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Flavonoid-Associated Direct Loss of Rotavirus Antigen/Antigen Activity in Cell-Free Suspension

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Abstract

Secondary plant metabolites, such as the flavonoids epigallocatechin gallate (EGCG) and proanthocyanidins (PAC) have been linked to numerous health promoting activities. Studies addressing flavonoid antiviral activity, however, have been directed towards testing infectivity and investigating inhibition of metabolic processes associated with viral growth in the host cell. Few studies have addressed the direct loss of viral activity by plant metabolites in a cell-free system. In the current study, the extent of antiviral activity by a structurally diverse group of flavonoids present in cranberry juice and grape juice (proanthocyanidins), citrus fruits (hesperidin, naringin, and diosmin), and green tea (catechins) was investigated using a quantitative antigen capture enzyme linked immunoassay (ELISA) in a cell-free system of simian rotavirus strain SA-11 (RTV). The RTV, representative of an enteric virus, was placed in MA104 cell cultures and tested by endpoint dilution. PACs at 100 and 200 μg/mL caused a direct loss of virus activity at 56% and 92%, respectively, as compared with the control. Use of EGCG at 80 and 160 μg/mL resulted in a loss of RTV infectivity of 45 and >98%. Individual tests with the PAC components catechin and epicatechin (EC) required increased concentrations (ca. 5000 to 10,000 μg/mL) to cause an RTV antigen loss approaching 50%. The combined use of catechin and EC had no significant synergistic effect on the loss of RTV capsid antigen. Tests with the flavanones (hesperidin and naringin) and the semisynthetic isoflavone (diosmin) displayed antiviral activity at a concentration of 1200 μg/mL. PAC extract at 200 μg/L inhibited RTV-induced hemagglutination, suggesting an inhibitory effect on the binding of RTV antigenic determinants to RBC receptors. Gold-labeled immunoelectron microscopy showed RTV particles entrapped or sequestered with PAC aggregates. These findings suggest two things: first, recognition that the potential antiviral efficacy of flavonoids is related to structural differences between groups and within subgroups, and second, antiviral (anti-RTV) activity occurs at the virus-flavonoid interface in the absence of the viral host cell.

INTRODUCTION

Numerous health promoting activities have been associated with the consumption of selected plant juices, such as cranberry and grape, and with

green tea (Howell, 2009; Cooper, 2012). Current evidence suggests that flavonoid (phenolic) components, especially epigallocatechin gallate and the proanthocyanidins, observed in a number of plant species may have a significant impact on human health (Aron and Kennedy, 2008; Bose et al., 2008; Higdon and Frei, 2003; Xiao et al., 2011). Flavonoid-associated antimicrobial effects have been the subject of a number of reports (Calzada et al., 2001; Gescher et al., 2011; Guay, 2009; 2010; Kim et al., 2010; Lipson et al., 2010; Lu et al.,2004; Mim and Hart, 2003; Mukhtar et al., 2008; Mukoyama et al., 1991; Nagi et al., 1992; 1995; Nair et al., 2002; Roh and Jo, 2011; Song et al., 2005; Suzutani et al., 2003; Thapa et al., 2012; Zakay-Rones, et al., 2004), proposing consumption of comestible plants and their products act on bacteria (*E. coli* in urinary tract infections), viruses (herpes simplex virus, human immune deficiency virus, respiratory syncytial virus, and hepatitis C virus among others, plus the parasites (*Entamoeba histolytica* and *Giardia lamblia*).

More specifically, the antiviral activity of flavonoids has been ascribed to several mechanisms, including blockage of viral antigenic determinants or cellular receptors, loss of viral/cellular enzyme integrity/function, inhibition of particle biosynthesis, and/or particle egress. Furthermore, antiviral and antimicrobial activity have been associated with specific flavonoid subclass groups, the catechins and proanthocyanidins of grapes, berries, and tea, the flavanones naringenin and hesperetin of citrus fruits, the flavonols kaempferol, quercetin in onions, kale, and blueberries. In addition, differences in "R" group substituents in the phenylbenzopyran A, B, or C rings of some of these molecules could affect antiviral and antimicrobial activity (Cushnie and Lamb, 2011; Lipson et al., 2012; Bae et al., 2000; Foo et al., 2000; Gharras, 2009; Goldwasser et al., 2011; Savi et al., 2010; Steinmann et al., 2013; Thapa et al.,2012; Tsai et al., 2011; Williamson and McCormick, 2006). Details on flavonoid structure, biochemistry, and effects on plant physiology have been summarized by Buer et al., (2010) and Haslam (1998).

Although significant, studies on flavonoid antiviral and antimicrobial activity at the cellular level essentially overlook interactions at the plant metabolite-virus interface. Except for the recent reports by Lipson and co-workers using proanthocyanidins and selected plant juices (Lipson et al., 2011; Lipson et al, 2012), virtually no investigators have addressed the direct antiviral activity by flavonoid subclasses in a cell free system. Moreover, no studies have addressed the effects of flavonoids on enteric virus mediated hemagglutination – an assay that could help in understanding the mechanisms in antiviral activity of flavonoid groups.

This study investigated the potential of direct antiviral activity of flavonoid subclasses as antiviral moieties in a cell-free system. The simian rotavirus SA-11 (RTV) was used throughout this study as a model enteric virus system.

MATERIALS AND METHODS

Secondary plant metabolites. The secondary plant metabolites used in study included flavonoids -(-) epicatechin), -(-) epicatechin gallate, -(-) epigallocatechin, and (-)- catechin (catechin), were purchased from Sigma-Aldrich (St. Louis, MO). Epigallocatechin gallate was purchased from Cayman Chemical Co. (Chicago, IL). Cranberry and grape proanthocyanidin extracts were kindly supplied by Dr. A. B. Howell (Marucci Center for Blueberry Cranberry Research, Rutgers University, Chatsworth, NJ; Foo et al., 2000; Howell et al., 2005). HI PAC 4.0, which is readily soluble in water-base diluents, was supplied by Decus Botanical Synergies, Carver, MA. Hesperidin, naringin, diosmin, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO).

For the experimental trials, the PACs (1000 μg were suspended in two mL sterile distilled H_2O and homogenized on ice for 10 min at high speed in an OMNI TH homogenizer (OMNI International, Kennesaw, GA). The flavonones hesperetin, naringin, and diosmin were solubilized in absolute methanol and >99% dimethylsulfoxide (DMSO), followed by dilution of the preparation to yield concentrations of 2.6% and 40% methanol and DMSO containing a flavanone concentration of 1200 μg.

Virus, cell culture, and enzyme immunoassay. Infectivity titration measurements were used to determine the number of infectious RTV particles (simian rotavirus

strain SA-11 ATCC VR-1565) (Manassas, VA) added to our experimental systems. Host cells consisted of African green monkey kidney epithelial (MA-104) cells, clone *Chlorocebus aethiops* grown with titrations in 96-well, flat bottom microtiter plates.

Growth media (GM) consisted of Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL of streptomycin, 100 units of penicillin, 1% L-glutamine, and 1% amphotericin B. The maintenance medium (MM) was the same as GM except that 2% FBS was added to the medium supplement. Prior to virus inoculation, the GM was aspirated, and each well was replaced with MM. Infectivity titers were determined by end-point dilution and expressed as a tissue cul- ture infective dose ($TCID_{50}$/mL) (Reed and Muench, 1938).

Direct loss of viral antigen by flavonoids in cell-free suspension was determined using a viral capsid protein 6 (VP6) quantitative antigen capture in an RTV enzyme-linked immunosorbent assay (ELISA) (Premier™ Rotaclone® Enzyme Immunoassay Assay; Cat. No. 696004; Meridian Bioscience, Inc., Cincinnati, OH). The RTV antigen capture ELISA technology was chosen due to recognition of the quantitative and qualitative determination of virus infectivity (antigen) both in the clinical and research settings (Dornai et al., 1993; Fang et al., 2009; Lipson et al., 2011; Knowlton et al., 1991; Raj et al., 2007; Stals et al., 1984; Tran et al., 2010). Readings were quantitatively determined using a Genesys 20 Spectrophotomer (ThermoFisher, Inc., Waltham., MA) at an absorbance of 450 nm as per instructions of the manufacturer specifications. All experiments and ELISA titrations were done in triplicate.

Synergistic effects of catechin and epicatechin on rotavirus activity. To determine possible synergistic activity, the effect of a catechin and EC mixture (50% each) at 313 and 2500 µg/mL was compared with each individual flavanol at 313, 625, 1250, and 2500 g/mL. The mixture of flavanols and each individual phytochemical were added to separate, but equal volumes of RTV and incubated in cell-free suspension for 30 min at room temperature (23°C). After the incubation period, viral activity was determined by the quantitative [capsid] antigen detection ELISA. Inoculum stock titer was 9.2×10^4 $TCID_{50}$/mL. The percent activity was determined by comparison with a control in which no catechin nor EC were added.

Microhemagglutination-inhibition (HAI). The inhibition of microhemagglutionation, was tested using a modification of the procedures of Swenson et al. (1992). A 7% guinea pig red blood cell concentra- tion, prepared daily was diluted (1 part blood: 67 parts buffer) with a phosphate-buffered saline solu- tion containing no Ca^{2+} or Mg^{2+} (PBS) for each experiment. An equal volume of PBS containing 200 µg/mL of the PAC was subsequently mixed with the PBS containing the blood cells for 30 min incubation at room temperature (23°C). At the end of the incubation period, 10 µL of the working strength RBC-PAC preparation was added to clean glass slides to which equal volumes of RTV at the tissue culture infective dose were added. Suspensions were mixed with a wood applicator and incubated at room temperature (23°C) for an additional 30 min.

In an effort to separate the effect of the PAC preparation on the virus and not the RBCs, the RTV was treated first followed by the immediate addition of untreated guinea pig RBCs. Careful attention was placed on the physical handling of control slides, which, except for the PAC being replaced with PBS, were identical to experimental slides. The negative control consisted of RBCs which were treated with PBS, alone. Additional HAI testing was done using herperidin, naringin, and diosmin. These flavonoids were dissolved in methanol or DMSO, diluted in 1X PBS to achieve a final concentration of 1200 µg/mL at 1.3 and 20%, respectively. Experimental and control preparations were observed by light microscopy (100X) and photographed.

Interaction of proanthocyandins and rotavirus. To visualize any interaction of cranberry proanthrocyandins (c-PACs) with rotavirus, transmission electron microscopy (TEM) was used. Immuno-gold-labeled rotaviruses in a cell free system were treated with high molecular weight C-PACs. The primary antibody, which consisted of a mouse monoclonal (3C10) specific to the viral antigen VP6 (Abcam®,

Cambridge, MA), was diluted 1 part to 20 parts in PBS. A volume of this antibody mixture was added to an equal volume of the rotavirus at a concentration of 9.2 X 10^5 $TCID_{50}$/mL. The mixture was incubated at 37°C for 30 min.

The secondary antibody consisted of a 6 nm gold-labeled goat-anti-mouse IgG (H+L) conjugate (Electron Microscopy Sciences, Hatfield, PA), diluted 1:10 in PBS. The diluted secondary antibody was added to an equal volume of the 3C10-rotavirus complex and incubated for an additional 30 min at 37°C. The positive control consisted of gold-labeled virus only, and the negative control consisted of C-PACs only. Rotavirus alone or 3C10-treated rotavirus in the absence of the secondary antibody, showed no artifacts. The gold-labeled immunoelectron microscopy was done using a Hatachi transmission electron microscope.

Cytotoxicity tests. Testing for cytotoxicity was done using a Toxilight® Non-Destructive Cytotoxicity Bioassay kit (Cat. No. LT37-619; Lonza, Rockville, MD). MA-104 cells were grown in a monolayer on culture flat bottom, 96-well, polypropylene cluster plates as per specifications of the manufacturer. Cellular membrane damage was quantified through the release of adenylate kinase (AK) driving (in part) the lucinferin/luciferase assay to produce an ATP "spark."

Catechin and EC at 5000 and 10,000 µg/mL in 1.3 and 2.6% methanol were added to the prepared MA-104 culture monolayers and incubated for 5, 30, and 60 min periods at room temperature (23°C). Cytotoxicity was evaluated after each incubation period. The positive control consisted of an ammonium chloride-based lysing reagent (Cat. No. LT07-517; Lonza, Rockville, MD) in the absence of methanol and in 1.3 or 2.6% methanol. The negative control consisted of phosphate-buffered saline containing either 1.3 or 2.6% methanol. Identical testing was done using 100 µg/mL cranberry-PACs (C-PACs), grape-PACs (G-PACs), and 160 µg/mL EGCG. Results were reported as relative light units (RLUs) employing a Modulus luminometer (Model No. 9200-003; Turner Biosystems/Promega Corp., Sunnyvale, CA).

RESULTS

Effect of flavonoids on the loss of rotavirus activity. EGCG and cranberry PACs (C-PACs) were effective anti-RTV plant secondary metabolites, with loss of RTV antigen activity (as determined by VP6 function) at 160 and 200 µg/mL to >98 and 90% of control, respectfully. Anti-RTV activity by selected catechin isomers, epicatechin (EC), epigallocatechin (EGC), and epicatechin gallate (ECG), was markedly less effective, requiring concentrations >2000 µg/mL to reduce viral activity reductions to ca. 38, 50, and 78%, respectively (Table 1 & Figure 1).

Table 1. Loss of rotavirus antigen structural integrity.

Plant metabolites	Concentration (µg/mL)[1]	Virus antigen (% loss)[2]	Significance (P-value)[3]
Proanthocyandin	100	66	0.002
Proanthocyandin	200	92	<0.001
Naringin	1200	0	-
Hesperidin	≤1200	0	-
Diosmin	1200	0	-

[1]Metabolites were solubilized in methanol to working concentration of 1.3%.
[2]Loss of antigen was determined by enzyme immunoassay.
[3]Student's t-test, $p<0.05$, $n=3$; value <0.95 were considered significant.

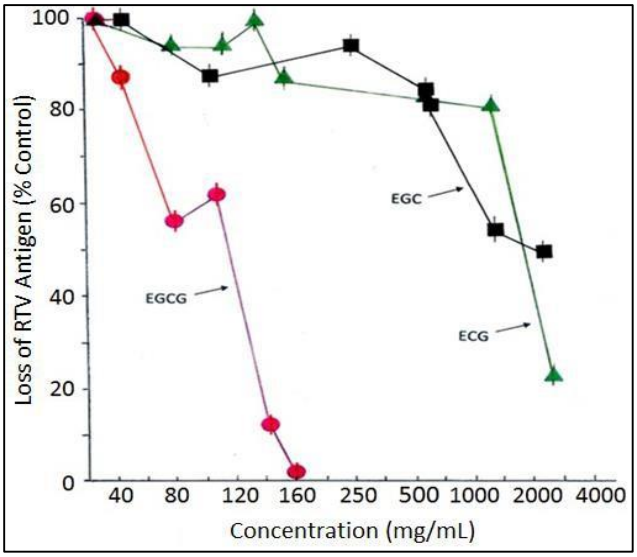

Figure 1. Effect of epigallocatechin gallate, epicatechin gallate, and epigallocatechin on rotavirus antigen loss.
Note: Experimental trials were done in a cell free suspension for 30 minutes at room temperature; loss of RTV activity was measured by the reduction of VP6 antigen levels as determined by an antigen-capture ELISA. EGCG = epigallocatechin gallate, ECG = epicatechin gallate, EGC = epigallocatechin.

The flavanones naringin, hesperidin, and the isoflavone diosmin had no direct effect on the loss of viral antigen activity in our experimental system at concentrations equal to or slightly greater than 1200 µg/mL. In the test system, higher flavanone concentrations (>2,400 µg/mL) precipitated from the methanol carrier upon mixing with maintenance medium or PBS. Markedly increased concentrations of 23 and 25 x 10^3 µg/mL of "HI PAC 4.0" were required to affect a loss of RTV activity that approximated 50 to 90% of the control, respectively (Figure 2). The high solubility of "HI PAC 4.0" (Anonymous, 2010) permitted use of the inordinately high concentrations of this commercially available product in our experimental cell- free assay system.

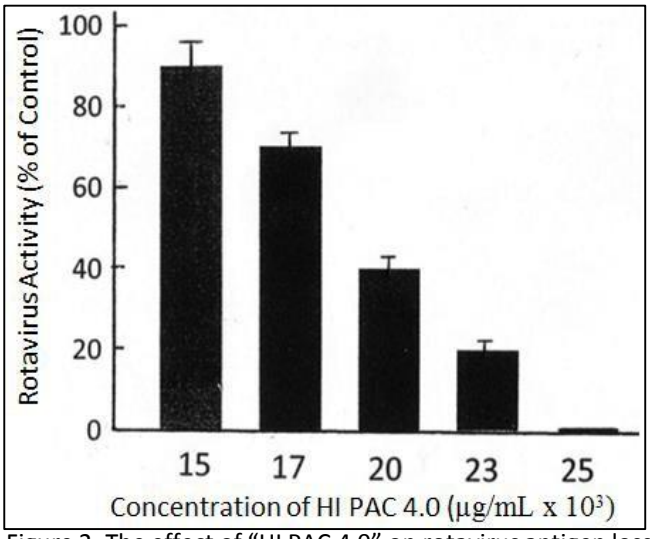

Figure 2. The effect of "HI PAC 4.0" on rotavirus antigen loss.
Note: RTV suspensions were added to equal volumes of "HI PAC 4.0" powder dissolved in phosphate buffered saline with rotavirus activity measured after 30 minutes. Rotavirus concentration was 9.2 x 10^4 $TCID_{50}$/mL. The results are reported as the mean ± SEM of three replicates

Synergistic effects of epicatechin (EC) and catechin on rotavirus activity. The flavanol molecular groups, EC and catechin (at a final concentration of 313 µg/mL) added alone or in combination to RTV in a cell-free suspension had no significant effect on the loss of RTV activity. Similarly, neither EC nor catechin at the same concentration, showed any antiviral activity. RTV activity was significantly reduced to 71% of control when treated with EC-catechin mixture at 2500 µg/mL, but did not approach that of catechin (625, 1250, or 2500 µg/mL) (Figure 3). The inordinately high catechin concentrations of 625, 1250, and 2500 µg/mL, although affecting RTV antigen reductions (loss of viral activity) to 65, 73, and 78% respec- tively, failed to approximate that of PAC or EGCG associated anti-RTV activity (92 to >98%, respective- ly) at markedly reduced levels.

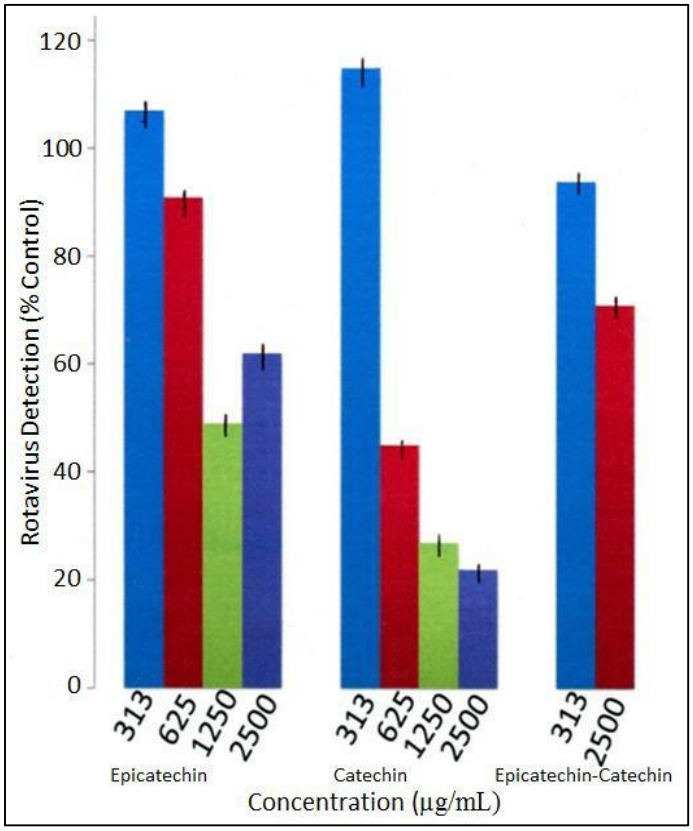

Figure 3. Effects of epicatechin and catechin on the loss of rotavirus activity in cell-free suspension.
Note: Concentrations of epicatechin, catechin, and the combination were incubated with equal volumes of rotavirus at room temperature for 30 minutes. Rotavirus concentration was 9.2 x 10^4 $TCID_{50}$/mL and the activity was quantified using the capsid antigen. The results are reported as the mean ± SEM of three replicates.

Cytotoxicity testing. Cytotoxicity was not observed with either the cranberry (C-PAC) or the grape (G-PAC) tested concentrations (100 µg/mL) nor by that of the catechin isomer EGCG at 160 µg/mL (Figure 4). C-PAC at 200 µg/mL produced no adverse effect on cellular integrity during cytotoxicity testing (data not shown). Similarly, EC and catechin, each tested at concentrations 5,000 and 10,000 µg/mL and containing 1.3 or 2.6% methanol, had no overt effect on MA-104 cell membrane integrity as determined by the cytotoxicity bioassay (Figure 5). The flavonoid and alcohol concentrations used in the

cytotoxicity testing approximated those used in the anti-RTV experimental system.

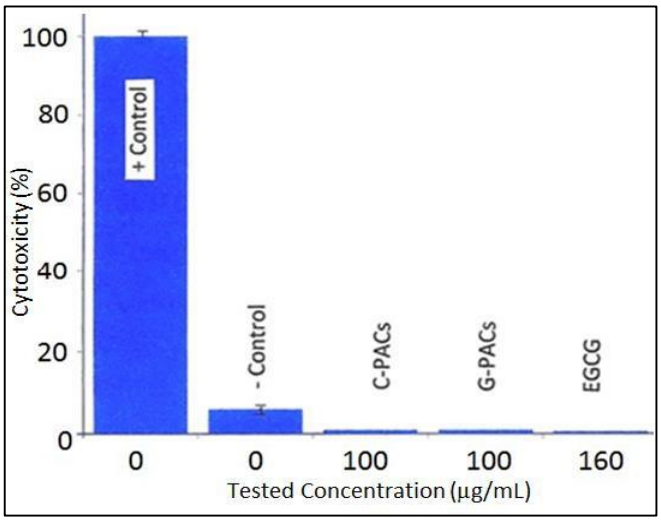

Figure 4. Cytotoxicity test.
Note: Adenylate kinase released by damaged cellular membranes resulted in ATP used in the lucinferin-luciferase Toxilight® Bioassay. Measures of cytotoxicity after 60 min at room temperature indicated the cranberry-PACs, the grape-PACs, and the epigallocatechin gallate protected cellular membranes. The positive control was NH_4Cl lysing reagent (Lonza, Rockville, MD). The negative control was a buffered phosphate buffered saline solution. The results are the mean ± SEM of three replicates.

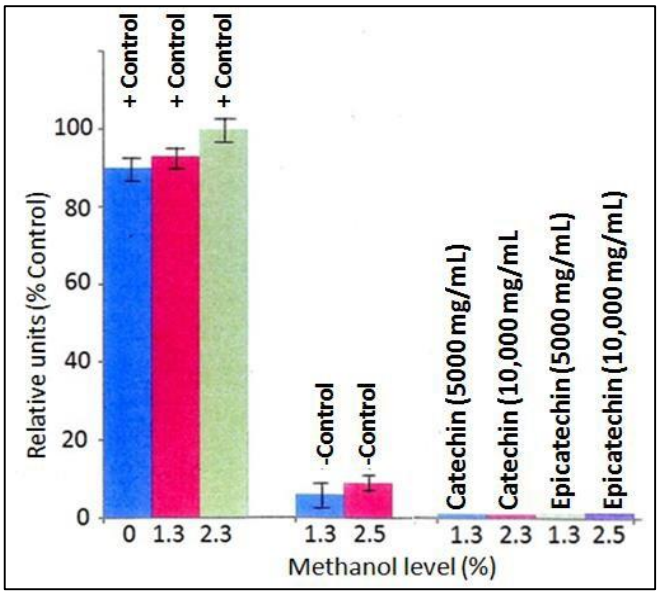

Figure 5. The effect of methanol on prevention of cytotoxicity by catechin and epicatechin.
Note: Catechin and epicatechin were added to MA-104 cell culture monolayers and incubated for 5, 30 (not shown), and 60 minutes at 37 ^{o}C in the presence of 1.3 and 2.5% methanol. The positive controls were NH_4Cl and the negative controls consisted of PBS + the indicated methanol level. No negative effects on cellular integrity due to methanol were noted. Results are means ± SEM of three replicates.

Microhemagglutination-inhibition. A microscopic examination (100X) of the rotavirus and red blood cells (RBCs) at 30 min, after each were pretreated with 200 µg/mL PACs at room temperature for 30 min, showed the pretreatment with the proanthocyanidins prevented cellular aggregation by RTV. Treatment of RTV in suspension by PACs followed by an immediate inoculation of RBCs, similarly inhibited cellular (RBC) aggregation. Herperitin, naringin, or diosmin at1200 µg/mL (in methanol or DMSO) displayed no microhemagglutination inhibition activity. Under identical conditions, but using an RTV-PBS preparation (positive control), hemagglutination was apparent (Figure 6). PBS alone had no effect on the RBC aggregation (not shown).

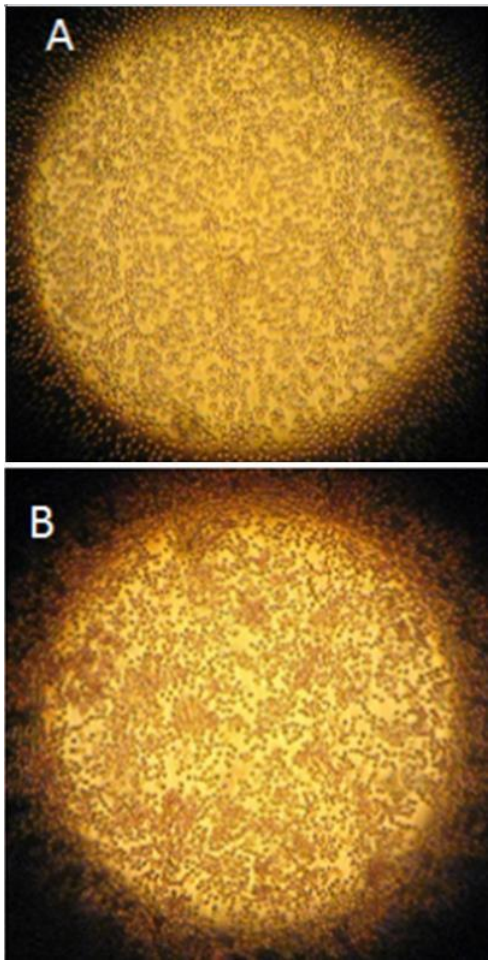

Figure 6. Microhemagglutination inhibition.
Note: A = inhibition of RBC aggregation following virus or pretreatment with PACs. B = a positive control consisting of RTV-PBS preparation showing RBC aggregates. The negative control, RBC treated with PBS, had no hemagglutination.

Electron microscopy. Electron microscopy of the incubated mixture of equal volumes of gold-labeled RTV (9.2 x 10^5 $TCID_{50}$/mL) and PAC preparations (200 μg/mL) showed morphological intact RTV particles were heterogeneously dispersed throughout the preparations. Virions, made visible by the gold-labeling, appeared to be sequestered or embedded within the PAC particulate fraction (Figure 7). Earlier attempts to differentiate non-labeled RTV in association with the flavonoid in question proved futile, due to the near opaque nature of the phenolic under transmission electron microscopy.

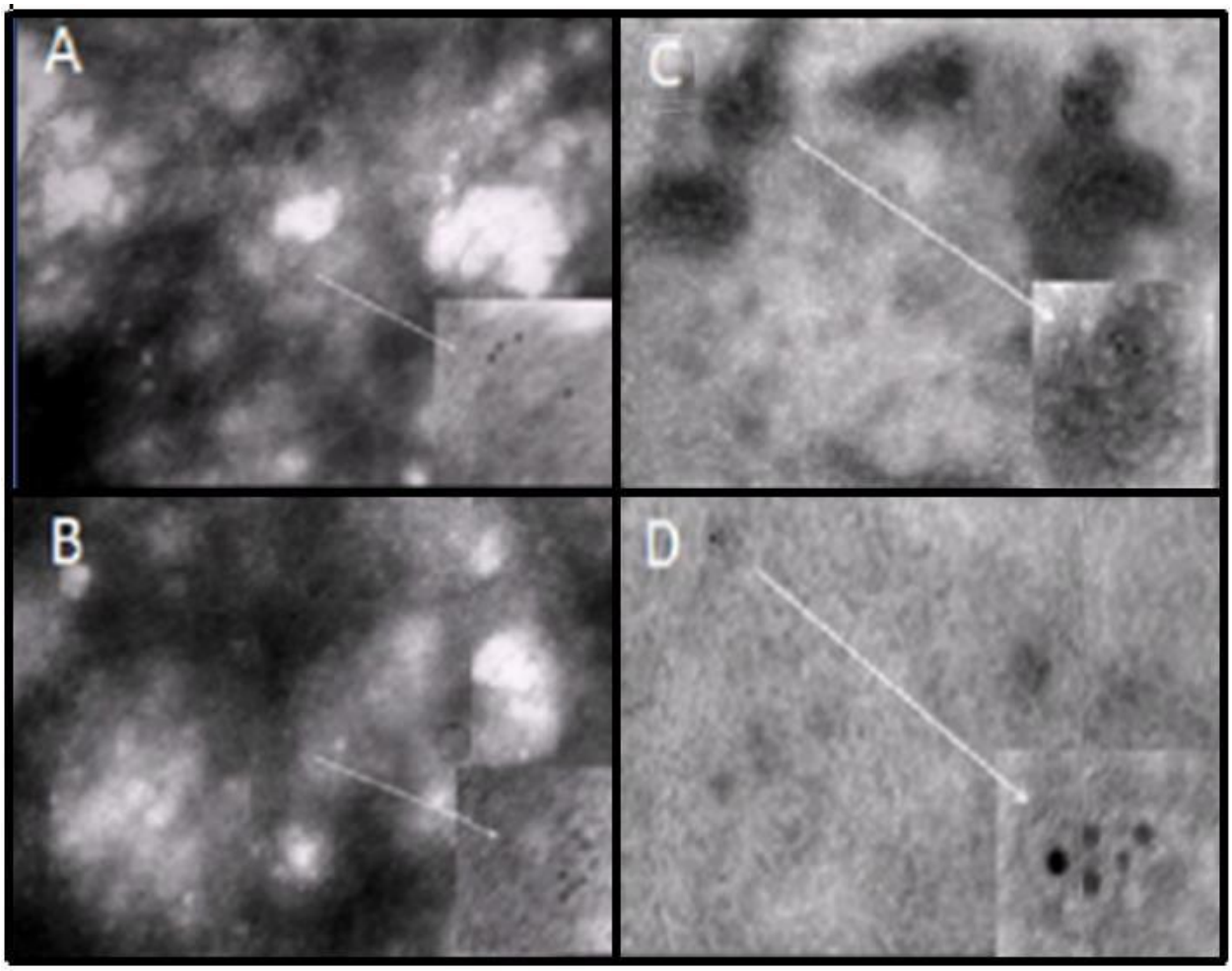

Figure 7. Entrapment of rotavirus by proanthocyanidin particulates.
Note: A & B = RTV sequestered with PAC particles; C = Positive control consisting of gold-labeled virus in the absence of PACs; D = Negative control consisting of PACs only. No artifacts similar to the 6 nm gold particles were observed (data not shown). RTV entrapment and envelopment by both PAC particulate and solubilized fractions occurred in the water column.

DISCUSSION

Among the catechin group of flavonoids tested, a direct loss of RTV antigen in cell-free suspension occurred in the of EGCG > ECG > EGC > EC = (-)-catechin. The catechin group of plant metabolites (EGCG, ECG, EGC, EC) are characterized in part, by the flavan-3-ol backbone skeleton. Importantly, these molecules become structurally discrete by location and differences in substituent side groups on the rings designated B and C (Figure 8). Specifically, the galloyl moiety bound to the EGCG and ECG at the benzopyrene-2 position (position C3 of the benzopyrene molecular component), appears to be associated with the increased anti-RTV activity of these two molecules. The galloyl moiety *per se*, however, cannot in itself, be considered with the enhanced anti-

RTV activity. The markedly enhanced direct anti-RTV activity fostered by EGCG, appears to be supplemented by a trihydroxylation of the aromatic B ring of the molecule (Cos et al., 2003; Zanchi et al., 2009).

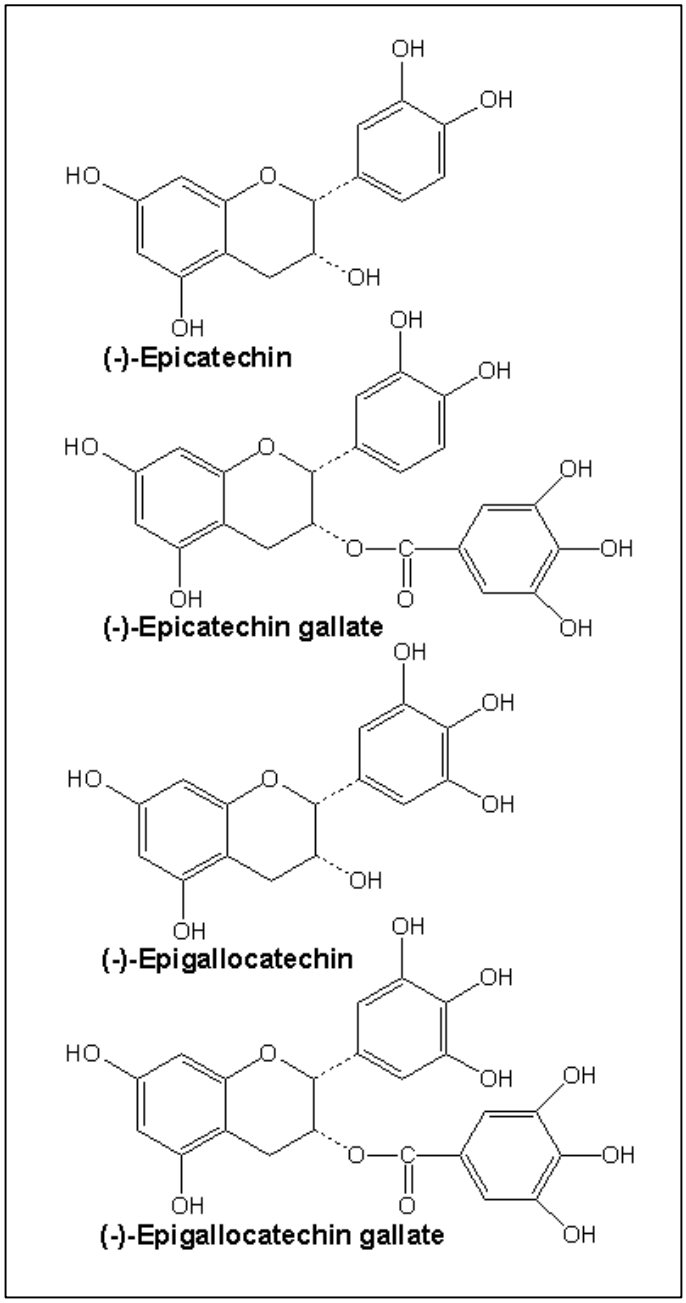

Figure 8. Major catechins in green tea (*Camellia sinensis*).
Note: Epigallocatechin gallate (EGCG) is the most abundant in green tea, but minimal in black tea due to the manufacturing process.

In support of our findings, comparative studies have ascribed the importance of individual catechin species, notably EC, EGC, ECG, EGCG, to alterations in viral structural integrity and then in turn infectivity. Using several influenza virus strains, Song, et al. (2005) reported a loss, with one excep- tion, of viral envelope integrity (neuraminidase), reduced RNA synthesis, and reduced infectivity following viral treatment by select catechins in the order EGCG>ECG>EGC. These findings were ascrib- ed to galloylation at the 3- hydroxyl of the flavon-3-ol catechin skeleton and to a lesser extent to the 5'-OH at the trihydroxylbenzyl -2 position. Additional studies were done to address the antiviral activity and structural significance of EGCG (using the herpes simplex virus type-1, HSV-1) in comparison with EC (dihydroxylated), EGC (trihydroxylated), and the di-hydroxyl galloylated gallocatechin molecular groups (Gescher at al., 2011). Comparative testing again ascribed the significantly enhanced (100-fold) anti-viral activity of EGCG to both trihydroxylation and galloylation of the B and C ring systems, respectively, of the molecules.

Galloylation and trihydroxylation at the C ring and B ring, respectfully, have been similarly recog-nized to explain enhanced EGCG activity against HSV-2 (Isaacs et al., 2008). Using the hepatitis C virus (HCV), Roh and Jo (2011) have reported a re-markable inhibition of HCV infectivity as determined by a reduction of the viral polymerase nonstructural protein, NS5B (an RNA dependent-RNA polymerase), following HCV treatment with EGCG. Other flavo-noids, including, but, not limited to naringenin, hesperetin, quercetin, and (-)-catechin, affected virtu-ally no anti-HCV (polymerase) activity. Similarly, EGCG concentrations at 70 to 100 µM inhibited expression of immediate-early and other Epstein-Barr virus (EBV) proteins using an EBV-positive Burkett's lymphoma cell line. Differences in flavo-noid antiviral activity in relation to other poly- phenolic structural characteristics, however, was not addressed (Chang et al., 2003).

Some two decades earlier, antiviral activity by EGCG of several human RTV and enterovirus strains not only showed a differential EGCG-mediated antiviral effect between human RTV strains and enterovirus groups, but differences in antiviral activity even between strains within the same

[enterovirus] group (Mukoyama et al., 1991). The current study complements recognized anti-[animal] virus activity by EGCG on the cellular level, demonstrating the ability of this flavonoid to directly impart an antiviral effect through the loss of RTV [VP6] antigen detection or by VP6 integrity in the cell-free assay system. Apparently, EGCG possesses broad antiviral activity on the cellular level and can directly compromise RTV capsid antigen function in the cell-free system.

Our findings indicate the unique chemical structure of EGCG (the galloylation and the trihydroxylation at the aromatic B ring) is responsible for the reduced detection levels of RTV [antigen] activity in the cell-free detection system. The apparent broad antiviral activity exhibited by green tea appears due to the abundant amounts of EGCG in the tea leaves (Henning et al., 2011) and the direct antiviral effect of EGCG on rotavirus in suspension. This observation supports the need to further address the application of this secondary plant metabolite at the organismic level.

The anti-RTV activity of EGCG in cell-free suspension prompted a comparison of this catechin isomer to the well characterized PAC extract from cranberries (*Vaccinium macrocarpon*), under identical conditions. Relatively low PAC concentrations (100 and 200 µg/mL) similar to that of EGCG, affected a significant reduction of RTV activity, as measured through the loss or compromising of RTV viral (VP6) capsid antigen. The mechanism associ- ated with the direct effect on RTV activity by PACs (in the cell-free system) is not fully understood. Electron microscopy, however, indicated the RTV viral capsid antigen was entrapped and enveloped by both PAC particulate and solubilized fractions in the water column, respectively (Lipson et al., 2011). The apparent tenacity of RTV to PACs suggests an attachment of this flavonoid to proline residues within the RTV capsid protein (Estes et al., 1984; Lipson et al., 2011).

Our proposed mechanism of PAC-RTV attachment is supported in part, by earlier PAC-protein system studies, demonstrating a preferential attachment of these PACs to proline (Charlton et al., 2002; Hagerman and Butler, 1981 Ricardo-da-Silva et al., 1991). Thus, suggesting a blockage of the V6 capsid proteins necessary for antibody attachment in the ELISA system, as well as a potential concomitant blockage of VP4/VP7 capsid [surface] antigenic determinants needed for viral attachment and subsequent infection of a host cell is not unreasonable. Direct PAC-associated antiviral activity being dependent on differences of capsid protein/amino acid composition is in agreement with other studies (Gescher et al., 2011). A marked affinity for proline-rich protein by EGCG has been described as well (Pascal, et al. 2007). On the molecular level, a hydrophobic-mediated stacking of proline side chains to specific EGCG ring molecules takes place.

The direct treatment of influenza virus by the related catechin isomer EGCG and theaflavin could affect viral aggregation/clumping and thus a reduction in hemagglutination activity and infectivity in host cell monolayer cultures (Nakayama et al., 1993). Of course, a joint or complementary effect on viral loss of infectivity by EGCG and the theaflavin in suspension on a cellular level is also possible (Imanishi et al., 2002). Viral aggregation and clumping with reduced attachment to the host cell are known to alter viral activity/infectivity (Bergey, et al., 1993; Young and Sharp, 1977).

The PAC extract in the current study was a complex of dimers, oligomers, and polymers, consisting of catechin, the isomer EC, and other flavanol units (Gescher et al., 2011; Pascal et al., 2007). Although similar in structure, catechin and epicatechin may have completely different stereochemical configurations at the C2 (trans) and C3 (cis) positions. Indeed, a synergistic or additive effect was noted by catechin and EC. High concentrations of catechin (>625 µg/mL), however, were significantly more effective than any catechin/EC complex.

Synergistic activity between flavonoid subclasses at the cellular level (cellular growth, apoptosis induction, and antiviral activity) have been reported (Amoros et al., 1992; Horie et al., 2005). Mechanistically, flavanol complex formation apparently affects discrete cellular pathways or events, such as increased caspase activity and extracellular H_2O_2 production, associated with physiological cell death (Horie et al., 2005) and/or

inhibition of the cell cycle by stopping the G0-G1 phase (Thakur et al., 2012). Although our system precluded any descriptive analysis at the cellular level, we propose that the anti-RTV activity among the flavanols is associated with steroisomerism and/or other modifications in molecule structure at the flavanol-virus interface (Niemeyer and Brodbelt, 2007; Gescher et al., 2011). An anti-RTV synergistic effect with catechin-EC complexes as compared with catechin alone, was expected, but did not occur. Indeed, an antagonistic effect between the catechin- EC complex reduced activity as compared with catechin (≥ 625 μg/mL). The evidence indicates that the combination of catechin and EC under ordinary chemical means in the cell-free system does not mimic that of anti-RTV activity seen in naturally occurring PAC molecule.

Attempts to induce a direct loss of RTV activity by using the flavanone glycosides hesperidin, naringin, and the isoflavone diosmin, proved inefficacious, suggesting that among these tested non-catechins RTV is not susceptible to direct treatment by these flavonoids. At the cellular level, antiviral activity has been reported among hesperidin, naringin, and diosmin (Amoros et al., 1992; Tang et al., 2012). The possibility also exists that the method of flavanone preparation is of significant importance, except for EGCG and, to a lesser extent PACs (Lipson et al., 2007, 2011). Flavones are generally immiscible in water-based solvents/diluents, such as the cell PBS cell culture medium.

The nonpolar nature of most flavonoids, the use of organic solvents or carrier molecules, such as DMSO, are necessary to bring these polyphenols into solution. The absence of a standardized flavonoid-alcohol or DMSO protocol and the lack of information on the alcohol/DMSO threshold levels for cell types used in cytotoxicity testing, probably explain the contradictory reports on flavanone/isoflavone antiviral activity in such infections as sindbus, dengue, and the human RTV Wa strain (Bae et al., 2000; Paredes, et al., 2003; Zandi et al., 2011).

The potential for virucidal activity also occurs when commonly used organic solvents are used to bring flavonoids to the target site. Cytotoxicity studies by Da Violante et al. (2002), observed no significant cell damage in culture monolayers of Caco-2 cells after some 2 h in medium supplemented with DMSO concentrations <10% (Da Violante et al., 2002). A DMSO concentration of 15% produced no visible cytotoxic effects on HeLa cells after a 96-h incubation period (Malinin and Perry, 1967). A recent study in which several Malasyian plant species were tested for anti-dengue virus infectivity in Vero cells used DMSO at a working strength of <1% (Tang et al., 2012). Anecdotal blogs also suggest DMSO concentration in cell culture systems should not exceed 0.1 to 0.5% (Protocol Online, 2006).

Identifying appropriate concentration of methanol and DMSO that did not prove virucidal or cytotoxic while retain a maximum flavonoid soluble working strength concentration upon dilution in PBS was relevant to our study. While methanol levels of 1.3% and 2.6% were not toxic, the concentration of 1.3% methanol proved effective in retaining naringin, hesperidin, an diosmin at 1200 μg/mL. While the naringin, hesperidin, and diosmin were shown to be soluble in DMSO, dilution of DMSO solubilized flavonoid preparations in PBS or culture medium concentrations >20% precipitated almost immedi- ately. To determine a real antiviral effect by an experimental flavonoid or other analyte, the carrier molecule must be benign. DMSO concentrations ≤ 20% proved non-toxic and non-virucidal.

Although the current study demonstrated direct anti-RTV activity at the virus-flavonoid interface with EGCG and PACs in a cell-free suspension, the data show the need for an inordinately high concentration HI PAC 4.0. The use of high flavonoid concentrations are not recommended due the potential of cytotoxicity in the in vivo system.

The inherently discrete physiochemical, molecular, and structural/functional differences among viral species, limits any broad behavioral and associated biotechnical issues for modifying virus activity, susceptibility to chemical treatments, and vaccine and/or drug development (Gerba et al., 1980; Herrmann et all, 1974; Hashiguhchi et al.,

2007; Krug and Aramini, 2009: Wigginton and Kohn, 2012).

Of course, among the large number of reported flavonoid-associated antiviral studies, some controversy could be expected. For example, the flavone glycoside hesperidin showed antiviral activity to the human RTV Wa strain, but not to Sindbis (SV) nor Enterovirus 71 (E71) and had no inhibitoroy effect on the replication of the unrelated Dengue virus (Bae et al., 2000; Paredes et al., 2003; Zandi et al., 2011). Behavorial differences (loss of activity/infectivity) among viral strains and strains within the same genus occur among hepatitis C virus (HCV) chimeric strains in association with the flavan-3-ol monomer, EGCG, and studies employing the HCV JFH1-ΔE1E2 construct have expounded the antiviral effect of EGCG through blockage of virion RNA replication (Chen et al., 2011). The same catechin subgroup failed to inhibit the HCV Luc-Je1 chimeric strain (Ciesek et al., 2011). Chen et al. (2012) have ascribed such differences in anti-HCV activity by EGCG to genetically modified strains, although differences in virus detection methodologies were also suggested. These studies indicate the need to address the effect of defined flavonoid classes, viral types/strains, and methodologies utilized in any evaluation of any plant metabolite for antiviral activity.

Our current research specifically supports the specificity or at least, the unique, direct association among flavonoid groups in animal antiviral (anti-RTV) activity. The data clearly indicate the antiviral activity of flavonoid types need to be addressed individually. The anti-RTV activity of the four catechin isomers was EGCG >>> ECG > EGC > EC. The markedly efficacious anti-RTV activity by EGCG (approaching 100%) may be ascribed to a trihydroxylation and galloylation at the B and C rings, respectively, of the core molecule, respectively.

PACs, under identical experimental conditions, affected a loss of RTV antigen of more than one order of magnitude. In general, no significant anti-RTV synergistic effect was recognized through the combination of EC and catechin PAC core components. In general, the envelopment or entrapment of RTV within colloidal PAC particulates in water, noted in the electron micrographs, may have inhibited the viral induced hemagglutination reaction. Products mandating inordinately high concentrations to promote an antiviral effect should not be considered candidates for additional testing. Possible PAC-associated loss of viral activity/antigen detection through a blockage of VP6 and probably outer capsid antigenic determinants should be considered candidate epitopes for additional testing. Further evaluation of flavonoids for antiviral activity must consider these molecules' unique structures.

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****Correction: Due to a typographical error in this published manuscript, the spelling of an author's name was corrected on June 2, 2015. The editors apologize for this error.*