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

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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]

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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)

- THf-AGEH: mixture of *A. gangetica* + *E. coccinea* + *H. rosasinensis* (1:1:1 w/w)
- THf-AMAE: mixture of *A. montanus* + *A. gangetica* + *E. coccinea* (1:1:1 w/w)
- THf-AMAH: mixture of *A. montanus* + *A. gangetica* + *H. rosasinensis* (1:1:1 w/w)
- THf-AMEH: mixture of *A. montanus* + *E. coccinea* + *H. rosasinensis* (1:1:1 w/w)
- QHf-AAEH: mixture of *A. montanus* + *A. gangetica* + *E. coccinea* + *H. rosasinensis* (1:1:1:1 w/w)

2.5. Measurement of radical scavenging capacities of herbal formulations

2.5.1. Superoxide radical

The herbal formulation scavenging capacity index (SCI) against O_2^- was measured according to the method of Nishikimi et al[51] with minor modifications. Briefly, 0.5 mL of different concentrations (10–1500 μg/mL) of the herbal sample was introduced into a test tube containing 6.0 μL of 0.1 mM EDTA (Mayer and Baker, England), 0.5 mL of 0.1 mM NaCN (NOAH Technologies Corporation, Texas, USA), 0.5 mL of riboflavin, and 0.5 mL of 150 mM nitroblue tetrazolium (NBT). The assay mixture was made up to the 3-mL mark by the addition of 0.1M phosphate buffer (pH = 7.4). The test tubes containing the test and control samples were illuminated with an incandescent lamp for 15 minutes. Absorbances of the samples were measured before and after illumination with a spectrophotometer (Digital Blood Analyzer; SPECTRONIC 20; Labtech, LabX, Bay Street, Midland, ON, Canada) at maximum absorptivity (ƛmax) = 530 nm. The herbal formulation superoxide radical scavenging capacity index (SORSCI) was calculated as follows:

$$\text{SORSCI\%} = 100 - \frac{\text{Absorbance of test medium}}{\text{Absorbance of control medium}} \times 100 \quad (1)$$

where absorbance of the medium = absorbance after illumination minus absorbance before illumination of the media.

The SORSCI% was expressed as SCI_{50}, which is defined as the concentration (μg/mL) of the herbal formulation required to scavenge 50% of O_2^-.

2.5.2. Hydrogen peroxide

The herbal formulation SCI against H_2O_2 was measured according to the method of Delpour et al.[52] A solution of 40 mM H_2O_2 was prepared in phosphate buffer (pH = 7.4). Next, 1.4 mL of different concentrations (10–1500 μg/mL) of the herbal formulations was added to 0.6 mL of the H_2O_2 solution. The assay mixture was allowed to stand for 10 minutes at 25°C and the absorbance measured against a blank solution at ƛmax = 230 nm. The herbal formulation hydrogen peroxide scavenging capacity index (HPSCI) was calculated as follows:

$$\text{HPSCI\%} = \frac{\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Blank}}} \times 100 \quad (2)$$

The HPSCI% was expressed as SCI_{50}, which is defined as the concentration (μg/mL) of the herbal formulation required to scavenge 50% of H_2O_2.

2.6. Nitric oxide radical

The herbal formulation SCI against NO^- was assayed by the method of Green et al.[53] An assay mixture composed of 2.0 mL 10 mM NaN_3 and 1.0 mL of different concentrations (10–1500 μg/mL) of the herbal sample was incubated for 50 minutes at 25°C. A 1.5-mL aliquot of Griess reagent [1% sulfanilamide, 2% H_3PO_4, and 1% *N*-(1 naphthyl) ethylenediamine dihydrochloride] was added to the incubated mixture and the absorbance measured at ƛmax = 546 nm. Quercetin was used for the control sample. The herbal formulation nitric oxide scavenging capacity index (NOSCI) was calculated as follows:

$$\text{NOSCI\%} = 100 - \frac{\text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100 \quad (3)$$

The NOSCI% was expressed as SCI_{50}, which is defined as the concentration (μg/mL) of the herbal formulation required to scavenge 50% of NO^-.

2.6.1. Hydroxyl radical

The herbal formulation SCI against OH^- was determined as a measure of inhibition of deoxyribose degradation in the presence of the herbal formulation according to the method of Ohkawa et al[54] with modifications.[38] Briefly, 100 μL of different concentrations (10–1500 μg/mL) of the herbal sample was added to a reaction mixture containing 200 μL of 2.8 mM 2-deoxy-D-ribose, 400 μL of 200 μM $FeCl_3$, 100 μL of 1.0 mM EDTA, 200 μL of 1.0 mM ascorbic acid, 200 μL of 1.0 mM H_2O_2, 20 mM phosphate buffer (pH = 7.4), and made up to a final volume of 3.0 mL with distilled water. The assay mixture was incubated at 37°C for 60 minutes. Next, 1.0 mL of 1% thiobarbituric acid (TBA) and 1.5 mL of 2.8% trichloracetic acid (TCA) were added and further incubated at 100°C for 30 minutes. After cooling to 25°C, the absorbance of the assay mixture was measured at ƛmax = 532 nm against a control containing deoxyribose and phosphate buffer. Inhibition of deoxyribose degradation (IDRD), which was equivalent to the herbal formulation SCI, was calculated as follows:

$$\text{IDRD\%} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100 \quad (4)$$

The IDRD% was expressed as SCI_{50}, which is defined as the concentration (μg/mL) of the herbal formulation required to scavenge 50% of OH^- radical.

2.6.2. 2,2-Diphenyl-1-picrylhydrazyl radical

Measurement of the herbal formulation SCI against the DPPH radical was by the method of Wang et al[29] with minor modifications according to Gyamfi et al.[55] Briefly, 0.1 mL of different concentrations (10–1500 μg/mL) of the herbal sample was added to 3.9 mL of DPPH (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) solution in 0.1 mmol/L of C_2H_5OH (BDH, UK) with 450 μL of 50 mM Tris–HCl buffer (pH = 7.4) and mixed quickly. The assay mixture was allowed to stand for 60 minutes at 37°C and the absorbance was measured at ƛmax = 517 nm. The blank sample consisted of DPPH in ethanol only. The herbal formulation DPPH scavenging capacity index (DSCI) was calculated as follows:

$$\text{DSCI\%} = \left(1 - \frac{\text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Blank}}}\right) \times 100 \quad (5)$$

The DSCI% was expressed as SCI_{50}, which is defined as the concentration (μg/mL) of the herbal formulation required to scavenge 50% of DPPH radicals.

2.7. Statistical analysis

The data collected were analyzed using the analysis of variance procedure whereas treatment means were separated using the

Table 1
Phytochemicals of leaf extracts of *Acanthus montanus*, *Asystasia gangetica*, *Emilia coccinea*, and *Hibiscus rosasinensis*.

	Concentration (mg/g dry sample)			
Samples	Alkaloids	Flavonoids	Saponins	Tannins
ACMO	$177.25 \pm 5.26^{b,c}$	561.00 ± 9.50^{a}	71.00 ± 6.55^{a}	$26.50 \pm 5.50^{a,b,c}$
ASGA	188.25 ± 4.56^{b}	450.50 ± 8.48^{b}	$44.50 \pm 7.59^{b,c}$	33.75 ± 5.19^{a}
EMCO	352.75 ± 8.17^{a}	345.00 ± 6.96^{d}	$43.50 \pm 7.94^{b,c,d}$	$29.50 \pm 3.70^{a,b}$
HIRO	70.00 ± 4.72^{d}	$425.00 \pm 6.46^{b,c}$	45.25 ± 9.05^{b}	$25.50 \pm 8.74^{a,b,c,d}$

Data are the mean ± SD of six ($n = 6$) determinations.
[a,b,c,d] Means denoted by the same letter are not significantly different at $p > 0.05$ according to LSD.
ACMO = *Acanthus montanus* (Nees) T. Anderson; ASGA = *Asystasia gangetica* L.T. Anderson; EMCO = *Emilia coccinea* (SIMS) G. Don; HIRO = *Hibiscus rosasinensis* L.; LSD = least significance difference; SD = standard deviation.

least significance difference (LSD) incorporated in the Statistical Analysis System (SAS) package version 9.1 (2006).

3. Results

Table 1 showed that among the four herbal samples, EMCO gave the highest concentration of alkaloids. The concentration of alkaloids of ACMO was not significantly different ($p > 0.05$) from that of ASGA. Specifically, $[\text{alkaloids}]_{ASGA} = 188.25 \pm 4.56$ mg/g sample and $[\text{alkaloids}]_{ACMO} = 177.25 \pm 5.26$ mg/g sample; $p > 0.05$. HIRO gave the lowest concentration of alkaloids, which was 5.03 folds higher than that of EMCO; $p < 0.05$. The concentration of flavonoids in HIRO was not significantly different ($p > 0.05$) from ASGA. ACMO gave the highest concentration of flavonoids, whereas EMCO registered the lowest concentration of flavonoids, which represented 38.50% less than that of ACMO; $p < 0.05$. Comparatively, the flavonoids were the most abundant phytochemical present in the four herbal samples. Saponin concentrations of ASGA, EMCO, and HIRO exhibited no significant difference ($p > 0.05$). Specifically, saponin concentrations of the four herbal samples were within the range of 45.50 ± 7.94 mg/g to 71.01 ± 6.55 mg/g sample. ACMO gave the highest concentration of saponin. The concentration of tannin in the four herbal samples varied within a relatively narrow range of 25.50 ± 8.74 mg/g to 33.75 ± 5.19 mg/g sample. In addition, tannin was the lowest phytochemical present in the four herbal samples.

Fig. 1 showed that SHfs SCI_{50} against superoxide (O_2^-) radicals (SOR) was within the range of 501.88 ± 13.11 μg/mL to 734.15 ± 11.23 μg/mL, which was in the order SHf-ACMO > SHf-HIRO > SHf-SHf-EMCO > SHf-ASGA; $p < 0.05$. Specifically, SHf-ASGA exhibited the highest SCI_{50} against hydrogen peroxide (HP); $SCI_{50:SHf\text{-}ASGA} = 208.09 \pm 8.41$ μg/mL, whereas SHf-EMCO gave the lowest SCI_{50} against HP; $SCI_{50:SHf\text{-}EMCO} = 102.01 \pm 9.61$ μg/mL. The SCI_{50} of SHf-EMCO against nitric oxide (NO^-) radicals (NOR) was 1.84 fold > $SCI_{50:SHf\text{-}ASGA}$; $p < 0.05$. Also, SHf-ACMO SCI_{50} against hydroxyl (OH^-) radicals (HR) was significantly different ($p < 0.05$) from that of SHf-ASGA. SHf-EMCO exhibited the lowest SCI_{50} against NO^-; $SCI_{50:SHf\text{-}EMCO} = 590.43 \pm 8.23$ μg/mL. The four SHfs SCI_{50} against DPPH radicals were in the order: SHf-HIRO = 675.85 ± 7.34 μg/mL > SHf-ACMO = 515.26 ± 6.41 μg/mL > SHf-EMCO = 404.67 ± 6.92 μg/mL > SHf-ASGA = 393.39 ± 5.11 μg/mL.

A cursory inspection of Fig. 2 showed that DHfs SCI_{50} against SOR was within a narrow variability (623.40 ± 10.92 μg/mL to 691.80 ± 12.62 μg/mL); except $SCI_{50:SHf\text{-}AGEC} = 553.23 \pm 11.45$ μg/mL; $p < 0.05$. DHf-AGAM and DHf-AGEC exhibited equal SCI_{50} against HP, which represented the highest capacity of the six DHfs to eliminate HP. The SCI_{50} of DHf-AGHR, DHf-AMEC, and DHf-ECHR showed no significant difference ($p > 0.05$) in their capacities to eliminate HP. Dhf-ECHR gave the highest SCI_{50} against NO^-. DHf-AGEC, DHf-AGHR, DHf-AMEC, and DHf-AMHR exhibited approximately equal capacities to eliminate NO^-. The six DHfs SCI_{50} was within the range of 909.60 ± 10.49 μg/mL to 1127.45 ± 14.95 μg/mL, which was in the order DHf-AGAM = 1127.45 ± 14.95 μg/mL > DHf-AGHR = 1065.68 ± 15.68 μg/mL > DHf-AMHR = 1028.95 ± 14.41 μg/mL > DHf-AMEC = 979.75 ± 11.45 μg/mL > DHf-AGEC = 929.45 ± 11.61 μg/mL > DHf-ECHR = 909.60 ± 10.49 μg/mL. DHf-AMHR exhibited the highest SCI_{50} against DPPH radicals, whereas $SCI_{50:DHf\text{-}AMEC}$ against DPPH radicals = 506.90 ± 6.49 μg/mL, which represented 1.21 folds < $SCI_{50:DHf\text{-}AMHR}$ against DPPH radicals.

Fig. 3 showed that QHf exhibited greater SCI_{50} than THfs except with respect to NO^-. The THfs SCI_{50} against SOR and HP were within narrow ranges of (603.63 ± 16.23 μg/mL to 665.04 ± 17.89 μg/mL) and (190.74 ± 11.45 μg/mL to 209.28 ± 9.28 μg/mL), respectively. The $SCI_{50:THf\text{-}AMEH} = 606.73 \pm 15.11$ μg/mL against NO^- was 4.25 folds > $SCI_{50:THf\text{-}AGEH}$. The THfs and QHf SCI_{50} against HR were comparatively higher than against other investigated radicals. Also, the THfs SCI_{50} against DPPH radicals were of approximate equal magnitudes, except that of QHf SCI_{50}. A general overview of Figs. 1–3 showed that the SHfs, DHfs, THfs, and QHf SCI_{50} against the investigated radicals followed the same order of HR > SOR > DPPH radicals > HP > NOR.

From comparative analyses of Figs. 1–3, herbal formulations SCI_{50} against SOR were within the range of 501.88 ± 13.11 μg/mL to 780.22 ± 21.41 μg/mL. The SCI_{50} of SHf-ASGA = 501.88 ± 13.11 μg/mL, SHf-EMCO = 580.73 ± 14.29 μg/mL, and DHf-AGEC = 553.23 ± 11.45 μg/mL against SOR were significantly low

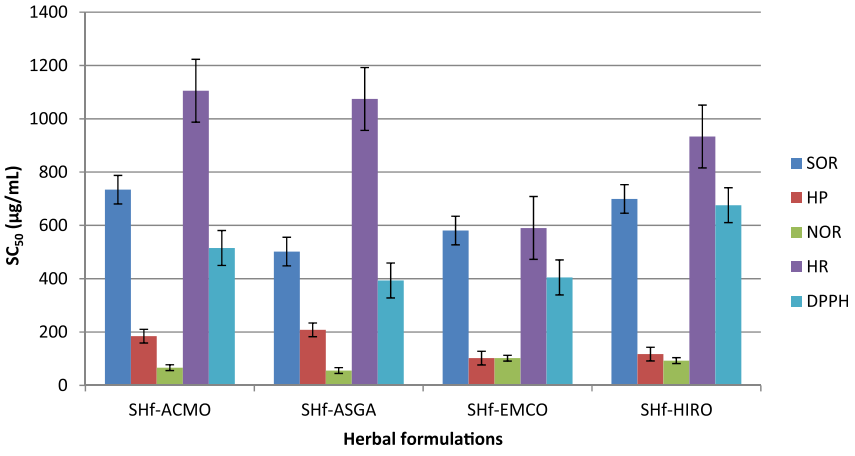

Fig. 1. Radical scavenging capacities of single herbal formulations.

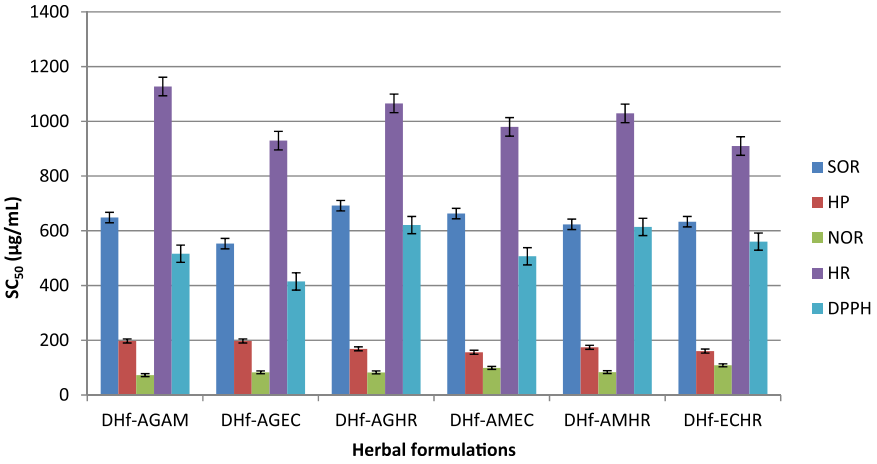

Fig. 2. Radical scavenging capacities of double herbal formulations. SOR: Superoxide radicals; HP: Hydrogen peroxide; NOR: Nitric oxide radicals; HR: Hydroxyl radicals; DPPH: 2, 2-diphenyl-1-picrylhydrazyl.

($p < 0.05$) compared to that of other herbal formulations (Figs. 1 and 2). Conversely, the SCI_{50} of SHf-ACMO = 734.15 ± 11.23 µg/mL and QHf-AAEH = 780.22 ± 21.41 µg/mL against SOR were comparatively high with respect to other herbal formulations; $p < 0.05$ (Figs. 1 and 3).

The SCI_{50} of QHf-AAEH = 334.67 ± 17.21 µg/mL against HP gave the highest value amongst the various herbal formulations, whereas SHf-EMCO = 102.01 ± 9.61 µg/mL was the lowest SCI_{50} against HP (Figs. 1 and 3). The SCI_{50} of SHf-HIRO = 117.22 ± 10.11 µg/mL against HP was not significantly different ($p < 0.05$) from that of SHf-EMCO = 102.01 ± 9.61 µg/mL. Furthermore, the SHf-EMCO and SHf-HIRO SCI_{50} against SOR and DPPH radicals were comparatively lower than that of SHf-ACMO and SHf-ASGA; $p < 0.05$. The DHfs SCI_{50} against NO^- radicals were comparatively of approximate magnitude; $p > 0.05$, expect that of DHf-ECHR. Generally, the variations in SCI_{50} of the SHfs and DHfs were comparatively of approximate magnitude, exemplified by the comparatively narrow range of their SCI_{50} values: (55.33 ± 6.11 to 83.58 ± 6.98 µg/mL) except SCI_{50} of DHf-ECHR = 108.45 ± 9.11 µg/mL (Figs. 1 and 2). The THf-AMEH peak SCI_{50} against NOR = 606.73 ± 15.11 µg/mL.

The variations in SCI_{50} of the DHfs and THfs were comparatively within a narrow range of 909.60 ± 10.4 µg/mL to 1132.50 ± 31.44 µg/mL (Figs. 2 and 3). The SCI_{50} of SHf-EMCO = 590.43 ± 8.23 µg/mL against HR gave the lowest value, whereas QHf-AAEH peak SCI_{50} against HR was the highest registered value compared to the other various herbal formulations. Also, SHf-HIRO SCI_{50} against DPPH radicals was the highest amongst the other herbal formulations. Conversely, SCI_{50} of SHf-ASGA = 393.39 ± 5.11 µg/mL was the lowest value compared to other herbal formulations. The herbal formulation SCI_{50} against the investigated radicals were in the order QHf > THfs > DHfs > SHfs (Figs. 1–3).

4. Discussion

From the present findings, the composite four leaf extracts of *A. montanus*, *E. coccinea*, *H. rosasinensis*, and *A. gangetica* used for the herbal formulations contained impressive levels of antioxidant phytochemicals. Previous reports have shown that the free radical scavenging property of alkaloids is associated with their anti-inflammatory, anticarcinogenic, antibacterial, and antiviral activities.[56,57] Furthermore, medicinal plants that are rich in alkaloids have been applied for the treatment of cardiovascular diseases (CVDs), cancer, and age-related conditions such as Alzheimer's disease or dementia because of the strong antioxidant activity of the alkaloids.[58,59] The presence of antioxidant phytochemicals, especially the flavonoids, in the leaf extracts contributed to the radical scavenging effects of the herbal formulations. The flavonoids have been confirmed to be one of the several phytochemicals

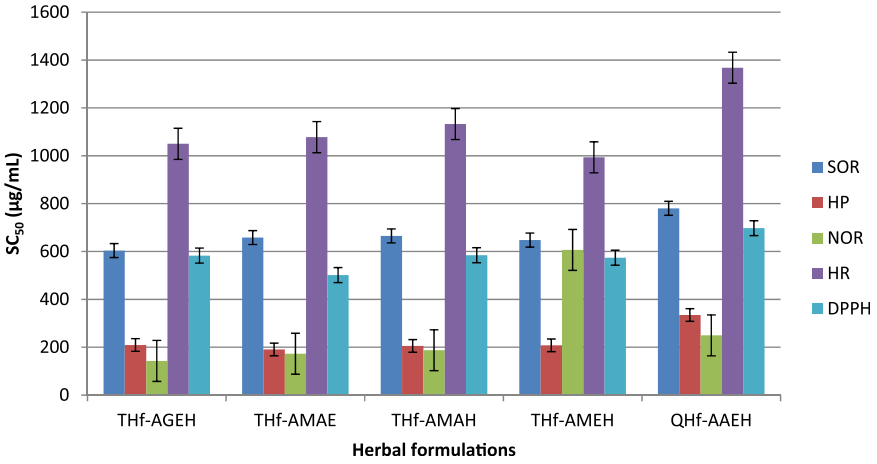

Fig. 3. Radical scavenging capacities of triple and quadruple herbal formulations. SOR: Superoxide radicals; HP: Hydrogen peroxide; NOR: Nitric oxide radicals; HR: Hydroxyl radicals; DPPH: 2, 2-diphenyl-1-picrylhydrazyl.

with antioxidant activity against deleterious RONS.[8,60,61] Furthermore, reports have suggested that flavonoids are the major active ingredients in plant materials that account for the ameliorative effects against pathophysiologic disorders such as diabetes mellitus,[62] tissue inflammation,[62] hemolysis,[61,63] and hypertension,[64] which are etiologically linked to oxidative stress.

Previous studies had shown that the saponin extract from *Hedera helix* L. (Araliaceae),[65] defatted kenaf (*Hibiscus cannabinus* L.) seed meal,[66] and *Chlorophytum borivilianum*[67] exhibited antioxidant activity *in vitro*. These findings suggested that radical scavenging properties of the experimental herbal formulations were connected with saponin content in the various plant extracts. The antioxidant activities of tannins have been reported elsewhere.[25,68–70] In a previous related study, Gu et al,[69] using the hydroxyl radical scavenging activities of 2-deoxyribose oxidation and salicylic acid systems, superoxide anion scavenging, and linoleic acid lipid peroxidation inhibition reactions, reported that tannic extracts from *Diospyros kaki* L. (persimmon) pulp exhibited antioxidant activity in a dose-dependent manner. By implication, the presence of tannins in the various experimental herbal formulations could have facilitated the radical scavenging activities as presently reported.

From general principles and by definition, the present study showed that the experimentally derived SCI_{50} was inversely proportional to the herbal formulation SCI against the radicals. An overview of the results in Figs. 1–3 showed that the various herbal formulations exhibited inconsistent and ambivalent antioxidant activities in terms of their comparative radical scavenging capabilities. For instance, the composite leaf extracts of the herbal formulations displayed synergy and increased effectiveness to neutralize the investigated radicals, exemplified by comparative decreased SCI_{50} of the integrated herbal formulations against the radicals. Specifically, the elevated SCI_{50} of DHf-AGAM = 1127.48 ± 14.95 μg/mL against HR relative to that of DHf-AGEC = 929.45 ± 11.61 μg/mL was an indication that DHf-AGEC had a greater SCI against HR than that of DHf-AGAM (Fig. 2). The additive and synergistic bioactivity of phytochemicals in polyherbal extracts have been described by previous authors.[8,24,26,36,41] According to Liu,[24] phytochemicals are potent antioxidant and anticancer compounds for which the nutraceutical benefits of diets rich in fruits and vegetables are attributed to the complex mixture of phytochemicals, which has been corroborated elsewhere.[8,71,72] They further emphasized that no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables to achieve therapeutic benefits.[24] Additionally, *Camellia sinensis* (green tea) mixed with some selected herbs exhibited synergistic antioxidant activity that was comparable to standard ascorbic acid against free radicals.[26] From another perspective, the display of antagonism in bioactivity potentials amongst phytochemicals could affect the efficacy of crude extracts in traditional medicine.[27] Specifically, Milugo et al,[27] noted that the antioxidant activity of *Rauvolfia caffra*, which was comparable to quercetin, was ironically reduced by the antagonistic effect of co-occurrence of alkaloids and saponins in the herbal extract. In that regard, they proposed that exclusion of saponins and alkaloids from the herbal extract would increase the efficacy of *R. caffra* extracts in ameliorating oxidative stress. Intuitively, the comparative lower capacity of QHf to scavenge HR than those of SHfs and DHfs (Figs. 2 and 3) were outcomes of antagonistic interactions amongst the phytochemicals of the constituent herbal extracts.

5. Conclusion

From the results of the present study, the display of synergy or antagonism by the composite herbal extracts to neutralize the investigated radicals depended on the type and number of individual herbal extract used in constituting the herbal formulations. Furthermore, a broad survey of 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*.

Conflicts of interest

All authors declare no conflicts of interest.

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