Supporting Information

*Centaurium Erythraea* Extracts Exert Vascular Effects through Endothelium- and Fibroblast-dependent Pathways

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1. Additional evidence for involvement of muscarinic receptors on the effects of MFCE (methanolic fraction from sequential extraction of C. erythrea aerial parts) on isolated rat ileum

In order to confirm the involvement of muscarinic receptors in the vascular effects of methanolic fraction from sequential extraction of C. erythrea aerial parts (MFCE), experiments were carried out using isolated ileum preparations, a classical model to study muscarinic responses.

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 1S.** Influence of the muscarinic receptor antagonists 1 μM atropine or 1 μM AQ-RA 741 on the concentration-response curves in rat ileum preparations to carbachol.
(A) and to MFCE (B). Results are shown as mean ± SEM (n = 4) and mean differences were tested for significance using the 2-way ANOVA test, followed by post hoc multiple comparisons Bonferroni’s test; *p < 0.05 significantly different from responses in the absence of atropine. #p < 0.05 significantly different from responses in the absence of AQ-RA 741.

Ileum fragments were dissected and strips of about 1 cm long were fixed vertically in an organ bath (10 mL volume) filled with KHS, the lower end fixed to a tissue holder and the upper end tied to an isometric force transducer, type DY2 (Ugo Basile), connected to a polygraph (Unirecord 7050, Ugo Basile) and kept under a 9.8 mN tension. The KHS was kept at 37°C and continuously oxygenated with a mixture of 95% O2 + 5% CO2. Tissue preparations were allowed to equilibrate for at least 60 min and tension adjusted until stabilization. Concentration-response curves to MFCE (0.015–1.0 mg/mL) or to carbachol (7.5 nM to 25 µM) were recorded before and 15 min after addition of the muscarinic receptor antagonists.
2. Additional evidence for an MFCE-induced release of NO: effects of MFCE on NO release detected by DAF-FM DA, a selective fluorescence probe, in MCF-7 cells.

In order to investigate the effects of MFCE on the release of NO, experiments were carried out in MCF-7 cell cultures using the selective NO fluorescence detector DAF-FM DA, a cell-permeable fluorescence probe accepted to be selective and highly sensitive to nitric oxide (Fig. 1S). Therefore, the increase in DAF-FM DA fluorescence intensity in MCF-7 cells caused by MFCE and its prevention by inhibition of nitric oxide synthase, likely reflects an increase in NO formation.

![Image of fluorescence intensity data](image)

**Fig. 2S.** Influence of MFCE on nitric oxide levels, detected with the fluorescence probe DAF-FM-DA, in the absence and in the presence of the nitric oxide synthase.
inhibitor L-NAME in MCF-7 cells. Panel A shows representative images. Panel B shows the average values of fluorescence intensity (mean ± SEM; n = 4 independent experiments); *p < 0.05 significantly different from responses in the absence of L-NAME; Student’s t-test.

MCF-7 breast cancer cells were cultured in DMEM (Sigma) supplemented with 10% heat-inactivated FBS and 1% of a mixture of penicillin/streptomycin (10000 U/mL and 10 mg/mL, respectively) and kept in 37°C with 5% CO₂. Cells were seeded at an initial cell density of 3 × 10⁴ cells/mL (100 µL per well) in 96-well plates. After 24 h of attachment, cells were subsequently incubated for 6 h, in the absence or in the presence of MFCE (0.3 and 1.0 mg/mL), with or without L-NAME (500 µM). Measurement of NO formation was carried out using the DAF-FM DA probe. Briefly, after treatments, cells were washed with medium and incubated with 5 µM of DAF-FM DA with FBS-free medium, for 30 min. Thereafter, cells were washed with PBS and incubated for an additional period of 30 min, in FBS-free medium. Fluorescence images were taken using the real-time fluorescence microscope Lionheart FX (BioTek Instruments). The mean fluorescence intensity was calculated using the software Gen 5.0.

References

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