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Radical scavenging potentials of single and combinatorial herbal formulations *in vitro*

Okey A. Ojiako [a], Paul C. Chikezie [b, *], Agomuo C. Ogbuji [c]

[a] Department of Biochemistry, Federal University of Technology, Owerri, Nigeria
[b] Department of Biochemistry, Imo State University, Owerri, Nigeria
[c] Department of Food Science and Technology, Abia State Polytechnic, Aba, Nigeria

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ABSTRACT

Reactive oxygen and nitrogen species (RONS) are involved in deleterious/beneficial biological processes. The present study sought to investigate the capacity of single and combinatorial herbal formulations of *Acanthus montanus*, *Emilia coccinea*, *Hibiscus rosasinensis*, and *Asystasia gangetica* to act as superoxide radicals (SOR), hydrogen peroxide (HP), nitric oxide radical (NOR), hydroxyl radical (HR), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical antagonists using *in vitro* models. The herbal extracts were single herbal formulations (SHfs), double herbal formulations (DHfs), triple herbal formulations (THfs), and a quadruple herbal formulation (QHf). The phytochemical composition and radical scavenging capacity index (SCI) of the herbal formulations were measured using standard methods. The flavonoids were the most abundant phytochemicals present in the herbal extracts. The SCI_{50} defined the concentration (μg/mL) of herbal formulation required to scavenge 50% of the investigated radicals. The SHfs, DHfs, THfs, and QHf SCI_{50} against the radicals followed the order HR > SOR > DPPH radical > HP > NOR. Although the various herbal formulations exhibited ambivalent antioxidant activities in terms of their radical scavenging capabilities, a broad survey of the results of the present study showed that combinatorial herbal formulations (DHfs, THfs, and QHf) appeared to exhibit lower radical scavenging capacities than those of the SHfs *in vitro*.

1. Introduction

Reactive oxygen and nitrogen species (RONS) or radicals and oxygen derived, nonradical reactive species (nRRS), referred to as pro-oxidants, are involved in deleterious/beneficial biological processes such as mutation, aging, carcinogenesis, degenerative diseases, inflammation, signal transduction, immune response, cellular regulatory events, and cell development.[1–9] Both RONS and nRRS are predictable products of aerobic metabolic pathways[10] that encompass membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase, lipoxygenase, cytochrome P-450, and xanthine oxidase activities.[9,11]

Numerous reports have shown that oxidative stress injuries are metabolic outcomes of noxious chemical agents[12,13] or impaired metabolic events,[14,15] which are characterized by disequilibrium between physiologic levels of oxidants and corresponding activities of antioxidant systems. The RONS include among other reactive oxides, the superoxide ion (O_2^-), nitric oxide (NO^-), hydroxyl (OH^-), peroxyl (ROO^-), and alkoxyl (RO^-), whereas the nRRS and their derivatives include hydrogen peroxide (H_2O_2), organic peroxide (ROOH), hypochlorous acid (HClO), Ozone (O_3), aldehydes (RCOH), peroxynitrite (ONOOH), and singlet oxygen ($^1O_2$).[5,9]

Depending on its prevailing environmental pH, superoxide may exist in two states as O_2^- (high pH) or hydroperoxyl ($HO_2^{\cdot}$) (low pH) ion, which defines its biologic properties.[5,16] Evidence showed that at acidic pH the most important reaction of O_2^- is dismutation.[5] The O_2^- is a powerful nucleophile, capable of attacking positively charged centers of array of biomolecules. As an oxidizing agent, O_2^- reacts with proton donors such as ascorbic acid and tocopherol. Conversely, when present in organic solvents, its ability to act as a reducing agent is increased.[5]

* Corresponding author. Department of Biochemistry, Imo State University, PMB 2000, Owerri 460222, Nigeria.
E-mail address: p_chikezie@yahoo.com (P.C. Chikezie).
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Spontaneous dismutation of O_2^- or/and superoxide dismutase (SOD) activity is the primary generator(s) of cellular H_2O_2.[5,17] The deleterious actions of H_2O_2 stems from its oxidizing potential and its ability to act as a substrate for the generation of other oxidizing species, such as OH^- and HClO.[18,19] The molecular bases of H_2O_2 toxicity include their capability to degrade heme proteins, inactivate enzymes, oxidation of DNA, lipids, and SH groups.[17,20]

The NO^- is produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine. The nitric oxide synthase (NOS) pathway is responsible for the biosynthesis of NO^- in a variety of tissues.[19] The presence of endotoxins and/or cytokines in mononuclear phagocytes induces NOS, the so-called iNOS, which elicits raised cellular levels of NO^-.[21] The NO^- derivative-ONOOH, elicits the depletion of SH groups and oxidation of biomolecules, engendering tissue damage similar to that caused by the actions of OH^-, such as DNA damage, protein oxidation, and nitration of aromatic amino acid residues in proteins.[22]

The formation of OH^- accounts for much of the damage done to biological systems by increased generation of O_2^- and H_2O_2.[23] The most important biological properties of OH^- are abstraction, addition, and electron transfer reactions.[19] Generally, OH^- is a fast reacting and powerful oxidizing agent. According to *in vitro* studies by Cohen,[12] certain cell toxins effect their deleterious actions on specific target cells through intracellular generation of OH^-. In physiologic systems, reactions of OH^- with biomolecules such as DNA, proteins, lipids, amino acids, sugars, and metals are the biochemical bases of several pathologic disorders and the ageing process.[6,24]

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical used for ascertaining the capacity of tissue extracts to act as free radical scavengers and to measure their antioxidant activity *in vitro*.[25–27] The reaction of DPPH with antioxidant of tissue extracts produces a corresponding reduced compound (hydrazine $DPPH_2$), which can be monitored by color change from purple to yellow with maximum absorptivity (ƛmax) within the range of 515–528 nm[28,29]

The medicinal usefulness of *Acanthus montanus*, *Emilia coccinea*, *Hibiscus rosasinensis*, and *Asystasia gangetica* has been reported elsewhere.[30–34] Most of the therapeutic benefits derivable from medicinal plants are hinged on their capability to ameliorate oxidative stress.[35–38] Furthermore, alleviation of oxidative stress-induced pathologic conditions following the administration of RONS antagonists from diverse plant species have been reported by several authors.[39–41] Accordingly, most ethnomedicinal practices presume that poly-herbal decoctions are more efficacious than mono-herbal formulae against pathologic conditions and physiologic disorders.[26,42–45] However, combinatorial herbal formulations have been reported to cause alterations in the pharmacologic properties and therapeutic outcomes of individual plant extracts.[26,29,45] The present study sought to investigate the capacity of single and combinatorial herbal formulations of *A. montanus*, *E. coccinea*, *H. rosasinensis*, and *A. gangetica* to act as RONS and nRRS antagonists using *in vitro* models.

2. Materials and methods

2.1. Collection and preparation of herbal samples

Fresh leaves of *A. montanus* (Nees) T. Anderson (ACMO), *E. coccinea* (SIMS) G. Don (EMCO), and *H. rosasinensis* L. (HIRO) were collected from uncultivated lands in Umuamacha Ayaba Umaeze, Osisioma Ngwa LGA (Local Government Area), Abia State, Nigeria, whereas fresh leaves of *A. gangetica* L.T. Anderson (ASGA) were collected from Ubowuala, Emekuku, Owerri North LGA, Imo State, Nigeria. The four herbs were identified and authenticated by Dr. M. Ibe, School of Agriculture and Agricultural Technology (SAAT), Federal University of Technology, Owerri. All the leaves were collected between the months of July and August, 2009.

The leaves of individual plants were washed with continues flow of distilled water for 15 minutes and allowed to dry at laboratory ambient temperature (24 ± 5 °C). A 500-g part of each herbal sample was weighed using a triple beam balance (OHAU 750-50; OHAUS Triple Beam Balance, Model TJ611, Burlington, NC, USA) and dried in an oven (WTC BINDER; 7200 Tuttlingen, Germany) at 60 °C until a constant weight was achieved. The dried leaves were packaged in dark polyethylene bags and kept in a cold room (7 ± 3 °C) for 24 hours before pulverization. Next, the separate dried leaves were pulverized using the Thomas-Willey milling machine (ASTM D-3182; India), after which the ground samples were stored in air-tight plastic bottles with screw caps pending extraction.

2.2. Extraction of herbal samples

A portion of 40 g of each pulverized dried sample of ACMO, ASGA, EMCO, and HIRO were subjected to repeated soxhlet extraction cycles for 2 hours using 96% C_2H_5OH (BDH, UK) as solvent to obtain a final volume of 500 mL of each herbal extract. The volumes of the extracts were concentrated and recovered in a rotary evaporator (Rotavapor R-200; Büch, BÜCHI Labortechnik AG, Flawil, Switzerland) for 12 hours at 60 °C under reduced pressure. The extracts were dried in a desiccator for 24 hours, wrapped in aluminum foil, and stored in air-tight plastic bottles with screw caps at ≤ 4°C. The yields were calculated to be as follows: ACMO = 16.35% (w/w), ASGA = 16.69% (w/w), EMCO = 17.99% (w/w), and HIRO = 17.23% (w/w). The separate herbal extracts were reconstituted in phosphate-buffered saline (PBS) solution, osmotically equivalent to 100 g/L PBS (90.0 g NaCl, 17.0 g $Na_2HPO_4 \cdot 2H_2O$ and 2.43 g $NaH_2PO_4 \cdot 2H_2O$). Portions of the individual extracts were also measured for phytochemical contents.

2.3. Phytochemical composition of herbal extracts

The flavonoid content was measured according to the method of Bohm and Koupai-Abyazani.[46] The concentration of alkaloids was measured by the method of Harborne.[47] The saponin content was measured according to the method of Harborne,[47] as reported by Obadoni and Ochuka.[48] The tannin content was estimated by the method of Van-Burden and Robinson,[49] as reported by Belonwu et al.[50]

2.4. Herbal formulations

The herbal extracts were single herbal formulations (SHf-ACMO, SHf-ASGA, SHf-EMCO, and SHf-HIRO), double herbal formulations (DHf-AGAM, DHf-AGEC, DHf-AGHR, DHf-AMEC, DHf-AMHR, and DHf-ECHR), triple herbal formulations (THf-AGEH, THf-AMAE, THf-AMAH, and THf-AMEH), and a quadruple herbal formulation (QHf-AAEH). All the herbal formulations were constituted in PBS, pH = 7.4.

- SHf-ACMO: *A. montanus*
- SHf-ASGA: *A. gangetica*
- SHf-EMCO: *E. coccinea*
- SHf-HIRO: *H. rosasinensis*
- DHf-AGAM: mixture of *A. gangetica* + *A. montanus* (1:1 w/w)
- DHf-AGEC: mixture of *A. gangetica* + *E. coccinea* (1:1 w/w)
- DHf-AGHR: mixture of *A. gangetica* + *H. rosasinensis* (1:1 w/w)
- DHf-AMEC: mixture of *A. montanus* + *E. coccinea* (1:1 w/w)
- DHf-AMHR: mixture of *A. montanus* + *H. rosasinensis* (1:1 w/w)
- DHf-ECHR: mixture of *E. coccinea* + *H. rosasinensis* (1:1 w/w)