 5-HT. The IC$_{50}$ for inhibition of $I_{5\text{HT}}$ when the cell was pre-exposed to proanthocyanidin for 30 s was 6.5±0.4 μg/ml and the Hill coefficient was 2.5±0.1 ($n=6$). After inhibition with different concentrations of proanthocyanidin, washout studies showed partial (20—30%) recovery from proanthocyanidin inhibition over 15 min. Extended time studies showed about 50% recovery from proanthocyanidin inhibition at 1 h (data not shown). Thus, the effect of proanthocyanidin on 5-HT-induced currents seems to be irreversible, which makes it necessary to change the cell after its exposure to proanthocyanidin every time.

**Drug Application Mode- and Time-Dependent Inhibition of $I_{5\text{HT}}$ by Proanthocyanidin** As shown in Fig. 1, pretreatment of proanthocyanidin before 5-HT application produced much stronger inhibition than did co-application with 5-HT. We further investigated the inhibitory effect of proanthocyanidin (5 μg/ml, near IC$_{50}$ for 3 μM 5-HT-induced currents) on $I_{5\text{HT}}$ using three different drug application modes: (1) simultaneous exposure of the cell to proanthocyanidin and 5-HT (co-application); (2) pre-exposure to proanthocyanidin before 5-HT but no co-application with 5-HT; (3) continuous exposure to proanthocyanidin before and during application of 5-HT (Fig. 2A). The proanthocyanidin had no significant effect on $I_{5\text{HT}}$ when it was applied simultaneously with 3 μM 5-HT, while the inhibition by proanthocyanidin was pronounced when the drug was exclusively applied 30 s prior to agonist-induced activation of the 5-HT$_3$ receptors and an equally high inhibition was seen when proanthocyanidin was applied both 30 s before and during 5-HT application. The effects of proanthocyanidin from the above three drug application modes can be expressed as % of control values of 98.0±0.8 ($p=0.238$), 72.7±3.7 ($p<0.001$) and 71.2±3.0 ($p<0.001$), respectively ($n=7$). Unlike the weak inhibition found during the first drug application mode, proanthocyanidin caused marked inhibition of $I_{5\text{HT}}$ following the second and the third drug application mode, and there were no significant difference between the second and the third drug application mode ($p=0.806$, $n=7$) (Fig. 2B). Thus, the second drug application method was adopted in all subsequent studies.

Figure 2C shows the time-dependent relationship for inhibition of $I_{5\text{HT}}$ by 5 μg/ml proanthocyanidin. Cells were pre-treated with proanthocyanidin for 5, 10, 30, 60, or 120 s before 5-HT was applied. The data indicate that proanthocyanidin produced negligible inhibition of $I_{5\text{HT}}$ when it was pre-applied for less than 10 s before 5-HT application, while inhibitory effects were nearly same after 30 s; maximal inhibition occurred after 1 min of pretreatment. Proanthocyanidin inhibited $I_{5\text{HT}}$ by 1.8±1.3, 4.2±1.0, 30.1±3.0, 39.7±4.5, and 42.7±2.6% after 5, 10, 30, 60, and 120 s pretreatment, respectively, before 5-HT application. As 30 s pre-exposure to proanthocyanidin produced a near-maximal inhibition of $I_{5\text{HT}}$, this protocol was used in all subsequent experiments.

**Effect of Proanthocyanidin on 5-HT$_3$ Receptor Activation and Desensitization** For these experiments, a concen-
Fig. 2. Drug Application Mode- and Time-Dependent Inhibition of \( I_{5-HT} \) by Proanthocyanidin

(A) Sample traces from different cells showing the inhibition of 5-HT (3 \( \mu \)M, open bar)-induced currents (black trace) by proanthocyanidin (5 \( \mu \)g/ml, closed bar) following three different modes of drug application (gray trace): co-application of proanthocyanidin with 5-HT (+ +), exclusive application for 30 s prior to 5-HT (+ -), continuous application 30 s before and during stimulation with 5-HT (+ +). Black line is almost invisible in the first sample trace. (B) Summary of proanthocyanidin effects on \( I_{5-HT} \) following three different drug application modes. Currents were normalized to the amplitude recorded under control conditions. Each value represents the mean±S.E.M. (n=7). Symbols show a significant difference from the control group (###) or from the corresponding labeled groups (n.s., ==), ### \( p < 0.001; == p < 0.01; \) n.s., not significant. (C) Pretreatment time-dependent relationship for inhibition of \( I_{5-HT} \) by 5 \( \mu \)g/ml proanthocyanidin. Cells were pre-treated with proanthocyanidin for 5, 10, 30, 60, or 120 s before 5-HT was applied. Each point represents the mean±S.E.M. (n=6).

Fig. 3. Effect of Proanthocyanidin on 5-HT (30 \( \mu \)M)-Induced Currents in NCB-20 Cells

(A) Sample traces of the effect of proanthocyanidin (30 \( \mu \)g/ml) on 30 \( \mu \)M 5-HT-induced currents (black trace). Proanthocyanidin (closed bar) was applied 30 s before application of 5-HT (open bar). Upper panel represents the original traces. To delineate effects of proanthocyanidin on the kinetics of 5-HT-induced currents, the currents induced by 5-HT alone and 5-HT with proanthocyanidin pretreatment were scaled up and superimposed (lower panel). (B) The dose-response curve for proanthocyanidin on the peak amplitude of currents induced by 30 \( \mu \)M 5-HT. (C) Summary of effects of proanthocyanidin on the rise slope of 30 \( \mu \)M 5-HT-induced currents. (D) Summary of effects of proanthocyanidin on the current decay time constant \( (t_{off}) \) of 30 \( \mu \)M 5-HT-induced currents. The data were normalized to the value of the currents induced by 30 \( \mu \)M 5-HT, which was taken as 100%. Each value represents the mean±S.E.M. (### \( p < 0.001; == p < 0.01, \) vs. control group, n=7).
tration of 30 μM 5-HT was chosen because it elicited sub-maximal and reproducible current responses to 5-HT. At 30 μM, 5-HT induced an inward current, which activated fast and desensitized rapidly during the application of the agonist for 5 s (Fig. 3A). Figure 3B shows the concentration-dependent inhibition of peak currents evoked by 30 μM 5-HT at different concentrations of proanthocyanidin (the IC50 was 22.1 ± 0.4 μg/ml, and the Hill coefficient was 2.4 ± 0.1, n = 7). In addition, pretreatment of cells with proanthocyanidin for 30 s significantly decreased the slope of 5-HT-induced currents at concentrations ≥3 μg/ml, giving % of control values of 93.9 ± 0.8 (p < 0.01), 59.6 ± 2.8 (p < 0.001), 10.1 ± 1.1 (p < 0.001) and 4.1 ± 0.3 (p < 0.001) at proanthocyanidin concentrations of 3, 10, 30, and 100 μg/ml, respectively (n = 7). Meanwhile, proanthocyanidin significantly prolonged the current decay time constant (τm) at concentrations higher than 30 μg/ml, with % of control values of 198.3 ± 22.0 (p < 0.01) and 245.1 ± 13.6 (p < 0.001) when cells were pretreated with 30 and 100 μg/ml proanthocyanidin for 30 s, respectively (n = 7). These effects of proanthocyanidin on both activation and desensitization of 5-HT3 receptors showed obvious concentration-dependency (Figs. 3C, D).

**Noncompetitive Inhibition of 5-HT3 Receptors by Proanthocyanidin** To evaluate the mechanism by which proanthocyanidin inhibits I5-HT, we analyzed the effect of 20 μg/ml proanthocyanidin (which is near the IC50 for inhibition of the effects of 30 μM 5-HT, Fig. 3B) on I5-HT evoked by different concentrations of 5-HT. Following pretreatment of cells with proanthocyanidin for 30 s, the dose–response curve for 5-HT (0.3—300 μM) was shifted to the rightward (EC50 from 2.7 ± 0.4 to 6.2 ± 0.5 μM and Hill coefficient, from 2.7 ± 0.1 to 2.1 ± 0.2, n = 6). Meanwhile, pretreatment of cells with proanthocyanidin (20 μg/ml) substantially inhibited I5-HT over a broad range of agonist concentrations, resulting in a reduced Emax to 5-HT. The inhibition of the peak amplitudes of 30 and 300 μM 5-HT-induced currents by 20 μg/ml proanthocyanidin were 37.5 ± 1.3 and 38.9 ± 4.7%, respectively (n = 6). The concentration–response curves for 5-HT with or without proanthocyanidin are shown in Fig. 4A, which suggest a noncompetitive inhibition of 5-HT3 receptors by proanthocyanidin.

**Current–Voltage Relationship of I5-HT in Response to Proanthocyanidin-Induced Inhibition** In experiments of current–voltage relationship, the proanthocyanidin-induced inhibition on I5-HT was measured with different holding potentials; −50, −30, −10, 0, +10, and +30 mV. The application of 30 μM 5-HT to cells induced inward currents at negative voltages and outward currents at positive voltages. Pretreatment with 20 μg/ml proanthocyanidin for 30 s before 5-HT application decreased both inward and outward currents. The reversal potentials from the current–voltage curve were near 0 mV in both paradigms, 5-HT alone and 5-HT after pretreatment with proanthocyanidin. The inhibitory effect of proanthocyanidin on I5-HT in NCB-20 cells was independent of the membrane holding potential (Fig. 4B). Proanthocyanidin inhibited I5-HT by 48.4 ± 2.1, 47.8 ± 2.4, 48.8 ± 3.4, 44.5 ± 2.7, 46.0 ± 1.6, and 39.5 ± 3.1% at −50, −30, −10, 0, +10, and +30 mV membrane holding potentials, respectively (n = 6).

**DISCUSSION**

In the present study, we demonstrated the effects of proanthocyanidin, a major active component of grape seed extracts, on the function of 5-HT3 receptors in NCB-20 cells. The function of these receptors was assessed by measuring the current induced by fast application of 5-HT under voltage-clamp conditions. The effects of proanthocyanidin on both I5-HT and the kinetics of activation and desensitization of 5-HT3 receptors were studied.

Co-application of 0.3–100 μg/ml proanthocyanidin produced a less than 10% reduction of I5-HT induced by 3 μM 5-HT, while pretreatment of cells with proanthocyanidin for 30 s before 5-HT application caused much stronger inhibition; under the latter conditions, the concentration dependence of the inhibition was apparent. The inhibitory effect of proanthocyanidin also showed a dependency on pretreatment time. These results suggest that proanthocyanidin is acting on a closed state of the receptor, or that the effect of proanthocyanidin is slower than the typical opening and closing kinetics of 5-HT3 receptor currents[20,21] as has been shown in our study: co-application of proanthocyanidin with 5-HT did not produce remarkable inhibitory effect because proanthocyanidin needs around 30 s to express its inhibitory effect.
and this time is too late to inhibit 5-HT-induced current whose peak appears in less than 0.5 s.

On the other hand, pretreatment with proanthocyanidin affected the kinetics of 5-HT$_3$ receptors. At concentrations $\geq$3 $\mu$g/ml, proanthocyanidin caused a pronounced concentration-dependent decrease of the rise slope of 5-HT-induced currents. At higher concentrations ($\geq$30 $\mu$g/ml), proanthocyanidin produced a significant deceleration of the current decay time constant ($\tau_{\text{dec}}$), which was also concentration dependent. Therefore, the data indicate that proanthocyanidin mainly produces a strong inhibition of activation at lower concentrations and inhibits the desensitization of 5-HT$_3$ receptors at higher concentrations.

We studied the mechanism by which proanthocyanidin inhibits 5-HT$_3$ receptors by measuring the effect of 20 $\mu$g/ml proanthocyanidin on the peak currents induced by different concentrations of 5-HT. In these competition experiments, we observed that the presence of proanthocyanidin caused a rightward shift of the dose–response curve without significantly changing the Hill coefficient. At the same time, the peak amplitude of the control current was inhibited consistently and the inhibitory effect was not relieved even at high 5-HT concentrations. All these results indicate a noncompetitive inhibitory mechanism of proanthocyanidin of the 5-HT$_3$ receptors. Prior studies suggested that proanthocyanidin is membrane-impermeable because of its size and polarity and that it seems unlikely that it is transported across the membrane at a sufficient rate to reach a potential intracellular target. Thus, the noncompetitive inhibition of 5-HT$_3$ receptor channel activity by proanthocyanidin indicates that proanthocyanidin has different binding or interaction sites through which it modulates the 5-HT$_3$ ion channel from the extracellular side.

Our data and the above reports suggest3,22,23,25 that proanthocyanidin inhibits 5-HT$_3$ receptors in a noncompetitive manner, and that there exits the possibility that proanthocyanidin acts as an open channel blocker at 5-HT$_3$ receptors. Therefore, we tested the voltage dependency of the inhibition by proanthocyanidin by measuring its effect on current–voltage relationships. As shown in Fig. 4B, the inhibition produced by proanthocyanidin was observed both at negative voltages and positive voltages and had a similar magnitude. These results indicate that proanthocyanidin might not act as an open channel blocker since inhibition by proanthocyanidin was not voltage dependent, whereas inhibition by open channel blockers shows strong voltage dependence owing to the charge they possess in a transmembrane electrical field.24–26

From the present study, we have demonstrated that (1) pretreatment of cells with proanthocyanidin before application of 5-HT causes much stronger inhibition of $I_{\text{max}}$ in NCB-20 cells than does co-application with 5-HT. The inhibition is irreversible and dose dependent; (2) at high concentrations, proanthocyanidin also inhibits the activation and desensitization of 5-HT$_3$ receptors in a dose-dependent manner; (3) inhibition of $I_{\text{max}}$ by proanthocyanidin is noncompetitive and voltage independent. The results of several studies have shown that proanthocyanidin has antinociceptive and antiemetic actions although the precise mechanism is unclear.11,13,10 On the other hand, the 5-HT$_3$ receptors mediate emesis and pain,27,28 and some antagonists of the 5-HT$_3$ receptors, like ondansetron, have been shown to prevent the development of pain and emesis.29–32 In addition, some antiemetic drugs like metoclopramide have been reported to be potent antagonists of 5-HT$_3$ receptors.21,22 Taken together, these findings suggest the possibility that proanthocyanidin-mediated regulation of 5-HT$_3$ receptors is therapeutically relevant although we currently do not have direct evidence that proanthocyanidin can be used as a therapeutic agent for the alleviation of 5-HT$_3$ receptor-related clinical symptoms such as vomiting and pain. To confirm this possibility, further studies are required to provide more insight into the effects and toxicity of proanthocyanidin in animals and humans.

Proanthocyanidin used in the present study was derived from grape seeds extract which also contains 3% monomeric bioflavonoids. Indeed, we consider that the inhibitory effects on 5-HT$_3$ receptors mainly result from the oligomeric proanthocyanidin. However, like most other studies using this compound, our study cannot completely exclude the possible role of monomeric bioflavonoids although their contents are very low.

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REFERENCES