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Comparison of metabolic profiles and bioactivities of the leaves of three edible Congolese *Hibiscus* species

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Abstract

Methanolic and dichloromethane extracts from the leaves of Congolese Hibiscus species were characterised by chromatographic and spectroscopic techniques and their *in vitro* biochemical activities against ROS production were evaluated in cellular models and on an enzyme, myeloperoxidase (MPO), involved in inflammation. *Hibiscus acetosella* has a chemical fingerprint different from *Hibiscus cannabinus* and *Hibiscus sabdariffa* both having similar fingerprints. Major compounds were polyphenols, represented mainly by caffeoyl-hydroxycitric acid for *H. acetosella* and neochlorogenic acid for the two other spiecies. All extracts displayed high cellular antioxidant activity with IC_{50} values ranging from 0.5 to 3 μg mL^{-1} using lucigenm on neutrophils. Dichloromethane extracts showed more efficient effects on extracellular ROS production and MPO activity. Antioxidant and anti-inflammatory activities of caffeoyl-hydroxycitric acid were significantly higher than those of neochlorogenic acid. The bioactivities of Hibiscus species were positively correlated with their phytochemical content and could justify the use as local nutraceutical resources and medicines.

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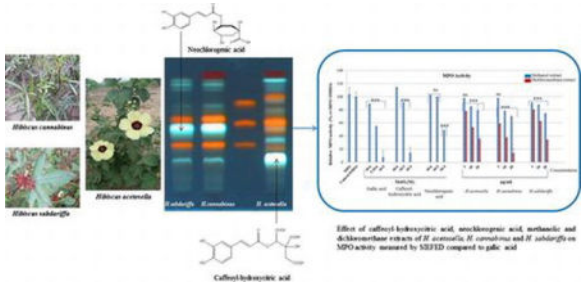
Supplementary material

Experimental details, figures and tables, relating to this, article are available online

Disclosure statement

No potential conflict of interest was report-ad by the authors.

Graphical abstract



Keywords

Caffeoyl-hydroxycitric acid; *Hibiscus acetosella*; *Hibiscus cannabinus*; *Hibiscus sabdariffa*; myeloperoxidase; neochlorogenic acid

Phenolic compounds such as quercetin-3-glucoside, kaempferol-rhamnoside, neochlorogenic acid are reported in the leaves from *H. canaabinus* (Pascoal et al. 2014). By comparison with standards, it was showed that *H. acetosella* contains chlorogenic acid, quercetin-3-galactoside and other nor-identified flavonoids, in accordance with results of Tsumbu et al. (2012). The two other species contained quercetin-3-rutinoside, quercetin-3-glucoside and kaempferol 3-rutinoside. Caffeic acid is present in all species but at high amounts in *H. acetosella.* TLC analysis of dichloromethane extracts showed the probable presence of terpenoid compounds (Figure S3). The main phenolic acid detected in *H. acetosella* was different from this detected *H. cannabinus* and *H. sabdariffa* (Figure S2). The isolation of main phenolic compounds was canied out with a Varian HPLC preparative chain. Spectroscopic analysis, including 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopy, combined with a comparison of their NMR data (Figure S4–S9) with those reported in the literature was realised on isolated compounds. MS analysis showed that molecular ions of major phenolic acid appeared at *m/z* 369.1169 $[M–5H]^{-}$ for the *H. acetosella* compound and 353.0893 $[M–5H]^{-}$ for the *H. cannabinus* and *H. sabdariffa* compound. Our results clearly showed that besides already described flavonoids, the main phenolic acid from *H. acetosella* is 2-O-trans-caffeoyl-hydroxycitrtc acid, while this from *H. cannabinus* and *H. sabdariffa* is neochlorogenic acid (Figure 1). These two species also contain caffeoyl-hydroxycitric acid in low quantities. Thus, these differences in the chromatographic fingerprints may be easily used to distinguish *H. acetosella* from *H. cannabinus* or *H. sabdariffa.*

2.2. Antioxidant activity

ABTS and DPPH assays showed that leaf methanolic extracts of the three Hibiscus species have the ability to scavenge free radicals with IC_{50} values comprised between 43 and 186 μg mL^{-1} (Table S1). The IC_{50} values showed that the extract of *H. acetosella* is the most active followed by *H. cannabinus* and *H. sabdariffa*. The dichloromethane extracts showed a weaker capacity to scavenge $ABTS^{+}$, while this capacity was null with DPPH. Indeed, dichloromethane allowed a better extraction of lipophilic compounds in comparison to methanol and it was shown that lipophilic compounds such as carotenoids did not scavenge

the DPPH radicals (Müller, Fröhlich, and Böhm 2011). Carotenoids were also reported in the leaves of hibiscus species (Raju et al. 2007). Beside, some coloured compounds car interfere at specific wavelengths of DPPH, leading to the underestimated antioxidant activity of extracts (Arnao 2001), Previous studies reported that extracts from parts of Hibiscus species such calyx of *H. sabdariffa* exhibited high scavenging activity (Sarkar et al. 2014) and the leaves of studied *Hibiscus* have shown a better DPPH scavenging activity (Andabati and Muyonga, 2014; Wang et al. 2014). Isolated caffeoyl-hydraxycitric acid and neochlorogenic acid, exhibited higher scavenging activities than the extracts but lower than gallic acid, used as positive control. Nevertheless, the antiradical activities of these phenolic acids can contribute to the global antioxidant capacity of the Hibiscus extracts.

Beside conventional cell-free antioxidant assays, it can be pertinent to evaluate the antioxidant and anti-catalytic potential of plant extracts in cellular models involved in ROS production and inflammatory responses. In a recent study, Zhen et al. (2016) showed the inhibitory capacity of the *H. sabdariffa* leaf extract on the nitric oxide synthase activity by LPS stimulated murine macrophage. In the present study, the lucigenin-dependent chemiluminescence (CL) and the intracellular fluorescent probe DCFH-DA were used to evaluate the extra- and intracellular ROS production resulting mainly from NADPH oxidase activity by stimulated neutrophil and HL-60 cells (Derochette et al. 2013). The addition of increasing concentrations of the leaf extracts (0.1–5 μg mL^{-1}), the isolated phenolic acids (10^{-6} to 5.10^{-5} M) resulted in a dose-dependent decrease of the neutrophils ROS production in comparison to the control test performed with DMSO, the solvent used to solubilize the extracts and phenolic acids (Figure S10). Our results showed that the cellular antioxidant activity of dichloromethane extracts is significantly higher ($p < 0.001$) than that of methanolic extracts in the following order: *H. acetosella* > *H. cannabinus* > *H. sabdariffa.* Caffeoyl-hydroxycitric acid is more active than neochlorogenic acid and gallic acid used as positive control.

The addition of increasing concentrations of the leaf Hibiscus extracts (10, 20 and 40 μg mL^{-1}), and the isolated phenolic acids (5.10^{-6} to 5.10^{-5} M) and gallic acid (10^{-5} to 10^{-4} M) resulted in a dose-dependent decrease of HL-60 ROS production in comparison to the control test performed with DMSO (Figure S11). With this cellular model, our results showed that the activity of methanolic extracts is significantly higher ($p < 0.05$) than that of dichloromethane extracts. In a previous study, Tsumbu et al. (2012) observed an inhibitory effect of the aqueous extract of *H. acetosella* on the intracellular ROS production in HL-60 cells suggesting that molecules released by polar extractions were more favourable for this cell model. Dichloromethane extracts were not or few active in this cell model and exhibited a significant pro-oxidant effect at the tested concentrations. In this cell model, neochlorogenic acid appeared less active than caffeoyl-hydroxycitric acid.

Indeed, methanolic extracts were active in the two cell-based assays, but dichloromethane extracts exhibited a higher anti-ROS activity in the lucigenin CL assay. Lucigenin seems to be a more specific probe for the detection of superoxide anions directly produced by the activity of NADPH oxidase (Franck et al. 2013).The high activity of dichloromethane extracts can be attributed to lipophilic compounds such as carotenoids that are known to be powerful scavengers of superoxide anions (Galano et al. 2010). However, the values

obtained with dichloromethane extracts on HL-60 cells showed that the extract of *H. acetosella* had a very weak effect on intracellular ROS production while the other extracts had rather a pro-oxidant effect. These results suggest that the compounds of dichloromethane extracts are not good intracellular ROS scavengers and could even amplify the activation cascade involved in ROS production. Further studies are needed to understand these observations. Nevertheless, Takamatsu et al. (2003) showed that carotenes did not inhibit intracellular DCF production in HL-60 cells, presumably because of a cell intake problem (Takamatsu et al. 2003). Our results suggest that both methanolic and dichloromethane extracts, as well as their major phenolic acids, without having any cytotoxicity are efficient as superoxide anion scavengers while only methanolic extracts appeared more efficient to inhibit intracellular ROS production.

2.3. Anti-inflammatory activity

In some acute and chronic pathologies, the uncontrolled stimulation of neutrophils could contribute to amplify or maintain the inflammatory response with the release of MPO, a pro-oxidant enzyme involved in secondary cell damage an d considered as a marker of inflammation (Franck et a1. 2013). All plant extracts and isolated phenolic acids exhibited a dose-dependent inhibitory effect on MPO activity. Dichloromethane extracts showed a stronger inhibition of MPO in comparison to methanolic extracts in the following order: *H. cannabinus> H. acetosella > H. sabdariffa.* The dichloromethane allowing a better extraction of lipophilic molecules may allow a better interaction of these molecules with the hydrophobic pocket at the entrance of the active site of MPO (Forbes et al. 2013). Caffeoyl-hydroxycitric acid and neochlorogenic acid are less efficient MFO inhibitors in comparison to gallic acid and they exhibited an inhibition higher than 50% at the highest concentration tested (10^{-4} M) (Figure 2). The SIEFED method used here to measure MPO activity allowed the detection of compounds that have a direct interaction with the MPO. Compared to gallic acid, caffeoyl-hydroxycitric acid and neochlorogenic acid are larger molecules that cannot enter easily into the active site of MPO and thus inhibit the enzyme (Franck et al. 2013).

Altogether the results showed that among the extracts tested, the leave extract of *H. acetosella*, which contains caffeoyl-hydroxycitric acid showed the highest acellular and cellular antioxidant activities but not the highest inhibition on MPO activity. As reported by Franck et al. (2013), the molecules having good antiradical activities are not necessarily appropriate to enter and inhibit the active site of the pro-inflammatory enzyme MPO. Metabolite profiling showed that studied *Hibiscus* species are rich in bioactive such as phenolic compounds and terpenoids, and they are well known as having high therapeutic interest. Polyphenols have the potential to confer benefit in diverse neurodegenerative disorders associated with oxidative damage (Vauzour, Kerr, and Czank 2013). The antioxidant and anti-inflammatory potentials of these phenolic acids as well as of Congolese *Hibiscus* leaves could have a beneficial health for Congolese people.

3. Conclusions

Metabolic profiles and biochemical activities of three Congolese *Hibiscus* species were determined and showed the important content of phenolic acids and terpenoids. The

metabolic profile of *H. acetosella* appears different to those of *H. sabdariffa* and *H. cannabinus* having both quite similar profiles. The chromatographic fingerprints may be used to distinguish *H. acetosella* from *H. cannabinus* or *H. sabdariffa.* Caffeoyl-hydroxycitric acid was found to be a major phenolic acid from *H. acetosella* and neochlorogenic acid the major phenolic acid from *H. cannabinus* and *H. sabdariffa*. Our comparative study on Congolese Hibiscus showed that the leaf extract of *H. acetosella* and its major phenolic acid, caffeoyl-hydroxycitric acid, have the best acellular and cellular antioxidant activities in comparison to the other species. In all species, the methanolic extracts appeared more efficient as radical scavengers and on the inhibition of intracellular ROS production by HL60 cells, while dichloromethane extracts appeared more efficient on the inhibition of the extracellular ROS production by PMN and on the activity of MPO. The antioxidant and anti-inflammatory activities of the leaves of the studied *Hibiscus* may have potential therapeutic interest and could justify their use in traditional medicine and local nutraceutical resources, but further studies are needed, especially *in vivo* studies, to demonstrate the benefit of these extracts and phenolic acids on nutrition and health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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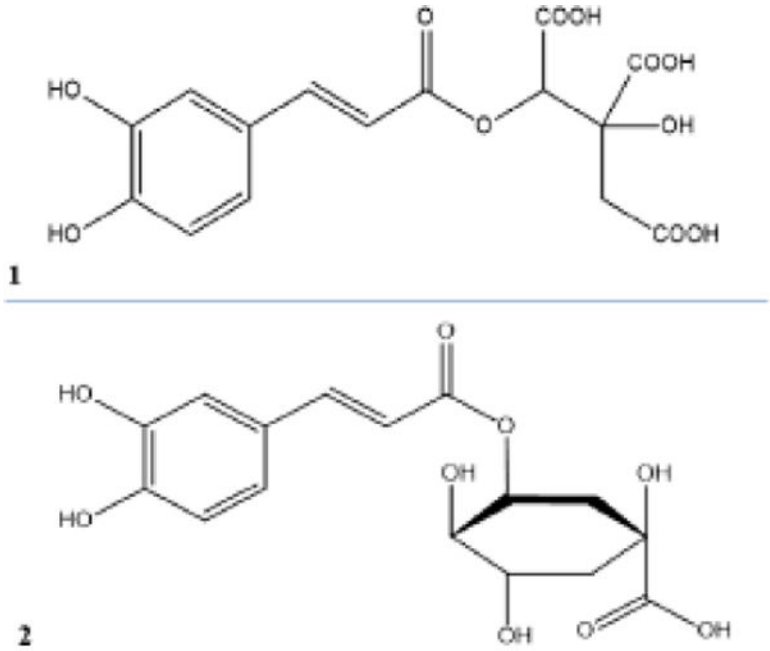

Figure 1.
Chemical structures of caffeoyl-hydroxycitric acid (1) and neochlorgenic acid (2).

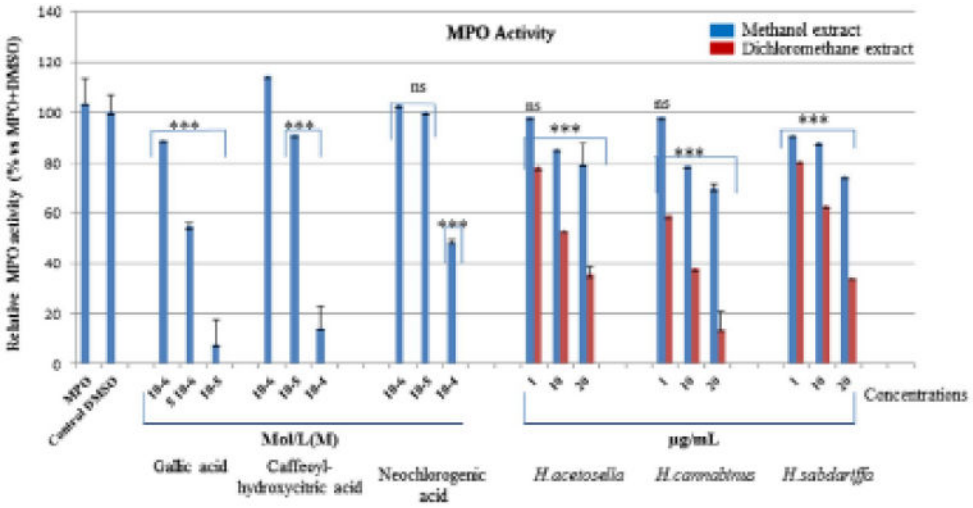

Figure 2.

Effect of gallic acid, caffeoyl-hydroxycritric acid, neochlorogenic acid and methanolic and dichloromethane extracts of *H. acetosella, H. cannabinus* and *H. sabdariffa* on MPO activity measured by SIEFED.

Notes: The percentage of inhibition was calculated for each sample concentration versus the corresponding control (MPG + DMSO), taken as 100% (mean ± SD. $n = 6$). Samples vs. Control DM5O:ns: no significance, p-values (****< p0.0001) calculated by two-way ANOVA indicated a significant effect vs. DMSO control set as 100% response. ns = not significant vs. DMSO control. There are statistical differences between dichloromethane and methanolic extracts of all Hibiscus species. Dichloromethane extracts are more active than methanolic extracts, caffeoyl-hydroxycitric and neochlorogenic acids are less active than gallic acid used as positive control. Extract of *H. cannabinus* are the most active, followed by those of *H. acetosella* and *H. sabdariffa*.