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Abstract

Combretum mucronatum (Schum. & Thonn.) is a scandent shrub whose leaves are widely used by Ghanaian herbalists for the treatment of both acute and chronic wounds. The aim of this study was to phytochemically investigate the extracts, isolate and characterize secondary metabolites and to determine their functional activity on skin cells, in order to assess the wound healing potential of *C. mucronatum*. Appropriate chromatographic methods (TLC, CC, HPLC, UPLC) and spectroscopic methods (ESI-MS, CD spectra, 1D-NMR, GC-MS) were used for the analysis, isolation and characterization. The functional activities of the aqueous extract, ethanol 50 % extract and the isolated procyanidins were investigated on HaCaT cell line, primary keratinocytes (pNHEK) and primary dermal fibroblasts (pNHDF) by determination of metabolic activity, proliferation and necrotic cytotoxicity by the MTT, BrdU and LDH assays respectively. The extracts and compounds were also investigated on cellular differentiation of keratinocytes by immunoflourescence staining and SDS-PAGE Western blots for involucrin and cytokeratin 10 expressions. Scratch assay was additionally performed for determination of the effect of the extracts on cellular migration.

Phytochemical analysis of the extracts showed the presence of flavonoids, proanthocyanidins, saponins and terpenes. Further purification, isolation and characterization of compounds from the ethyl acetate soluble fraction of the ethanol 50 % extract indicated procyanidins as a major class of compounds present in the extract. Specific compounds isolated and characterized were one flavan-3-ol and oligomeric procyanidins; epicatechin (**1**), procyanidin B2 (epicatechin(4β→8)epicatechin) (**2**), procyanidin B5 (epicatechin(4β→6)epicatechin) (**3**), procyanidin C1 (epicatechin(4β→8)epicatechin(4β→8)epicatechin) (**4**), procyanidin D1 (epicatechin(4β→8)epicatechin(4β→8)epicatechin)-(4β→8)epicatechin) (**5**). UPLC spiking experiments of the ethanol 50 % extract with reference compounds identified isovitexin (**6**) and vitexin (**7**). With respect to the aqueous extract, D-glucose (**35**) was found as the only sugar present by capillary zone electrophoresis.

Analysis of the petroleum ether extract of the leaves by GC-MS showed that it was composed of squalene (**12**) (54 %) as the major component, palmitic acid (**8**) (12 %), oleic acid (**9**) (11 %), linoleic acid (**10**) (3 %) and stearic acid (**11**) (2 %). The following compounds were also

identified after enrichment on silica gel, heptacosane (**13**), hexanedioc acid bis (2-ethylhexyl) ester (**14**), margaric acid (**15**), eicosanoic acid (**16**), α-linolenic acid (**17**), cyclodecane (**18**), palmitelaidic acid (**19**), and myristic acid (**20**). The methanol soluble fraction of the dichloromethane extract contained diethylene glycol (**21**), glycerol (**22**), butanedioc acid (**23**), propanoic acid (**24**), malic acid (**25**), butanal (**26**), 4-aminobutyric acid (**27**), threonic acid (**28**), arabinonic acid (**29**), xylitol (**30**), ribitol (**31**), protocatechuic acid (**32**), mannitol (**33**) and inositol (**34**).

On the functional aspect, the aqueous extract did not affect cellular viability and proliferation of HaCaT keratinocytes at concentrations between 0.1 to 10 µg/mL, but significantly reduced viability and the rate of proliferation at 50 and 100 µg/mL. Cellular viability was reduced to 87 ± 4.4 % and 85 ± 5.5 % while proliferation was reduced to 69 ± 7 % and 22 ± 3 % respectively. However over the tested concentration range (0.1-100 µg/mL) the aqueous extract caused a significant dose dependent reduction in LDH leakage, implying a dose dependent cytoprotective effect. The aqueous extract induced pNHEK differentiation at 0.1 µg/mL. On pNHDF, the aqueous extract significantly stimulated cellular viability to 146 ± 2.4 % at 10 µg/mL.

The treatment of HaCaT cells with epicatechin had no effect on viability and on the rate of proliferation but caused a dose dependent decrease in LDH leakage over the concentration range of 0.3 to 344 µM. The immunoflourescence staining seemed to show an induction of involucrin and cytokeratin 10 expression (0.1 to 100 µM) but this was not supported by the SDS-PAGE Western blot method. Procyanidin B2 at 0.1 and 1 µM stimulated cellular proliferation of HaCaT to 129 ± 6 % and 148 ± 4 % respectively. There was also a significant dose dependent reduction in LDH leakage (0.1 to 172 µM). Procyanidin B2 also stimulated pNHEK to undergo cellular differentiation at 1 and 10 µM concentrations. Procyanidin B5 moderately increased cellular proliferation a concentration of 86 µM (110 ± 9 %) and was also associated with a dose dependent decrease in LDH leakage. Procyanidin B5 did not induce pNHEK to cellular differentiation at the tested concentrations (0.1 to 100 µM).

Procyanidin C1 significantly induced proliferation of HaCaT at 57 µM to 130 ± 3 % and was also associated with a dose dependent reduction in LDH leakage from the cells but also did not induce cellular differentiation. Procyanidin D1 significantly induced cellular proliferation of HaCaT keratinocytes to 140 ± 2 % at 8 µM, with a significant dose dependent decrease in

LDH leakage (0.08 to 86 µM). pNHEK treated with procyanidin D1 at 0.1 and 1 µM were not induced to cellular differentiation.

On pNHDF, equimolar concentrations of epicatechin, procyanidins B2, B5, C1 and D1 all showed a dose dependent increase in cellular viability with corresponding decrease in proliferation and LDH leakage. At concentrations of 1 µM procyanidin B5 and at 10 µM epicatechin and procyanidin C1 significantly stimulated pNHDF to increased collagen formation.

An ICH-conform validated UPLC method was also developed to simultaneously quantify epicatechin, procyanidin B2, vitexin and isovitexin in the ethanol 50 % extract. *C. mucronatum* leave samples showed variations in their content with plant materials harvested from different sites, maturity of the leaves and temperature of drying.

In conclusion, the aqueous extract of *C. mucronatum* triggered primary keratinocytes into cellular differentiation at an optimal concentration of 0.1 µg/mL and significantly stimulated viability of pNHDF at 10 µg/mL to 146 ± 2.4 %. Toxicity was observed for HaCaT cells at 50 to 100 µg/mL of the extract. The lead compound that could be responsible for cellular differentiation is possibly procyanidin B2, which induces the cellular differentiation of pNHEK at optimal concentrations of 1 and 10 µM. The other procyanidins stimulated HaCaT cell proliferation at specific concentrations but did not have any induction on cellular differentiation. The *in vitro* effects of the aqueous extract on the skin cells rationalized the remedial effect in wound healing and possibly accounts for the reason why this plant may be widely used for this purpose. On the basis of this study, *C. mucronatum* therefore has potential for the treatment of skin conditions that are characterized by hyperproliferation of keratinocytes, as for example psoriasis and atopic dermatitis.