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Phyllis C. Braun
Fairfield University, pcbraun@fairfield.edu

Nikki A. Calabrese

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The Effects of Tea Polyphenols on *Candida albicans*: Inhibition of Biofilm Formation and Proteasome Inactivation

Nikki A. Calabrese* and Phyllis C. Braun*

*Fairfield University, Fairfield, Connecticut, USA

Address Correspondence to:
Phyllis C. Braun. Ph.D.
Department of Biology
Fairfield University
1073 North Benson Road
Fairfield, Connecticut 06824
USA
pcbraun@mail.fairfield.edu
Telephone: 203.254.4000 x2111
Fax: 203.254.4253

ABSTRACT

The adherence of *Candida albicans* to each other and to various host and biomaterial surfaces is an important prerequisite for the colonization and pathogenesis of this organism. Cells in established biofilms exhibit different phenotypic traits and are inordinately resistant to antimicrobial agents. Recent studies have shown that black and green tea polyphenols exhibit both antimicrobial and strong cancer-preventive properties. Experiments were conducted to determine the effects of these polyphenols on *C. albicans*. Standard growth curves demonstrated a 40% reduction in the growth rate constant (K), with a 2 mg mL^{-1} concentration of Polyphenon 60, a green tea extract containing a mixture of polyphenolic compounds. Cultures treated with 1.0 μM –(–)epigallocatechin-3-gallate (EGCG), the most abundant polyphenol, displayed a 75% reduction of viable cells during biofilm formation. Established biofilms treated with EGCG were also reduced, by 80%, as determined through XTT colorimetric assays. Identical concentrations of epigallocatechin (EGC) and epicatechin-3-gallate (ECG) demonstrated similar biofilm inhibition. Further investigations regarding the possible mechanism of polyphenol action indicate that *in vivo* proteasome activity was significantly decreased when catechin-treated yeast cells were incubated with a fluorogenic peptide substrate that measured proteasomal

41 chymotrypsin-like and peptidyl-glutamyl peptide-hydrolyzing activity.
42 Impairment of proteasomal activity by tea polyphenols contributes to
43 cellular metabolic and structural disruption that expedites the inhibition of
44 biofilm formation and maintenance by *C. albicans*

INTRODUCTION

Candida species are ubiquitous fungi and the most common fungal pathogens affecting humans. *C. albicans* is the primary etiologic agent of candidiasis, a disease that can vary from superficial mucosal lesions in the immunocompetent host to systemic or disseminated infection in the immunocompromised individual (Ahern, 1978; Calderone, 2002). *Candida* infections are usually associated with biofilm formation occurring on indwelling catheters and bioprosthetic surfaces such as dental implants, heart valves, and artificial joints. Mortality due to invasive candidiasis can be as high as 40% (Crump & Collignon, 2000; Dolan, 2001; Raad, 1998; Ramage *et al*., 2002).

As a consequence, considerable attention has been given to *C. albicans*, an opportunistic yeast, and its ability to adhere to implanted inert materials. In the case of *C. albicans,* these adhesive properties may be facilitated by intrinsic cell-surface hydrophobicity. Biofilm formation proceeds through several distinct developmental phases that transform adherent blastospores to well-defined cellular communities surrounded by a mannose polysaccharide matrix (Chandra *et al*., 2001; O'Toole *et al*., 2000; Ramage *et al*., 2000). The position and quantity of surface cell wall mannoproteins contribute to the hydrophobic state of *C. albicans,* (Braun,

1999; Braun, 1994) and the initial attachment of these cells to a solid surface is followed by proliferation and biofilm formation. Many reports have demonstrated that sessile *C. albicans* cells in biofilms display characteristics that are different from those of their free-living counterparts. A paper published by Kumamoto (2005) has shown that a cellular contact-activated, signal transduction kinase, Mkc1p, accumulates in active form when *C. albicans* attach to polystyrene wells. This cellular integrity signal transduction pathway, induced by contact-dependent responses, signals biofilm development. The resultant growth of *C. albicans* biofilm greatly enhances this organism's resistance to antifungal agents and protects it from host defenses, both of which have important clinical and therapeutic implications (Baille & Douglas, 1998; Chandra *et al*., 2001; Hawser & Douglas, 1995; Ramage *et al*., 2001).

All teas derived from the dried leaf of the *Camellia sinensis* plant contain a variety of biologically active compounds, including polyphenols, methylxanthines, essential oils, proteins, vitamins, and amino acids (Balentine *et al*., 1997; Hara, 1997; Yamamoto, 1997; Yam *et al*., 1997). Green tea is manufactured with minimal processing and has the greatest concentration of polyphenolic compounds; it is the most "natural" tea. Oxidation is employed to turn green leaves brown, and in the process

produce the flavor varieties of black teas. Most of the reported biological actions of tea—including hypolipidemic, anti-inflammatory, antimicrobial, anticancer, and anti-oxidant effects—are related to the polyphenol fraction, the tea catechins (Fujiki, 1999; Hsu *et al*., 2002; Nam *et al*., 2001; Okabe *et al*., 1997). The major polyphenol components of tea are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), and epicatechin-3-gallate (EGC). Collectively, these substances constitute about 15% (dry weight) of green tea.

Proteasomes are structures found in all eukaryotic cells. The ubiquitin-proteasome enzymatic pathway functions in the cell cytoplasm and is responsible for the specific degradation of abnormal, short-lived, and regulatory proteins found in the nucleus and cytosol. Nam *et al*. (2001) reported that interference with proteasome activity *in vivo* progresses to eukaryotic cell death.

The purification and characterization of the multicatalytic complex of the *C. albicans* 20S proteasome has been elucidated previously (Fernandez-Murray *et al*., 2000). It has been reported that the *C. albicans* proteasome moderates three major proteolytic activities: chymotrypsin-like, trypsin like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities. Proteasomal enzymes are used by *C. albicans* to regulate its metabolism and respond

appropriately to environmental signals. This proteasomal process controls the temporal regulation of the type and quantity of expressed cellular proteins.

The purpose of this study was to determine the consequences of the major polyphenols of green tea (ECGC, EGC, and ECG) on the ability of *C. albicans* to grow, as well as, form and maintain a biofilm community. Further investigations were done to determine the effects of these catechins on the *in vivo* activity of *C. albicans* proteasomes.

METHODS

Organism and Conditions of Culture. *C. albicans* 4918 (Manning and Mitchell 1980) and ATCC 10231 (both serotype A, (Mercure et al. 1996) were used in the course of this study. The culture was maintained on brain heart infusion (BHI) slants at 4°C.

Effect of Polyphenon 60 on Mean Growth Rate Constant. Twenty-four hour cultures of *C. albicans* were suspended in 100 mL of Sabouraud dextrose broth (pH 6.0; SDB Difco) at a concentration of 5 $X10^4$ cells per mL. Polyphenon 60 was added to the cultures at concentration of 0.1, 1.0, 2.0 and 5.0 mg mL^{-1}. The cell cultures were incubated at 37° C (150 rpm) for 48 h. At various times sample aliquots were removed and cells were quantified using Bright-line hemocytometer (Hausser Scientific). *C. albicans* growth rates were determined mathematically as described by Madigan and Martinko (2006).

Biofilm Formation. The system of Ramage, *et al*. (2001) was used with some modification. Cells were harvested from BHI slants which were incubated for 24 h at 37°C. Cells were washed and calibrated in sterile, phosphate-buffered saline (PBS, 0.05 M, pH 7.2) and resuspended in SDB, to a cellular density equivalent to 1.0 x 10^6 cells per mL using a Bright-line hemocytometer. Biofilms were formed on presterilized, polystyrene, flat-

145 bottom, 96-well microtiter plates (Corning Inc.) Biofilms were produced by
146 pipetting standardized cell suspensions (100 μL of the 10^6 cells mL^{-1}) into
147 selected wells of the microtiter plate, finalizing volumes to 200 μL using
148 SDB, and incubating microtiter plates for 48 h at 37°C.

149 **Polyphenol Treatment.** Ten-millimolar solutions of highly purified
150 polyphenols (–)–epigallocatechin-3-gallate (EGCG, >95%), (–)–
151 epigallocatechin (EGC, >98%), and (–)–epicatechin-3-gallate (ECG, >98%)
152 (Sigma) were used. To determine the effects of these polyphenols on biofilm
153 formation, each microtiter well contained a 100 μL of yeast cell suspension
154 (1.0 x 10^6 cells mL^{-1} of SDB), 20 mM EGCG, EGC, or ECG at various
155 concentrations to a maximum of 2.5 μM. Volumes were adjusted to 200 μL
156 total volume using SDB. Biofilms were subsequently incubated for 48 h at
157 37°C, and results were analyzed by XTT reduction assay (described below).

158 The effect of polyphenols on preformed biofilms was demonstrated by
159 using 48 h biofilms, removing the media, twice washing biofilms with PBS,
160 and adding one of the polyphenols to each biofilm. Volumes were adjusted
161 to 200 μL using a sterile Basal salt solution (0.5% w/v, (NH_4) SO_4, 0.02%
162 w/v Mg SO_4, 0.5% w/v NaCl, 0.0001% w/v Biotin, 4 mM glucose). These
163 cultures were immediately incubated for 24 h at 37°C (50 rpm) before the
164 XTT-reduction assay. After biofilm formation or disruption, the medium

was removed and nonadherent cells were removed by washing biofilms three times in sterile PBS before the XTT assay.

XTT-Reduction Assay. The quantity of biofilm in each microtiter well was calculated by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay (Sigma). Briefly, XTT was prepared in a saturated solution at 0.5 g/L in PBS, aliquoted, and stored at –70°C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (Sigma; 10 mM, prepared in acetone) was added to a final concentration of 1μM. A 100-μL aliquot of the XTT-menadione solution was then added to each prewashed biofilm and to control wells (for the measurement of background XTT-reduction levels and untreated cells.) The plates were then incubated in the dark for 2 h at 37°C. A colorimetric change in the XTT-reduction assay, which relies upon the mitochondrial dehydrogenases of live cells to convert XTT-tetrazolium salt into a reduced formazan-colored product, was then measured in a microtiter plate reader (Benchmark Microplate Reader; Bio-Rad)) at 490 nm.

Formation of Protoplasts. The methods for protoplast formation have been described previously (Braun, 1999). Yeast cells grown at 37°C to the point of early exponential growth phase were centrifuged and washed twice with PBS. Cell pellets were resuspended in 35 mL of 0.5 M sodium

thioglycolate in 0.1M Tris buffer (pH 8.7) and incubated with shaking at 37°C for 30 min. The cells were centrifuged (4°C) and washed with PBS containing 0.6 M KCl as an osmotic stabilizer. The cells were then suspended in 4.5 mL of the same buffer to which 0.5 mL of β-glucuronidase (Sigma) was added. After transfer to flasks, cells were incubated with shaking (100 rpm) at 37°C for 60 min. The degree of protoplast formation was assessed microscopically for osmotic sensitivity. Preparations yielding less than 99% protoplasts were not used. Protoplasts were washed three times in PBS-KCl prior to use.

Inhibition of Proteasome Activity in Whole Cells and Protoplasts. The system of Nam *et al*. (2001) was used with some modification. Whole cells or protoplasts, 100 μL of suspension (1.0×10^6 cells mL^{-1} PBS or PBS-KCl) were placed in microtiter plates and incubated in the dark for 24 h at 37°C (50 rpm) with various concentrations of EGCG, EGC, or ECG, followed by an additional 6-h incubation period with 2μL of a 20-μM fluorogenic peptide substrate (Calbiochem) containing either Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), benzyloxycarbonyl (Z)-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity), or Z-Gly-Arg-AMC (for proteasomal trypsin-like activity). All volumes were adjusted to 200 μL using PBS-KCl prior to incubation. After

the 6-h incubation, proteasomal enzyme activity was determined by the measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a Tecan™ spectrophotometer with an excitation filter of 380 nm and an emission filter of 460 nm. Controls for these experiments included untreated cells with or without fluorogenic peptide substrate and polyphenol-treated cells with no fluorogenic peptide substrate.

Statistical Analysis. Statistical analyses were performed on repeated experiments that had multiple samples. The mean, the standard deviation of the mean, and one-way analysis of variance (ANOVA) results were used to analyze the data. When the various biofilm data were compared, a *P*-value of •0.05 was considered to indicate a significant difference between the groups.

Experimental results demonstrated no statistical differences between *C. albicans* ATCC 10231 and 4918. All data presented in this paper are the results of experiments conducted on ATCC 10231.

RESULTS

Effect of Polyphenon 60 on Growth Rate Constant (K). Initial experiments were conducted to determine whether tea polyphenols had an effect on *C. albicans* growth rate patterns. Standard 24-h growth curves were

225 performed for *C. albicans* growing in Sabouraud dextrose broth. Treated
226 cultures were incubated with various concentrations of Polyphenon 60, a
227 green tea extract containing a mixture of polyphenolic compounds to a
228 minimum of 60% total catechins. As concentrations of Polyphenon 60
229 increased, the rate of growth as expressed by the growth rate constant (K)
230 continuously decreased. At the maximum experimental concentration of 5
231 mg mL^{-1} of this extract, *C. albicans* generation time was inhibited by 43%
232 **(Table 1)**.

233 **Inhibition of *C. albicans* biofilms with tea polyphenols.** Individual
234 polyphenols were used to determine their discrete affect on the ability of
235 both strains of *C. albicans* to produce biofilms. Both strains incubated with
236 either 1.5 or 2.5 micromoles of any one of the polyphenols for 48 h
237 demonstrated a reduction of biofilm formation that averaged 72% when
238 compared to untreated control cultures **(Figure 1)**. One-way ANOVA was
239 used to analyze the data, and it was determined that the effect of each of
240 these three polyphenols was significant ($P<0.001$) in the prevention of
241 biofilm formation with EGCG more potent than EGC or ECG at 2.5 μM
242 concentrations. However, it was determined that there were no significant
243 differences ($P>0.05$) in biofilm decrease when the polyphenol solutions
244 were between 1.0 μM and 3.0 μM. Further experiments were performed

using various polyphenol concentrations, up to a maximum concentration of 3.0 mM. The amount of biofilm formation as determined by the XTT-reduction analysis was not directly proportional to catechin concentation. From 1.0 μM to 3.0 mM, biofilm formation was reduced 75%.

Established 48-h biofilms were subjected to the same polyphenols; ECGC, EGC and ECG at concentrations between 1.0 μM to 3.0 μM for 24 h. The polyphenol-treated biofilms were disrupted by as much as 60%, as determined by the XTT reduction assay **(Figure 2)**. Statistical analysis using one-way ANOVA indicated a significant difference (P<0.001) between treated and control cultures; however, the level of disruption was not concentration-dependent up to 3.0 mM. Again, EGCG produced a statistically greater disturbance to the preformed biofilms than the other two catechins.

Effect of ECGC, EGC, and ECG on Proteasomes. The effect of the three catechins on *C. albicans* 20S proteasomes was investigated. Whole cell cultures of *C. albicans* treated with one of the polyphenols for 24 h were further incubated with one of the fluorogenic substrate peptides that would test for chymotryptic, PGPH, or tryptic activity. The fluorogenic substrates were not transported across the cell walls in the untreated control cultures. However, polyphenolic treated cells contained these fluorogenic peptides in

their cytoplasm (results not shown). Subsequently, protoplasts of *C. albicans* were created and the experiments were repeated. When proteasomal chymotrypsin-like activity was investigated, we observed a 70% decrease in chymotrypsin activity in the polyphenol-treated cultures, as compared with controls **(Figure 3)**. The inhibitory effect of each of the three catechins on the proteasome was not significantly different, and there was no difference in the amount of inhibition based on polyphenol concentration. Further experiments determining the effects of polyphenols on proteasomal PGPH activity revealed that EGCG had a concentration-dependent inhibitory effect on PGPH activity, with 1.0 mM inhibiting approximately 70% of the proteasomal PGPH activity and 2.5 mM inhibiting >80% of activity **(Figure 4)**. EGC also demonstrated a concentration-dependent inhibition; however, proteasomal PGPH activity was inhibited by this catechin by only 20% and 55% at 1.0 and 2.5-mM, respectively. ECG, on the other hand, displayed a lack of concentration dependence in PGPH inhibition; both 1.0- and 2.5-mM concentrations of ECG inhibited PGPH activity by 25% when compared to control cultures. The individual addition of these three polyphenols to *C. albicans* protoplasts had no significant effect on the proteasomal trypsin-like activity (results not shown).

DISCUSSION

Biofilms are a protective environment for *C. albicans.* This may become a serious public health problem because of the increased resistance of this organism to antifungal agents and the potential to cause infections in patients with indwelling or bioprosthetic medical devices. It has been reported that *C. albicans* biofilms are not a collection of randomly dividing cells, but instead are organized communities that are dependent on the activation of signal transduction pathways (Davies *et al*., 1998; Kumamoto, 2005) and on quorum sensing abilities (Braun, 2005; Ramage *et al*., 2002) These strategies for biofilm growth and development benefit the *C. albicans* community by preventing and controlling overpopulation and competition for nutrients. These conditions may be crucial for *C. albicans* dissemination and the establishment of infection at distal sites.

The present study demonstrates that tea polyphenols substantially causes metabolic instability to *C. albicans* cultures. The catechins are shown to retard the growth and the ability to form and maintain biofilms by *C. albicans*. Once a biofilm has been established, micromolar concentrations of all three tested polyphenolic compounds, EGCG, EGC, and ECG, were able to disrupt a preformed biofilm community within a 24-hour period as determined by the mitochondria-dependent XTT reduction assay. EGCG

was found to be the most potent of the three catechins. These results were not strain-specific and the outcome of all our experiments conducted on *C. albicans* 4918 and ATCC 10231 demonstrated no statistically significant differences.

To determine a site of action of the tea polyphenols on *C. albicans,* we investigated the possibility of proteasome activity disruption. Nam *et.al.* (2001) report that the same polyphenols destroyed the enzymatic activity of proteasomes in intact human tumor cells, contributing to the death of these cells. Although accumulating evidence indicates that tumor and normal cells behave differently in response to proteasome inhibition there is no well-defined mechanism to explain tumor cell susceptibility and normal cell resistance to proteasome inhibitors. A comparison of proteasome inhibition in normal and cancer cells was reviewed by Yang *et al*. (2008). In that report, many different types of proteasome inhibitors are described, which include several natural compounds such as tea, grape, and soy polyphenols, as well as Bortezomib, a drug approved by the US Food and Drug Administration (FDA). Although no well define mechanism has been elucidated to explain the differences in proteasome susceptibility of cancer cells by these inhibitors, it has been suggested that the suppression of cancer cell proteasome activity results in the abnormal accumulation of

transcriptional factor NF-κB and cyclin-dependent kinase inhibitors (CKI). Increased cellular CKI has been found to arrest cell cycle progression and in cancer cells stimulate apoptotic activity. Recently, CKI have been reported to regulate morphogenesis in *C. albicans* (Sinha *et al*., 2007) and it is very possible that the accumulation of CKI may disrupt morphogenesis and cell cycle activities in this organism as well.

The results of our experiments on *C. albicans* demonstrate suppressed proteasomal enzyme activity at similar tested concentrations as those used on human cancer cells. Our study suggests that the metabolic instability produced by the catechin-induced proteasome inactivation was a contributor to the decrease in the growth rate constant as well as biofilm formation and maintenance. Our experiments also suggest that other target sites of the polyphenols may also involve the cell membrane, cell wall, or perhaps both. In our attempt to use whole cells of *C. albicans* to perform these experiments, we found that the untreated controls were not able to transport the fluorogenic peptide substrates, but the 24 h polyphenol treated cells could. This observation suggests that incubation of *C. albicans* with any one of the three catechins either permeabilized the cell membrane or disrupted enough of the cell wall structure to allow the transport of the peptide substrates into the cell. The capacity of the polyphenols to cause outer cell

345 structure disruption contributes to their abilities to enter *C. albicans* and
346 exert intracellular antifungal activities. Further investigation is needed to
347 determine the nature of this catechin-induced cell disorganization.

348 Greater concentrations of the polyphenols were necessary to inhibit
349 proteasome activity than were needed to inhibit biofilm formation or disrupt
350 established biofilm structure. As others reported, (Nam *et al*., 2001) greater
351 concentrations of proteasome inhibitors might be needed to reach their *in*
352 *vivo* cellular target, the proteasome. Also, it is very possible that the
353 fluorogenic peptide assay was not sensitive enough to detect minor
354 proteasome disturbances induced by the lower concentrations of the
355 catechins that prevented biofilm formation and support.

356 To consider the possible antifungal effect of the tea polyphenols on *C.*
357 *albicans*, one must consider the concentrations of these molecules, which are
358 found physiologically in tea drinkers. Previous studies indicate that EGCG
359 or other catechins are present in low (1-10) micromolar ranges in the plasma
360 and saliva of human volunteers (Yang, 1999; Yang *et al*., 1999). Our
361 experiments also found that physiological polyphenol concentrations as low
362 as 1.0 μM significantly inhibited biofilm formation and disrupted established
363 biofilms. What also needs to be taken into consideration is the enhanced

antifungal effect on *C. albicans* of a sustained concentration of these polyphenols over time in tea-drinking individuals.

In summary, we provide evidence demonstrating that physiologic concentrations of polyphenols not only prevent biofilm formation, but also reduce the yeast population of established organized communities. Our results indicate that the green tea polyphenols significantly decreased the ability of *C. albicans* to grow and sustain biofilms. Further investigation on the possible effect these polyphenols had on *C. albicans* metabolism implicated proteasome involvement. It was determined that EGCG, EGC, and ECG polyphenols inhibited *C. albicans* proteasomal chymotrypsin-like and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities *in vivo.* The tea catechins also may affect other metabolic pathways besides proteasome disruption in *C. albicans* preventing the normal signals for growth and development.

Biofilm infections are an increasingly formidable problem, and often represent a therapeutic challenge because of their intrinsic resistance to conventional antifungal therapy. New treatment modalities to combat these infections are needed. Tea polyphenolic compounds are novel substances that have the potential to be used either alone or in combination with other

antifungal drugs to combat *C. albicans* infections. Further investigations are warranted.

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Legends

Table 1: Effect of Polyphenon 60 on Mean Growth Rate Constant (K).

C. albicans 4918 and ATCC 10231 cultures were grown for 24 h in Sabouraud dextrose broth containing various concentrations of Polyphenon 60. Values represent the average of 5 experimental growth curves. Data were analyzed using a Student's *t* test. When growth comparisons were determined, a *P*-value less than 0.05 was considered to indicate a significant difference.

Figure 1: Effect of Polyphenols on Biofilm Formation.

The effect of various concentrations (1μM to 3 μM) of EGCG**,** EGC, and ECG was investigated to determine biofilm inhibition after a 48-h incubation period. Values represent the average of eight replications from several separate experiments (n=48). ANOVA test was used to analyze the data. Error bars represent the standard deviation.

Figure 2: Effect of Polyphenols on Preformed Biofilms.

The disruptive effect of various concentrations (1μM to 3 μM) of EGCG**,** EGC, and ECG was investigated on established 48-h biofilms. Values represent the average of eight replications from several separate experiments

(n=48). ANOVA test was used to analyze the data. Error bars represent the standard deviation.

Figure 3: Specific Inhibition of Proteasome Chymotrypsin-Like Activity by Polyphenols in *C. albicans* Protoplasts. The graph represents the inhibitory effect of 1 mM and 2.5 mM of EGCG, EGC, and ECG on proteasome activity as determined by a decrease in Suc-Leu-Leu-Val-Tyr-AMC hydrolysis. Values represent the average of quadruple samples from several experiments (n=16). ANOVA test was used to analyze the data. Error bars represent the standard deviation.

Figure 4: Specific Inhibition of Proteasome PGPH Activity by Polyphenols in *C. albicans* Protoplasts. The graph represents the inhibitory effect of 1 mM and 2.5 mM of EGCG, EGC, and ECG on proteasome activity as determined by a decrease in benzyloxycarbonyl (Z)-Leu-Leu-Glu-AMC hydrolysis. Values represent the average of quadruple samples from several experiments (n=16). ANOVA test was used to analyze the data. Error bars represent the standard deviation.

Table 1: Effect of Polyphenon 60 on Mean Growth Rate Constant (K)

Polyphenon 60 (mg/mL)	Growth Rate Constant (K)	% Decrease
0	0.968	—
0.1	0.754	22
1.0	0.722	25
2.0	0.577	40
5.0	0.549	43

563 **Figure 1**
564

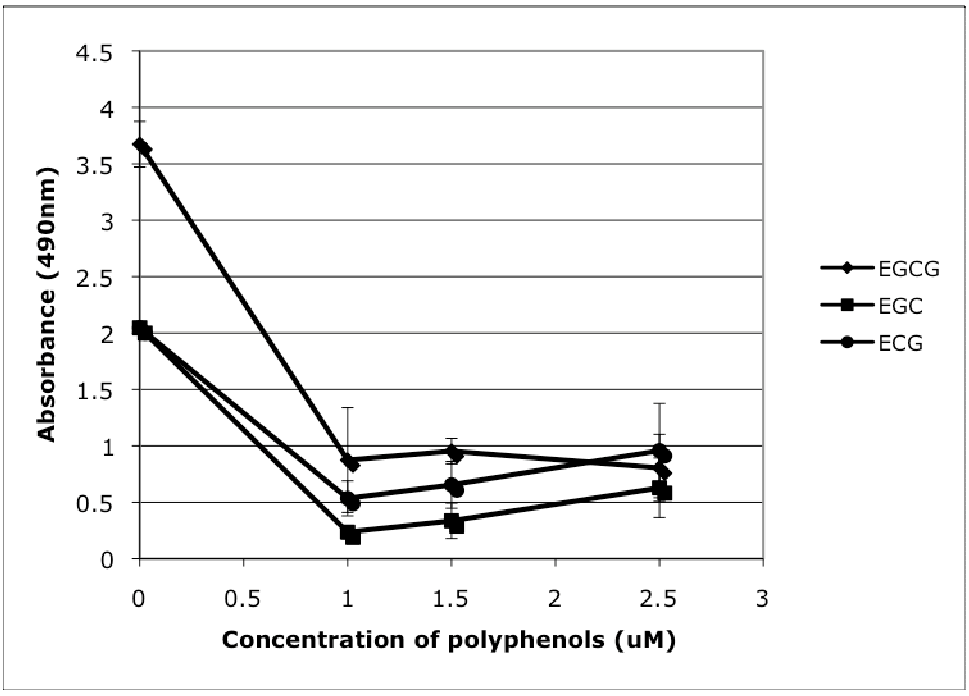

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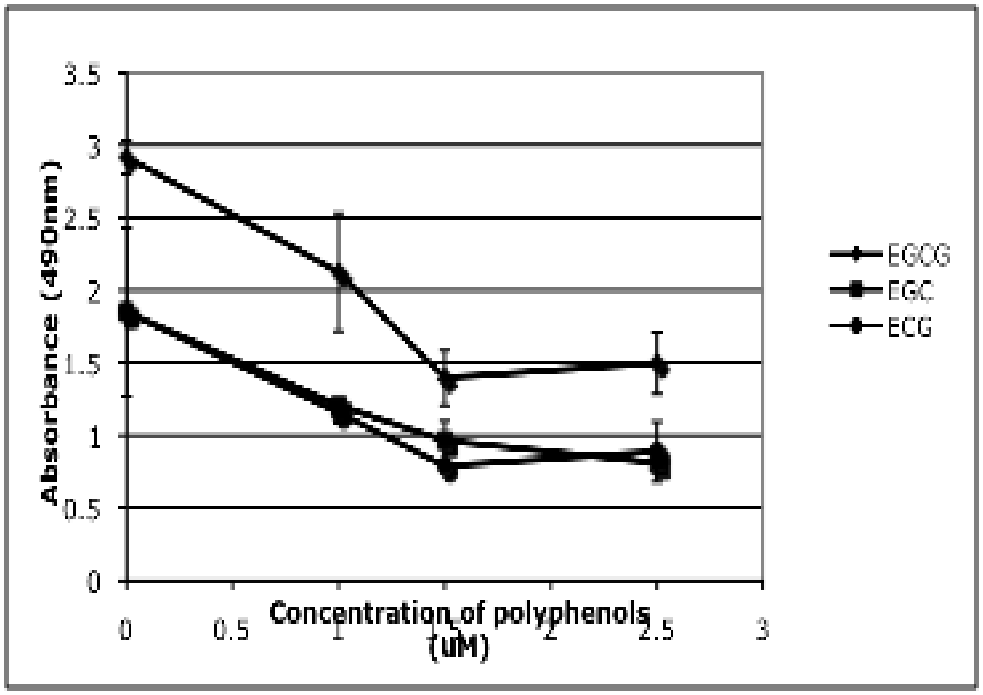
Figure 2


Absorbance (490nm)
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
2
2.5
3
Concentration of polyphenols (uM)
EGCG
EGC
ECG

573 **Figure 3**
574

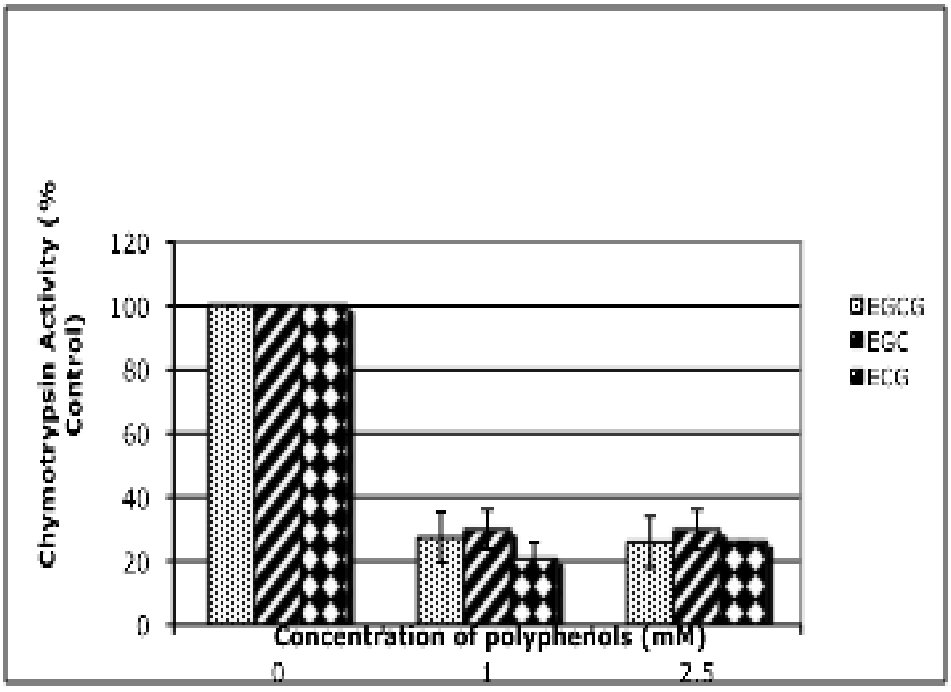


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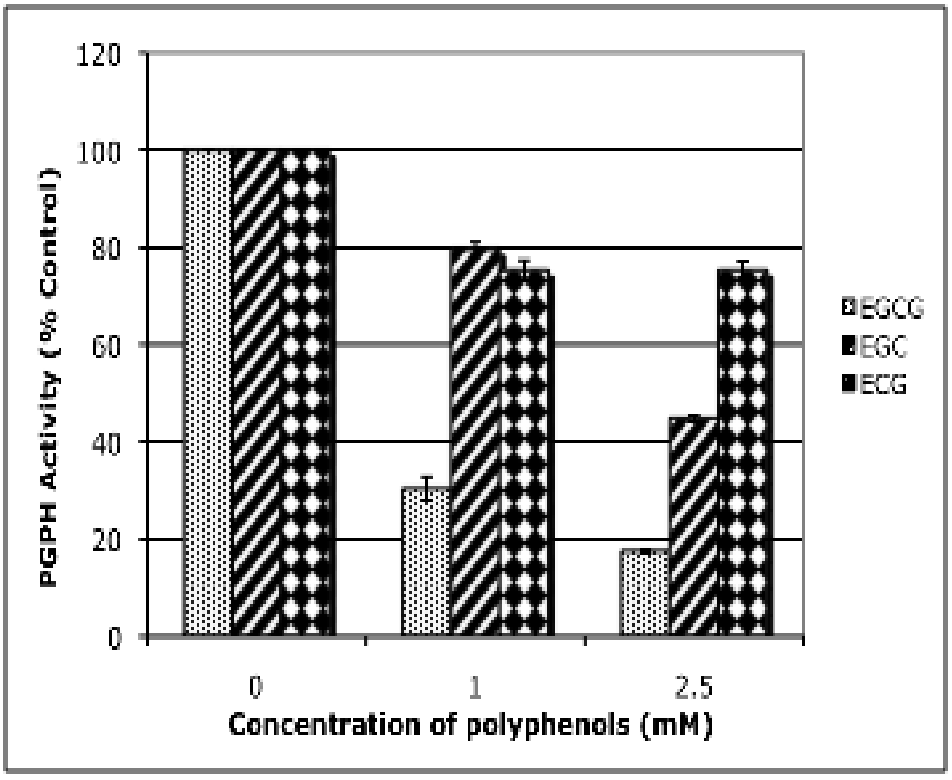
Figure 4


120
100
80
60
40
20
0
PGPH Activity (% Control)
0
1
2.5
Concentration of polyphenols (mM)
EGCG
EGC
ECG