Abstract

**ABSTRACT**

Ayurveda, traditional Indian medicine, is recognized by the European Union as non-conventional medicine and it includes more than 7000 plants used for therapeutic purposes in complex formulations poorly investigated under a chemical and biological point of view. On this basis and as part of a broader planning (PRIN2009LR9YLF, founded by MIUR of Italy) involving Research Unit of the University of Ferrara, Parma and Cosenza, the PhD project was set entirely on the chemical and biological characterization of traditional preparations formulated starting from plants used in the Ayurvedic tradition. The preparations (decoction, DEC, and hydro-alcoholic extract, HE) of *Azadirachta indica* A. Juss (leaves), *Boerhaavia diffusa* L. (roots), *Convolvulus pluricaulis* Choisy (whole plant), *Curculigo orchioides* Gaertn. (roots) and *Hemidesmus indicus* (L.) R.Br. Ex Shultz (roots) were phytochemically investigated with different chromatographic approaches (GC-FID, GC-MS, HPLC and HPTLC) and NMR, with the aim of identifying their main chemical compounds and verifying the repeatability of their fingerprinting – i.e. standardisation - through the detection and quantification of the most characterising molecules. The study of biological activities was driven both by the ethnomedical traditional uses of the species in the Ayurvedic culture and by those aspects related to modern western phytotherapeutic culture concerning the prevention of the oxidative stress (antioxidant activity), the evaluation of both genotoxic (safety) and antigenotoxic potential, and cytotoxicity against cancer and normal human cell lines trying to give a contribute to the never ending research about new tools for anti-neoplastic treatments and prevention, minimising drug-resistance phenomena. The main results are reported below plant species by plant species: *Azadirachta indica*: identification and quantification of flavonoids (rutin, isoquercitrin, quercitrin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside and quercetin) was carried out in DEC and HE by HPLC. Rutin quantification was also performed by HPTLC Visualizer (0.360±0.011 mg/ml in DEC and 0.587±0.009 mg/ml in HE) and the data compared with the HPLC-DAD results (0.330±0.003 mg/ml in DEC and 0.453±0.014 mg/ml in HE). Gas chromatographic analyses performed on phytocomplexes obtained by different polarity extractions permitted the identification and the quantification of stigmastanol and β-sitosterol. The hydro-alcoholic extract exhibit a higher antioxidant activity than DEC and each flavonoid showed IC\text sub{50} value lower then positive control. D7 and SOS-Chromotest did not highlight any genotoxic activity, confirming the safety of the preparations but they did not showed any noteworthy antigenotoxic capacity neither.
Quercetin was the only compound showing antigenotoxic activity in the SOS-Chromotest, but it did not reach the 50% inhibition. The study about the cytotoxicity against all human cancer cell line (A549, CaCo-2, MCF7, LoVo and HepG2) showed data far from those stated by the American National Cancer Institute (30 µg/mL for extracts, and 4 µg/mL for pure compounds, Suffness & Pezzuto, 1991), and therefore not significant for applicable further investigations.

*Boerhaavia diffusa*: ferulic acid (the most abundant compound: 6.803±0.022 µg/ml in DEC and 4.644±0.288 µg/ml in HE) and vanillin (1.833±1.387 µg/ml in DEC and 3.447±0.408 µg/ml in HE) were identified and quantified in DEC and in HE. However, HE showed the presence of other two characterising molecules: boeravinone B and eupalitin. β-sitosterol was identified and quantified in both preparations, but resulted more abundant in DEC. The evaluation of the antioxidant activity highlighted the radical scavenging capacity of ferulic acid against ABTS•⁺ higher than positive control, and the activity of eupalitin towards all the different assays. Preparations and pure compounds did not show any noteworthy antigenotoxic activity towards two compounds taken as reference for their known mutagenic properties. The study of cytotoxicity using DEC, HE and pure molecules against human cancer cell lines (A549, CaCo-2, MCF7, LoVo and HepG2), showed once more data far from those stated by the American National Cancer Institute (Suffness & Pezzuto, 1991).

*Convovulus pluricaulis*: the Ayurvedic preparation, DEC, was characterised by the presence of phenolic acid (caftaric acid, caffeic acid, p-coumaric acid, iso-ferulic acid and tr-ferulic acid). Stigmasterol, β-sitosterol, lupeol and vanillin isomers and derivatives (2H4MB, 3H4MB and acetovanillone) were also detected in the same preparation. HE showed the presence of p-coumaric acid, vanillin, 2-hydroxy-4-methoxybenzaldehyde, acetovanillone, and vanillic acid but the absence of phytosterols. The antioxidant activity evaluation indicated DEC as the phytocomplex that exhibited the best efficacy in each. HE showed instead an higher activity than DEC in the ABTS assay, no activity against DPPH radical and a lower capacity of blocking the radical propagation than DEC in the β-carotene bleaching test. Regarding the cytotoxic capacity of the considered preparations against A549, CaCo-2, MCF7, LoVo and HepG2 cancer cell lines, the growth inhibition experimental data, compared with the IC₅₀ threshold stated by the American National Cancer Institute (<30 µg/ml), are not significant. The tests carried out with DEC and HE extracts against two leukaemia cell lines, drug-sensitive (CCRF-CEM) and multi-drug-resistant (CEM/ADR5000), showed, instead, interesting results: DEC CHCl₃
extract and the HE soxhlet extract exhibit experimental data in line with the one stated by the American National Cancer Institute against the CCRF-CEM cell line, but respectively about 2 and 3 fold bigger in the tests against CEM/ADR5000, showing cross-resistance phenomena.

Curculigo orchioides: Curculigoside A (7.19±1.43 µg/ml in DEC and 40.62±6.57 µg/ml in HE) and orcinol-β-D-glucoside (72.93±0.43 µg/ml in DEC and 232.04±8.49 µg/ml in HE) were identified and chosen as characterising compounds of the preparations, therefore quantified through HPLC methods. β-sitosterol was also identified and quantified using GC-FID. The HE samples showed the highest antioxidant activity in every performed test. Regarding the antigenotoxic activity, DEC resulted slightly more active than HE, but the highest activity was showed by orcinol-β-D-glucoside, even if it did not reach the 50 % inhibition at the maximum concentration tested. The evaluation of the cytotoxic activity against human cancer cell line exhibited the same trend, pointing out DEC as more active than HE for A549, CaCo2, and HepG2. HE was instead more active against CCRF-CEM and CEM/ADR5000, but the IC_{50} data were far from those stated by the American National Cancer Institute. Pure molecules did not exhibit any activity.

Hemidesmus indicus: the two phytocomplexes were characterised by the main presence of vanillin isomer and derivatives: 2-hydroxy-4-methoxybenzaldehyde (2H4MB: 1.72±0.09 µg/ml in DEC and 214.54±5.17 µg/ml in HE), 3-hydroxy-4-methoxybenzaldehyde (3H4MB: 26.85±0.92 µg/ml in DEC and 57.10±1.39 µg/ml in HE) and 2-hydroxy-4-methoxybenzoic acid (2H4MBAc: 23.54±0.23 µg/ml in DEC and 32.51±1.23 µg/ml in HE). These molecules were quantified and showed their higher concentrations in HE than in DEC. The GC-MS analyses of different polarity extractions highlighted the presence of lupeol, lupeol acetate, β-sitosterol and β-amyrin acetate. They resulted more concentrated in DEC than in HE and a different grade of the extraction specificity of the used techniques was observed: lupeol and β-sitosterol were major constituents of soxhlet and CHCl_{3} extractions; while the acetylated compounds (lupeol acetate and β-amyrin acetate) were more abundant in the supercritical CO_{2} extraction. The two preparations showed radical scavenging activity and capacity of blocking the radical propagation. HE showed an higher antioxidant capacity than DEC and 3H4MB was the most active compound. The genotoxicity test confirmed the safety of the phytocomplexes, but did not highlight any antigenotoxic activity against the reference mutagens. 2H4MB showed to reduce the activation of the SOS system in the SOS-Chromotest indicating a weak antigenotoxic activity, without reaching the 50 % inhibition at the maximum concentration tested. Both
preparations were tested for their cytotoxic activity against human cancer cell lines (Ca-Co2, A549, MCF7, CCRF-CEM, CEM-ADR5000, HepG2 and LoVo) but the data were not noteworthy, being far from the concentration values stated by the American National Cancer Institute. The phytocomplex obtained after separation on gravimetric column, and the one obtained by soxhlet extraction, starting from HE, showed a promising activity with IC\textsubscript{50} values, respectively, of 27.93±0.58 µg/mL and 2.46±0.28 µg/mL against CCRF-CEM, and 69.70±1.22 µg/mL and 5.76±0.01µg/mL against CEM/ADR5000.

In conclusion, this research provided initial starting point response to the chemical and biological fingerprinting of Ayurvedic crude drugs and preparations focused on health uses.