

1 **Correlation between catechin content and NF-κB inhibition by infusions of black and green tea**

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8 **Short title:** Tea extracts as modulators of gastric inflammation

9 **Keywords:** *Camellia sinensis* L., Nuclear Factor-κB, antioxidant, radical scavenger, black and green
10 tea, catechins

11 **Abstract**

12 This study investigates whether infusions of green and black tea inhibit the NF-κB driven transcription in
13 human epithelial gastric AGS cells. Water extracts were prepared from different brands of green and
14 black tea available on the Italian market. Teas with or without caffeine were studied. An industrially
15 prepared freeze-dried water extract of green tea was also tested. Catechin and caffeine contents were
16 measured by HPLC analysis. The decrease in phenol and catechin content 3 months after the expiry date
17 was also investigated. The NF-κB driven transcription and the free radical scavenger activity were
18 inhibited, and this effect was related to catechin levels. The potency of epigallocatechin 3-gallate in
19 inhibiting NF-κB driven transcription is so great that tea extracts low in epigallocatechin 3-gallate are still
20 highly active. In one decaffeinated sample of green tea, the phenol and catechin content was very low,
21 probably as a consequence of caffeine removal. The decrease in catechin levels after 3 months did not
22 reduce the inhibition of NF-κB driven transcription by tea infusions. This is the first paper reporting the
23 inhibitory effect of NF-κB of commercial green and black infusions at the gastric level, evaluating their
24 stability as well.

25 **Introduction**

26 Tea obtained from the dried leaves of *Camellia sinensis* L. (family Theaceae) is one of the most widely
27 consumed beverages in the world, apart from water. Among teas produced and consumed in the world,
28 approximately 78% are black, 20% are green, and 2% are oolong teas [1]. The consumption of green tea
29 (GT) has increased exponentially in recent years because of its claimed beneficial effects on health. Black
30 tea (BT) is also valued for its fragrance produced by the fermentative process, which the leaves undergo.
31 GT contains many bioactive compounds and is particularly rich in flavonoids, including catechins and
32 their derivatives, which may constitute up to 30 % of the dry weight. Epigallocatechin 3-gallate (EGCg)
33 is the most abundant catechin in GT, representing around 60% of the total catechin content. *In vivo*
34 evidence of the health benefits of GT consumption include reduction of blood pressure [2-4] and serum
35 cholesterol [5, 6], protecting low density lipoprotein (LDL) from oxidation [7, 8], increased antioxidant
36 and free radical scavenger activity [9], and decreased risk of cardiovascular diseases and cancer. For
37 reviews on these topics, see [10-12]. A systematic review [13] on plant food supplements evaluated
38 twenty-nine human intervention studies of the anti-inflammatory effects of GT and BT infusions, but the
39 conclusions were contradictory. In addition, impact on polyphenol concentration in faeces, blood, and
40 urine after green and black tea consumption by humans has been reported [14]. Among Western
41 populations, dyspeptic disorders including gastritis and peptic ulcer are widespread and the consumption
42 of tea has been suggested for both prevention and treatment of the associated symptoms. Gastritis and
43 ulcer are often due to infection with *Helicobacter pylori* (*H. pylori*) [15], which is a serious public health
44 concern – the WHO classifies this bacterium as a Type 1 carcinogen. Inflammation caused by the
45 bacterial infection, chemical injury or stress, is associated with a massive production of cytokines by
46 immune cells (i.e. tumour necrosis factor alpha: TNF α) and gastric epithelium (i.e. IL-8); this response
47 depends on activation of NF- κ B (Nuclear Factor-kappaB), a critical regulator of genes involved in
48 inflammation, cell proliferation, and apoptosis.

49 Some studies have reported a positive effect of cold water extract or hydroalcoholic extract from green
50 tea (GT) or black tea (BT) on gastric inflammation [16], ethanol-induced gastric mucosal injury [17] and
51 the inhibition of *H. pylori* growth *in vitro* and *in vivo* [18-20]. Although several papers have studied the

52 composition of green tea as infusions, the most widely used form of consumption of green and black teas
53 [21], until now no studies have been evaluated the effect of this preparation on NF- κ B driven
54 transcription. This study investigated whether infusions of GT and BT inhibit NF- κ B driven transcription
55 in the human epithelial gastric cell line. Because different forms of commercial production of GT and BT
56 affect the content of active principles in the final herbal preparation, we investigated whether different
57 forms would affect NF- κ B activity differently. Infusions were therefore prepared of different brands of
58 green and black tea, with or without caffeine, available on the Italian market. For comparison, an
59 industrially prepared freeze-dried aqueous extract of green tea (GTE) was also tested. For each sample
60 catechin and caffeine content was evaluated by HPLC, as well as the content of phenols and catechin 3
61 months after the expiry date. Inhibition of NF- κ B driven transcription and free radical scavenger activity
62 were investigated and correlated with catechin levels.

63 **Materials and methods**

64 *Reagents*

65 Methanol and water were purchased from Romil Pure Chemistry (Cambridge, UK); formic acid and
66 Folin-Ciocalteu reagent were supplied by Merck (Darmstadt, Germany) and acetonitrile by VWR
67 International (Geldenaaksebaan, Leuven, Belgium). Pure standards EGCg, epicatechin (EC), epicatechin
68 gallate (ECg), caffeine, gallic acid and all the reagents for cell culture, including the culture medium
69 Dulbecco's modified Eagle's medium F12 (DMEM F12), and trypsin, were supplied by Sigma Aldrich
70 (Milan, Italy). Penicillin, streptomycin, and L-glutamine were from Gibco (Grand Island, NY). All
71 reagents used for the analytical determinations and for the biological assays were of HPLC purity grade.

72 *Preparation of green and black tea infusions and percent recovery*

73 Batches of brands of GT and BT with different expiry dates were purchased from various drugstores in
74 Italy. Four GT, three decaffeinated green tea infusions (GTID), two BT and one decaffeinated black tea
75 (BTID) were included. For each brand, one sachet was chosen randomly, and the content of each sachet
76 was infused for 3 minutes with deionised boiling water in the ratio herb/water 1.5g/200 mL. The solution

77 was then cooled, filtered through a 0.45- μ m Millipore filter (Billerica, Massachusetts, USA) and
78 lyophilized. The % recovery (w/w) was calculated. Sample were then stored at -20°C until the analysis.
79 GTE was provided by Martin Bauer Group (Martin Bauer GmbH & Co, Vestenbergsgreuth, Germany).

80 *Analysis of tea infusions by HPLC-UV*

81 The HPLC for quantitative analysis used two pumps PU-1580, the UV detector 975-UV, an autosampler
82 AS-2059 plus (Jasco Tokyo, Japan) and a column of pentafluorophenyl reverse-phase kinetex 2.6 μ m,
83 100 Å, 100 x 4.6 mm (Phenomenex, Torrance, CA, USA). Data were analysed by Borwin software
84 (Jasco, Tokyo, Japan).

85 The linear gradient elution for GT used mobile phases of water:formic acid (99.5:0.5 v/v) (phase A) and
86 acetonitrile:formic acid (99.5:0.5 v/v) (phase B). The gradient program was: 0-25 min, from 96 to 83% A;
87 25-30 min, from 83 to 25% A; 30-35 min, 25% A; 35-40 min, from 25 to 96% A. To optimize the
88 separation of catechins, the gradient for BT analysis was slightly different: 0-25 min, from 96 to 85% A;
89 25-30 min, from 85 to 30% A; 30-35 min, from 30 to 25% A; 35-40 min, from 25 to 96% A. Sample
90 injection volume was 20 μ L. Flow rate was 0.8 mL/min and the post-run time 10 minutes. The UV
91 detector was set at 280 nm. The content of caffeine and catechins (EGCg, EC and ECg) was measured.
92 Peaks were identified by comparison of retention times with those of pure reference compounds: stock
93 solutions of standards in methanol were sonicated for 1 min before injection and calibration curves were
94 obtained by injecting 40-2000 ng catechins and caffeine. The calibration curves were linear ($R^2 > 0.990$).

95 Total phenol content was measured by the Folin-Ciocalteu colorimetric assay using a Cary 50 Scan UV-
96 VIS spectrophotometer (Varian, Palo Alto, CA, USA) and expressed as mg of gallic acid equivalents
97 (GAE)/g of dry drug weight. The polyphenol concentration in samples was calculated using a standard
98 curve of gallic acid ranging from 1 to 50 μ g/mL ($R^2 = 0.990$).

99 *Stability of catechins in tea infusions*

00 GT and BT sachets were stored in the original packaging at 25°C in the dark. GT and BT were infused
01 and catechin content was measured at month 0 (corresponding to the expiry date) and 3 months later.

02 *DPPH free radical scavenging assay*

03 The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was applied as previously
04 described [1, 22], with slight modifications. Aliquots of 0.5 mL of tea infusions or extract were added to 1
05 mL of DPPH (5 mg/100 mL methanol) and after 30 min at room temperature the absorbance of the
06 solution at 517 nm was measured using methanol as blank. As negative control, 0.5 mL methanol was
07 added to 1 mL DPPH solution. The degree of free radical scavenging was expressed as mg of gallic acid
08 equivalents (GAE)/g dry drug. The calibration equation for gallic acid was $y = -0.1275x + 0.685$ ($R^2 =$
09 0.997).

10 *Cell culture and NF- κ B assay*

11 Human adenocarcinoma cells AGS (ATCC, Manassas, VA, USA) were grown at 37°C in DMEM F12
12 supplemented with 100 units penicillin/mL, 100 mg streptomycin/mL, and 10% heat-inactivated fetal calf
13 serum (FCS) (Euroclone SpA, Pero, Italy), in a humidified atmosphere containing 5% CO₂. At
14 confluence, cells were plated in 24-well plates (30,000 cells/well) and transfected after 48 h by the
15 calcium-phosphate method with a plasmid containing the luciferase reporter gene under the control of
16 NF- κ B promoter [23]. Cells were placed in a medium deprived of FCS, and treated for 6 h with the
17 compounds and TNF α 10 ng/mL as pro-inflammatory stimulus. GT and BT infusions were tested at 1-25
18 μ g/mL, GTE at 0.1-10 μ g/mL and the individual pure compounds at 0.1-10 μ M. After 6 h cells were
19 harvested and a luciferase assay was applied using Britelite™ Plus reagent (PerkinElmer Inc.
20 Massachusetts, USA) according to the manufacturer's instructions. Parthenolide was used as reference
21 inhibitor of NF- κ B driven transcription (-85% at 10 μ M) [24].

22 *Cytotoxicity assay*

23 The integrity of the cell morphology before and after treatment was assessed by light microscopy. Cell
24 viability was measured by MTT [25] and Trypan Blue methods. No sign of cytotoxicity was observed in
25 cells treated with infusions/extract (0.1-25 μ g/mL) or individual compounds (0.1-10 μ M).

26 *Statistical analysis*

27 The results are the mean \pm sd of at least three experiments performed in triplicate. IC₅₀s were calculated
28 with GraphPad Prism 4 software. The significance was set at $p < 0.05$ and was calculated using one-way
29 analysis of variance followed by Bonferroni's post hoc test.

30 **Results and Discussion**

31 *Composition of tea infusions*

32 The recovery of both green and black tea infusions (GTI and BTI, respectively) ranged from 20.6 to 32.5
33 %, calculated on the dried weight, with no significant difference between GT and BT (25.4 ± 5.24 and
34 24.7 ± 2.15 , respectively; $p = 0.83$). Table 1 shows the content of total phenols, catechins and caffeine.
35 There was good linear correlation between total catechins and total phenol content ($r = 0.86$). Fig. 1 shows
36 the HPLC profile of a GT (A) and BT infusions (B). Peaks 1, 2, 3 and 4 were identified as EGCg, ECg ,
37 EC and caffeine respectively; quantities are reported in Table 1. GT contained far more catechins than BT
38 (mean: 60.2 vs. 7.1 mg/g, respectively), whereas caffeine content was not significantly different. Green
39 tea is not fermented as black tea is, so that polyphenol oxidase, which degrades catechins, is not
40 developed. This in part explains the higher catechin content in GTE than in BTE [26]. However,
41 geographical origin, harvesting time, and quality of herb could also affect the abundance of catechins and
42 other phenols. As expected [27, 28]. EC was not detectable in BT infusions. Caffeine ranged from 29.4 to
43 47.6 mg/g (Table 1) in both GTI and BTI samples, but was undetectable in decaffeinated samples, as
44 expected. One of the decaffeinated teas (GTID-1) showed a total phenol and catechin content 6- to 10-
45 fold lower than other GTIs, possibly ascribable to the decaffeinating method used [29]. GTE showed the
46 highest content of phenols, catechins and caffeine.

47 *Effect of tea infusions and pure compounds on NF- κ B driven transcription in AGS cells*

48 All the infusions exhibited a concentration-dependent inhibition of the NF- κ B driven transcription that
49 had been induced by TNF α . IC₅₀ ranged from 2.47 to 18.9 μ g/mL (Table 2). Infusions from black tea
50 inhibited the transcription less powerfully than those from green tea (5.14-11.8 vs. 2.47-4.31 μ g/ml,
51 respectively). GTE was the most active (IC₅₀: 0.98 μ g/ml). Among the catechins, EGCg showed the

52 highest activity on the NF- κ B driven transcription induced by TNF α (IC₅₀: 146 \pm 32 nM, mean \pm sd).
53 ECG showed 30% inhibition at 10 μ M, whereas EC and caffeine, at this concentration, were inactive.
54 When incubated without TNF α , the extracts/individual compounds showed no inhibition of the NF- κ B
55 driven transcription; this excludes a direct effect of the extracts on the target. Green and black tea both
56 inhibit TNF α -induced NF- κ B driven transcription *in vitro* and the effect on the inflammatory cellular
57 response correlated closely with their total catechin content; correlation coefficient r was 0.84, thus
58 indicating a strong relationship between catechin content and inhibition of NF- κ B driven transcription
59 (additional data are given in online resource 1). Because the potency of EGCg in inhibiting NF- κ B driven
60 transcription is so high, tea extracts even with low content of EGCg (i.e. BTI) are still very active. The
61 EGCg content in 25 μ g/ml tea extracts ranged from 0.37 to 3.10 μ M for green tea and from 0.15 to 0.41
62 μ M for black tea. Taking into consideration its IC₅₀ on NF- κ B activity (0.146 μ M), it is apparent that
63 EGCg is the major component responsible for the overall *in vitro* effect of the extracts. If the IC₅₀ are
64 expressed per total catechin content, black teas exhibit IC₅₀ from 4 to 5 times lower than green tea
65 infusions. One possibility to explain this observation is that the relationship between catechin content and
66 NF- κ B inhibition is linear at lower concentrations whereas, at higher concentrations, inhibitory activity is
67 not linear. Although the *in vivo* relevance of the present findings needs to be further investigated, the
68 inhibitory effect on NF- κ B *in vitro* strongly suggests that green and black tea infusions could help to
69 prevent/attenuate gastric inflammation.

70 *Free radical scavenging activity of green and black tea*

71 It has been extensively reported that inflammation leads to a massive production of reactive oxygen
72 species (ROS), including H₂O₂, and the oxidation of NF- κ B by ROS inhibits its DNA-binding ability
73 [30]. Table 2 shows that green tea infusions have high free radical scavenging capacity, whereas that of
74 black tea infusions is four-fold lower. Correlation between antioxidant activity and catechin content was
75 significant (r=0.56) but not as high as correlation between catechin content and NF- κ B inhibition

76 (additional data are given in online resource 2). This observation indicates that other compounds, in
77 addition to catechins, contribute to the antioxidant activity of infusions.

78 It has been documented that green teas show strong antioxidant activity [31]; EGCg is an effective free
79 radical scavenger at concentrations of 1-10 μ M, which are easily reached in GT infusions [32, 33]. GTE
80 showed the most efficient radical scavenger properties, probably because of its higher content of
81 antioxidants.

82 *Stability of catechins in green tea*

83 These experiments were performed to assess the stability of catechins in sachets of green tea kept beyond
84 the expiry date declared by the seller, and whether the decrease in catechin content would affect its
85 scavenger activity. Fig 2 shows the results. Three months beyond the expiry date, the amount of catechins
86 in GTI-1 was 37% lower whereas in the decaffeinated sample (GTID-2) the difference was almost
87 negligible (-6%). The stability of catechins in tea (either in bulk or in sachets) has rarely been
88 investigated. Friedman et al. reported [34] that the average overall loss of total catechin content in eight
89 teas commercially available was 32% after 2 months' storage, and no further loss was seen after 4 months;
90 however, the study did not consider samples stored beyond the expiry date. The antioxidant activity did
91 decrease in both the expired samples (-62% and -51% for GT-1 and GTD-2, respectively), independently
92 of the catechin levels, indicating that other compounds contribute to the antioxidant activity. The effect of
93 green tea on NF- κ B driven transcription did not change with aging: IC₅₀ values 2.91 and 3.22 for GTI at
94 M0 and M3 respectively; 2.97 and 2.37 for GTID at M0 and M3, respectively. For each sample, IC₅₀
95 values were not statistically different, which suggests that EGCg still had the concentration necessary to
96 affect NF- κ B activity. However, in GTI-1 the decrease of catechin content was associated with a slightly,
97 though not significantly, higher IC₅₀.

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:01 <http://www.plantlibra.eu>). This report does not necessarily reflect the Commission's views or its future
:02 policy on this area.

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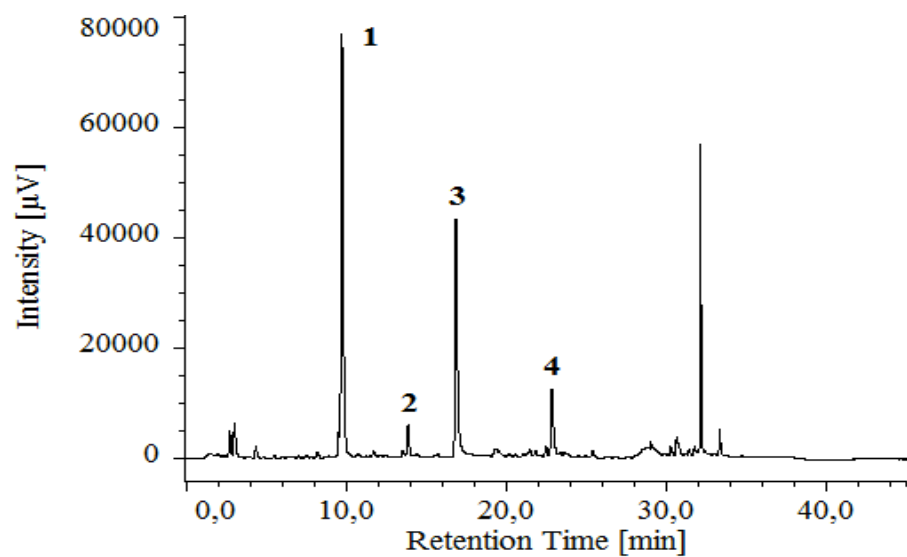
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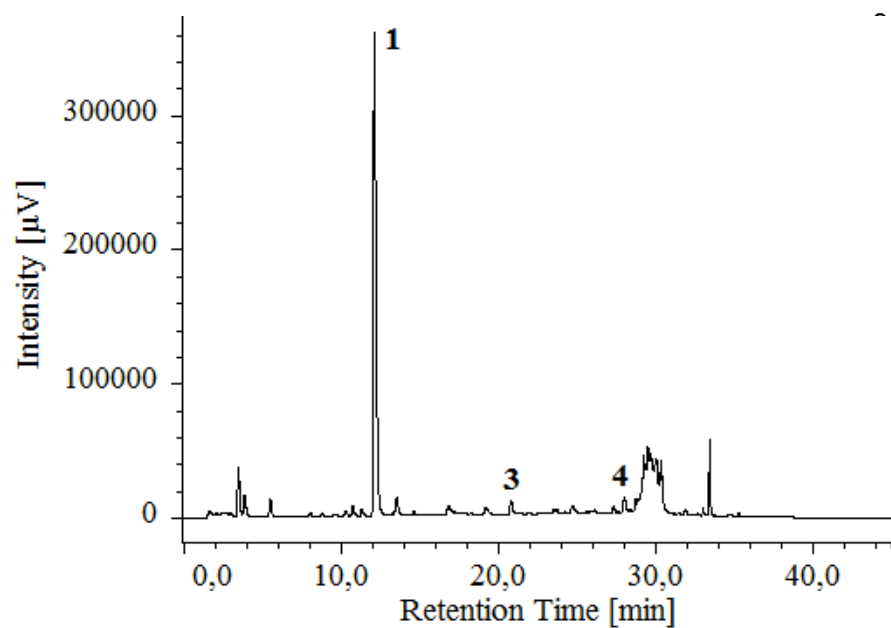
9 **A**

Figure 1

290



1 **B**



2

3 **Figure 1:** Representative HPLC profile of green (GT-1, panel A) and black tea (BT-2, panel B)

4 infusions. Peaks are caffeine (1), EC (2), EGCg (3) and ECg (4). Different gradients were used for

5 A and B, as described in the Materials and Methods section.