# The effects of tetrahydrocurcumin compared to curcuminoids on human platelet aggregation and blood coagulation *in vitro*

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Curcuminoids (diferuloylmethane [curcumin], demethoxycurcumin [DMC], and bisdemethoxycurcumin [BDMC]) are bioactive components of the spice, turmeric, that are believed to have anti-inflammatory, anti-oxidant, anti-carcinogenic and anti-thrombotic properties [1]. The most convincing evidence for a therapeutic role for curcumin comes from studies showing its ability to inhibit platelet aggregation *in vitro* [2]. Furthermore, a single study has shown remarkable effects on coagulation *in vitro*, with both curcumin and BDMC (at concentrations of 50 μM) able to prolong prothrombin time (PT) and activated partial thromboplastin time (APTT) by 2-3 fold [3]. However, these doses are often unable to be achieved *in vivo*, as curcumin exhibits notoriously low bioavailability; with poor solubility, low absorption, rapid metabolism and efficient elimination [4]. This has led to tetrahydrocurcumin (THC) becoming the focus of investigation, as the major plasma metabolite of curcumin, it is more soluble at physiological pH exhibits a longer half-life in plasma at 37$^{o}$C, and has demonstrated higher antioxidant, anti-inflammatory and anti-cancer activity in some biological settings [4]. However, the potential effects of THC on the coagulation system or platelet aggregation have not been investigated.

With widespread availability and consumption of turmeric supplements, any potential anticoagulant and/or antiplatelet effects of curcuminoids could have profound effects if consumed in combination with antithrombotic medications prescribed for these purposes. Alternatively, any confirmed pharmacologic features could be exploited for therapeutic use. Thus we tested the effects of curcumin, DMC, BDMC, and THC on platelet aggregation and coagulation *in vitro*.

Blood was collected from healthy adult donors after receiving written informed consent. Anyone regularly taking acetylsalicylic acid, nonsteroidal anti-inflammatory drugs, curcumin supplements or who had consumed curcumin containing foods within the past 24 h was excluded. The study was approved by the Hunter New England Research Ethics Committee.

Powdered (≥98% pure by HPLC) curcumin, DMC, BDMC and THC were purchased from Sigma-Aldrich (St Louis, MI, USA). Stock solutions of 50 mM (equivalent to 15 – 19 mg/ml) were prepared in dimethyl sulfoxide (DMSO).

Whole blood platelet aggregation was performed on individual blood samples (collected in >15 μg/ml hirudin) from 4 different healthy volunteers. Stock solutions of individual curcumin components (or DMSO as a control) were added directly to aliquots of each whole blood (1/250 dilution) before being mixed 1:1 with saline for a final concentration of 100 μM of curcumin components and 0.2% DMSO. After a 5 min incubation at 37°C, 20 μl of agonist (Collagen Reagent, Helena Laboratories, Beaumont, Texas, USA; ASPItest Reagent or ADPtest Reagent, Roche Diagnostics, GmbH, Mannheim, Germany) was added to each 600 μl test for final concentrations of 3.3 μg/ml collagen, 500 μM arachidonic acid (AA), and 6.7 μM adenosine diphosphate (ADP), respectively. Aggregation was recorded by impedance aggregometry on a Multiplate 5.0 analyser (Verum Diagnostica GmbH, Munich, Germany). All tests were run in duplicate.

Coagulation tests were performed using commercially purchased standard human plasma (Siemens Healthcare Diagnostics, Marburg, Germany) or freshly prepared normal pooled

plasma, spiked with various concentrations of individual curcuminoids as indicated. Pooled plasma was prepared from peripheral blood samples collected into 3.2% trisodium citrate from ≥4 healthy donors following separation of the plasma from the cellular fraction by centrifugation at 2100*g* for 10 min at room temperature.

Global coagulation tests were performed on a Sysmex CS-2000i (Sysmex Corporation, Chuo-Ku, Kobe, Japan) using Innovin (PT) and Actin FS (APTT) reagents from Siemens Healthcare Diagnostics (Marburg, Germany), and on a STA Compact (Diagnostica Stago, Asnières-sur-Seine, France) using Neoplastin (PT) from Diagnostica Stago and Actin FS (APTT) from Siemens Healthcare Diagnostics. Both normal and abnormal controls were performed with each set of tests.

The percent (%) change in platelet aggregation compared to the diluent DMSO control were assessed by ANOVA and post hoc Tukey tests. Differences in PT and APTT results for each curcuminoid compared to DMSO control was assessed by Wilcoxon matched pairs signed rank test. All calculations were performed using Statistica v10.0 (StatSoft, Tulsa, OK, USA) or STATA v11 (StataCorp LLC, College Station, Texas, USA) using two-tailed tests with p-values $<0.05$ considered statistically significant.

Baseline platelet aggregation results were all within normal range for the 4 individual participants tested, with average area under the curve values of 92.0 ± 3.7 U for platelet aggregation induced by collagen, 92.5 ± 13.4 U for AA, and 63.7 ± 5.0 U for ADP (data not shown). Reaction to all curcuminoids was highly variable between individuals as illustrated by the large standard deviations in **Figure 1**. All curcuminoids with the exception of

curcumin were able to reduce platelet aggregation induced by AA, with THC being the most potent (-67.1 ± 13.3%, p=0.0004). Curcumin itself significantly increased aggregation by ADP (42.9 ± 9.0%, p=0.0002), but there was no significant effect on platelet aggregation induced by collagen.

Initial investigations of the effects of curcuminoids on coagulation were performed using 50 μM of curcuminoids based on published literature [3]. However, in our experiments there was no effect on PT or APTT when this amount was spiked into standard human or pooled plasma (**Table 1**). We considered that the colour of the plasma, which became quite dark when spiked with the pigmented curcuminoids, may be interfering with optical clot detection on the Sysmex CS-2000i. In order to circumvent this issue, a Stago STA Compact with mechanical clot detection was used to repeat the experiments at higher curcuminoid concentrations. However, even at concentrations of 100 μM and 200 μM, there was no significant difference in PT or APTT for any curcuminoid or THC containing plasma compared to DMSO control.

Curcuminoids are believed to modulate platelet aggregation by altering eicosanoid metabolism through the cyclooxygenase (Cox) pathway to reduce the formation of pro-inflammatory thromboxanes [2, 5, 6]. It is not surprising that platelet aggregation induced by AA is most susceptible to inhibition by curcuminoids [2] and our results are consistent with these findings. We also concur with previous reports where the $IC_{50}$ for curcumin inhibition of platelet aggregation could differ by almost two fold depending on the platelet donor [6]. This high variability may be due to differences in the lipid content of blood from our non-fasting donors, and possibly contributed to the unexpected finding that curcumin consistently and significantly increased aggregation by ADP in our small cohort.

Alternatively, the use of pure diferuloylmethane in combination with whole blood may produce biochemical effects on blood cells that independently promote platelet aggregation in a way that is not reproduced in the majority of studies that use crude turmeric extracts (consisting of approximately 77% diferuloylmethane, 18% DMC, and 5% BDMC) in combination with cell depleted platelet rich plasma preparations.

THC differs from the curcuminoids in that it lacks an β,α-unsaturated carbonyl moiety and is thus unable to form Michael adducts with intracellular proteins, an attribute that is believed to contribute to the biological activity of curcuminoids [4]. However, others have shown that a similar metabolite, hexahydrocurcumin, also shares these properties [7], suggesting that this function is not important for inhibition of platelet aggregation. This is consistent with recent *in silico* predictions of curcumin interacting with Cox-2 via hydrophobic interactions as well as hydrogen bonding with Ser530, the target of acetylation by aspirin [8]. Given that Cox-1 has nearly identical substrate binding sites and catalytic residues, the potential exists for production of potent alternative Cox inhibitors.

We were unable to reproduce the coagulation results reported by Kim *et al*. [3], finding no change in PT or APTT clotting times using any of our curcuminoid or THC preparations in standard commercial or pooled normal human plasma. This did not appear to be due to our detection methods, one of which was non-optical mechanical end point detection similar to the semi-automated Thrombotimer (Behnk Elektronic, Germany) used by Kim *et al*. Our alternative source of curcuminoids may have been a factor, and we were unable to access curcumin from TCI Korea (Tokyo Chemical Industry Co. Ltd. Seoul, South Korea) for comparison. However, the platelet aggregation results suggest that our preparations contained active compounds. It is possible that the curcumin effects on platelet aggregation

are independent of that required for inhibition of coagulation. Others have speculated that curcumin degradation products, produced spontaneously under physiological conditions, may well be responsible for any observed biological effects [9]. Further studies will be required to resolve these apparent discrepancies.

*In vivo* studies are currently limited by the poor bioavailability of curcumin and its derivatives. Our experiments utilised a concentration of 100 μM, equivalent to 30-37 μg/ml of curcuminoids/THC in plasma. Although human clinical trials have shown that oral ingestion of up to 12 g of curcumin per day is well tolerated, even such high doses have failed to raise plasma levels above 50 ng/ml [10]. Newer formulations to increase bioavailability have been promising [11], with a recent report of a single 500 mg dose of Cureit$^{TM}$ producing maximal plasma concentrations of 74.3 ng/ml curcumin and 42.8 ng/ml THC [10].

We conclude that curcumin derivatives show antiplatelet but not anticoagulant effects *in vitro*. However, their low bioavailability makes it impossible to achieve such high plasma concentrations *in vivo* with standard oral preparations. Hence ingestion of currently available curcumin supplements is unlikely to cause any clinically significant anti-platelet effects. Newer formulations may hold therapeutic promise and will require further study.

**Statement of Contribution: LL, KC, FS, AA,** experimental work; **ES, AKE**, literature review; **LL, AKE,** project conception and design; **LL** supervision and data analysis; **All authors,** manuscript preparation.

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**Figure 1. Effects of curcumin, DMC, BDMC and THC on whole blood platelet aggregation induced by collagen, arachidonic acid (AA) or adenosine diphosphate (ADP).**

Results are presented as mean ± standard deviation (n=4 individual donors). *p<0.05 compared to 0.2% DMSO control

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**Table 1. Effects of curcumin, DMC, BDMC and THC on plasma PT and APTT *in vitro*.**

**Sysmex CS-2000i (optical clot detection)**

| Sample (50 μM, n=3) | PT (sec) | APTT (sec) |
|---|---|---|
| Control (DMSO) | 12.5 ± 1.1 | 28.2 ± 2.9 |
| Curcumin | 11.0 ± 1.3 | 27.6 ± 2.4 |
| DMC | 12.0 ± 1.5 | 27.6 ± 2.6 |
| BDMC | 11.0 ± 1.5 | 27.4 ± 2.6 |
| THC | 12.0 ± 1.4 | 27.7 ± 2.6 |

**Stago STA Compact (mechanical detection)**

| Sample (100 μM, n=4) | PT (sec) | APTT (sec) |
|---|---|---|
| Control (DMSO) | 13.3 ± 0.7 | 30.0 ± 1.6 |
| Curcumin | 13.4 ± 0.6 | 30.5 ± 1.5 |
| DMC | 13.3 ± 0.6 | 30.7 ± 1.2 |
| BDMC | 13.3 ± 0.6 | 30.5 ± 1.6 |
| THC | 13.4 ± 0.6 | 30.1 ± 1.6 |

| Sample (200 μM, n=2) | PT (sec) | APTT (sec) |
|---|---|---|
| Control (DMSO) | 12.7 ± 0.4 | 28.5 ± 0.4 |
| Curcumin | 12.8 ± 0.2 | 28.8 ± 0.7 |
| DMC | 12.7 ± 0.4 | 28.8 ± 0.1 |
| BDMC | 12.8 ± 0.4 | 28.8 ± 0.3 |
| THC | 12.8 ± 0.4 | 28.6 ± 0.2 |

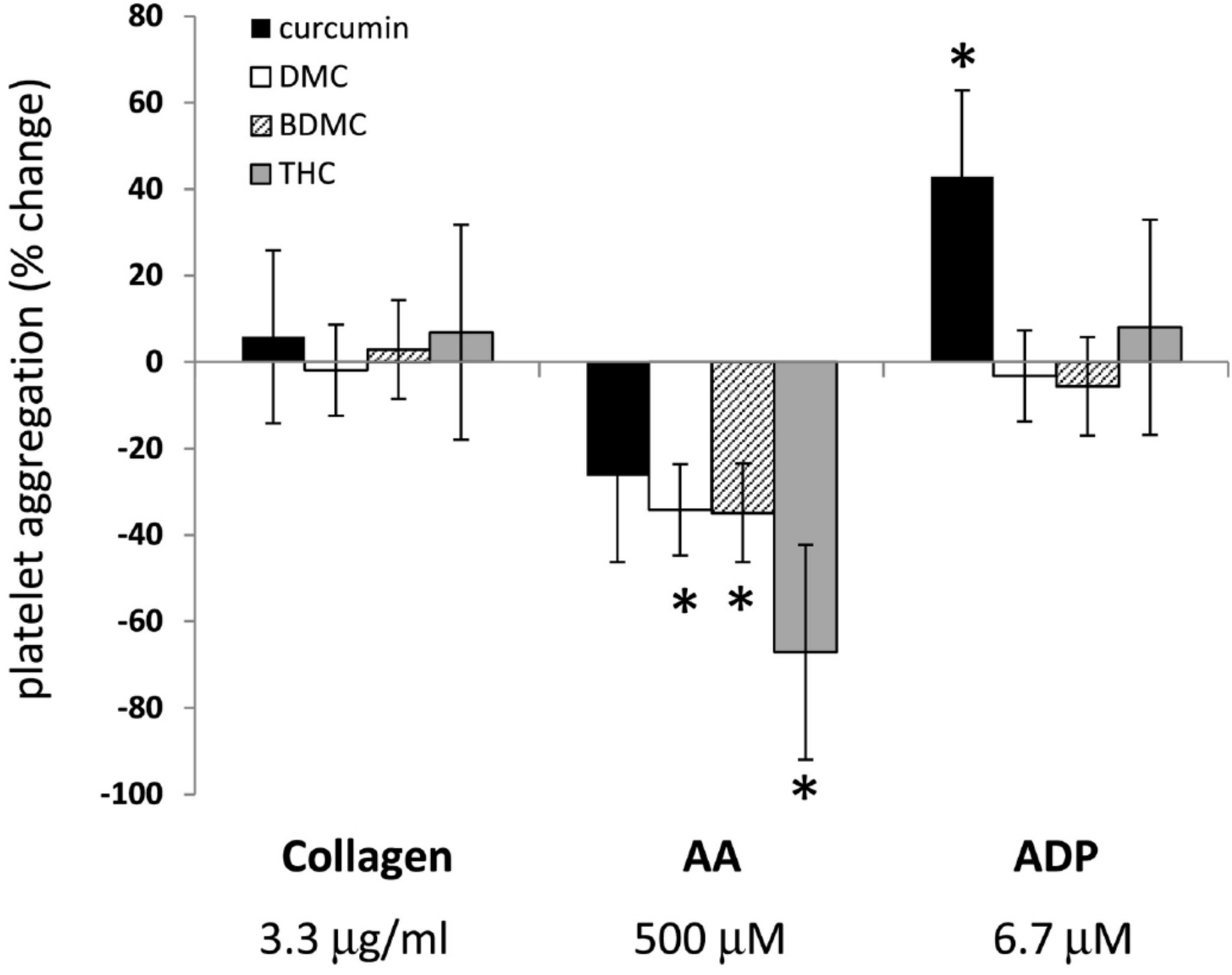
80
60
40
20
0
-20
-40
-60
-80
-100
curcumin
DMC
BDMC
THC
platelet aggregation (% change)
*
*
*
*
Collagen
AA
ADP
3.3 µg/ml
500 µM
6.7 µM

Figure 1