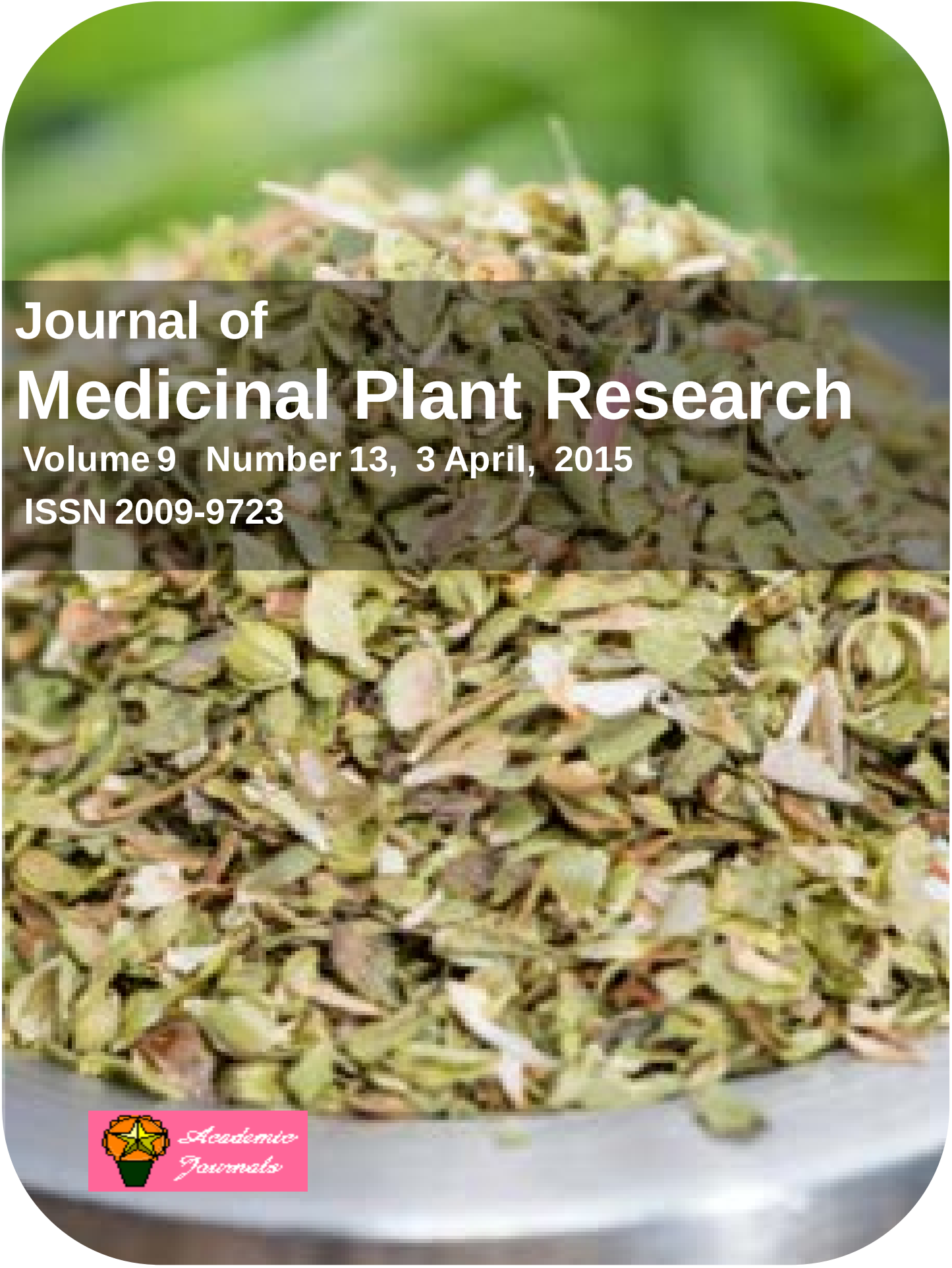

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Table of Contents: Volume 9 Number 13, 3 April, 2015

ARTICLES

Research Articles

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Full Length Research Paper

Morelloflavone and its semisynthetic derivatives as potential novel inhibitors of cysteine and serine proteases

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This article reports the three biflavonoids isolated from the fruit pericarp of *Garcinia brasiliensis* Mart. (Clusiaceae): Morelloflavone-4```-glycoside (compound 1), (±)-Fukugiside (compound 2), and Morelloflavone (compound 3). Structural modifications by acylation and alkylation reactions were performed on the natural biflavonoid (±) morelloflavone to obtain three semisynthetic compounds: Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (compound 4), Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (compound 5), and Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (compound 6). The inhibitory effects of these naturally isolated biflavonoid-type compounds and three semisynthetic derivatives on the activity of the cysteine proteases papain and cruzain, and on the serine protease trypsin were investigated. The potential inhibitory IC_{50} of natural bioflavonoids compounds 1, 2, and 3 were 11.0 ± 3.0, 23.0 ± 4.0, and 10.5 ± 0.3 µM, respectively, for papain; 0.86 ± 0.12, 106 ± 7, and 3.8 ± 0.1, 50 ± 2, 119.5 ± 5, and 9.6 ± 1.0 µM, respectively, for cruzain. On the other hand, the semisynthetic biflavonoids compounds 4, 5, and 6 were more efficient in the inhibition of enzyme activity with IC_{50} values 0.60 ± 0.02 µM (papain) for biflavonoids compound 4, 1.64 ± 0.11 µM (trypsin) for biflavonoids compound 5, and 8.1 ± 0.6 µM (cruzain) for biflavonoids compound 6. Compound 4 is more active owing to the carbonyl group in the structure; perhaps, this modification could favor a higher nucleophilic attack by serine and cysteine proteases. However, the semisynthetic compound 5 (IC_{50} = 15.4 ± 0.7 µM for papain), which has no carbonyl group in structure, was less active in the inhibition. Interestingly, structure–activity relationships (SARs) were confirmed by flexible docking simulations. Likewise, the potential usefulness of natural compound 1 as an antioxidant compound was strengthened by our results concerning the antiproteolytic activity.

Key words: *Garcinia brasiliensis*, biflavonoids, proteases, antiproteolytic activity.

INTRODUCTION

The Guttiferae family, also known as Clusiaceae, belongs to the angiosperm phylogeny group and is characterized by the conspicuous presence of latex in most of their species. This family consists of 47 genera such as *Vismia, Garcinia, Clusia, Cratoxylum, Harungana, Mesua, Hypericum* and *Kielmeyera,* and more than 1000 species that are grouped in six subfamilies widely spread all over Brazil (Piccinelli et al., 2005; Derogis et al., 2008). The *Garcinia brasiliensis* species is native to the Amazon and is cultivated throughout Brazilian territory where it is known as "bacupari." It is a tree of medium size that blooms from August to September. It has yellow fruit with a white and edible mucilaginous pulp used in folk medicine as a wound healing agent and for peptic ulcer, urinary, and tumor disease treatments (Corrêa, 1978; Santa-Cecília et al., 2011).

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). In this system, enzymes are given an Enzyme Commission number (EC). However, proteases do not comply easily with the general system of enzyme nomenclature due to their broad structural and functional diversity. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Rawlings and Barett, 1995). Proteases or peptidases are enzymes that catalyze reactions on peptide chains, hydrolyzing them into short fragments. Endopeptidases split the peptide bond between amino acid residues placed within the ribbon, while exopeptidases split the residues at the polymeric backbone end. These enzymes can be further joined according to the reactant groups that are present in the catalytic site, as serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic acid (EC 3.4.23), metalo (EC 3.4.24) and threonine (EC 3.4.25) proteases. Papain (EC 3.4.22.2), a plant cysteine protease isolated from *Carica papaya* latex, preferentially cleaves peptide chains on either Arg and Lys residues or hydrophobic Phe residues (Brocklehurst, 1987; Martins et al., 2009). Trypsin (E.C. 3.4.21.4) has specificity for peptides containing Arg and Lys residues, and its catalytic triad is composed of the amino acids serine, histidine and aspartate, which is similar to other serine proteases (Martins et al., 2009; Beynon, 1989). Each amino acid of the triad has a specific role in the peptide bond cleavage of the substrates. The carboxylate group of the aspartic acid forms a hydrogen bond to the amine nitrogen atom of the histidine residue, contributing to increase the electronegativity of the imine nitrogen within the same heterocyclic side chain. In this way, the free electron pair of this last imidazolyl nitrogen atom is devoted to accepting the hydrogen from the serine hydroxyl group, thereby enhancing the nucleophilic attack by this serine residue on the carbonyl carbon atom of the peptide bond that is properly oriented into the enzymatic active site.

Cruzain is a member of a large family of closely-related isoforms found in the parasite *Trypanosoma cruzi*. It is involved in intracellular replication and differentiation, and is essential at all stages of the parasite's life cycle (Campetella et al., 1992; Lima et al., 1994). Since currently available therapeutics are both ineffective against the acute phase of the disease and are too toxic prolonged administration, efforts have been made to identify novel therapeutic targets, among which the proteolytic apparatus of *T. cruzi* is a possible candidate (McKerrow et al., 2009).

In this work, the bioactivity of three natural and three semisynthetic biflavonoid compounds were reported as part of our continuous study of *G. brasiliensis* (Gontijo et al., 2012a, b). The potential enzymatic inhibitory effects of the natural and semisynthetic compounds were evaluated against papain, trypsin, and cruzain. In addition, flexible molecular docking simulations with papain, trypsin, and cruzain were performed.

MATERIALS AND METHODS

Plant materials

Fruit pericarps of *G. brasiliensis* Mart. were collected at the campus of the Federal University of Viçosa-MG, Brazil, in February (summer) 2009. Botanical identification was performed in the Horto Botânico of the Federal University of Viçosa by Dr. João Augusto Alves Meira Neto. A voucher specimen (number VIC2604) was deposited at the Herbarium of the Federal University of Viçosa.

General considerations

Reagents were used as received from commercial suppliers or dried by standard methods and recondensed or distilled before use. The reactions were extracted with CH_2Cl_2. The organic phase was separated and dried over Na_2SO_4. If necessary the residue was purified by column chromatography on silica, eluting with CH_2Cl_2 (100%). NMR spectra were measured on a multinuclear FT-NMR spectrometer (Bruker AVANCE DRX-400 or Bruker AVANCE DPX-200 MHz and DRX-500 MHz). ^{1}H and ^{13}C chemical shifts are δ values and given in ppm relative to Me_4Si. Coupling constants refer to H–H (^{1}H NMR) or (^{13}C NMR) unless stated otherwise. Molecular masses where determined by MALDI-TOF mass spectrometry (LCMS-2020EV) equipped with an ESI probe (Shimadzu), which was connected to the circuit after the UFLC SPD-20A UV detector.

Analyses were detected at 220 and 272 nm. Melting points were

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determined in sealed capillaries and uncorrected. Elemental analysis was performed using a TruSPEC (CHN-S) Analyzer by Leco Instruments, as described by Gontijo et al. (2012b). Protein Preparation Wizard protocol, Schrödinger Suite (Schrödinger Suite, 2009a).

The natural compounds were obtained from the ethyl acetate (EtOAc) extract of the fruit pericarp of *G. brasiliensis;* they were purified either by column chromatography on silica, eluting with hexane/ethyl acetate (60:40) or by Sephadex LH-20 eluting with MeOH. The isolated biflavonoids were identified as morelloflavone-4```-glycoside (compound 1) (Gontijo et al., 2012a), (±)-Fukugiside (compound 2) (Elfita et al., 2009) and morelloflavone (compound 3) (Li et al., 2002).

The three semisynthetic derivatives of morelloflavone (compound 3) (Figure 1) (compounds 4, 5 and 6) were obtained from acylation and alkylation reactions.

Enzymes

The enzymes cruzain and rCPB2.8 (recombinant cysteine protease type B) were generously supplied by Dr. Luiz Juliano (Department of Biophysics, Federal University of São Paulo, Brazil), trypsin and papain were from Merck (Darmstadt, Germany), TLCK (N-alpha-tosyl-L-lysinyl-chloromethylketone), E-64 (1-[[(Ltrans-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane), and fluorogenic substrate Z-FR-MCA (carbobenzyloxy-Phe-Arg-(7-amino-4-methylcoumarin) were obtained from Sigma-Aldrich Sigma (St. Louis, USA). Substrate hydrolyses were monitored in a spectrofluorometer F2500 Hitachi, and the enzymatic molar concentrations were estimated by titration according to kinetic parameters (Martins et al., 2009).

Inhibition assays

Compounds 1 to 6 were tested for their inhibitory potential on papain, cruzain, and trypsin by spectrofluorometric method. Enzyme inhibition was expressed as the compound concentrations causing a 50% decrease in enzyme activity (IC_{50} values). IC_{50} values were calculated by time-course, dose-response curves using inhibitors at different concentrations, and the data were analyzed in Grafit 5.0 software using Equation 1.

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}} \right)^{s}} \quad (1)$$

In Equation 1, Range is the fitted uninhibited value, *y* is the enzyme activity, *x* is the inhibitor concentration, and *s* is a slope factor. The equation assumes that *y* reduces with increasing *x*.

Inhibition reversibility assays for enzymes were carried out in 100 mM sodium acetate buffer, pH 5.5, 20% glycerol (to stabilize the enzymes), 5 mM EDTA, 5 mM dithiothreitol (DTT) (as cysteine protease papain and cruzain activator), and the enzyme solutions pre-activated for 5 min, at 37°C. The trypsin assays was carried out in 100 mM Trizma base HCl buffer, pH 7.5, 10% glycerol (to stabilize the enzyme). The substrate Z-FR-MCA was used as fluorogenic probe, and the hydrolysis of the substrate was monitored in a spectrofluorometer Hitachi F2500 at wavelength λ_{Ex} = 360 nm (excitation) and λ_{Em} = 480 nm (emission); the reference inhibitors used were E-64 for papain and cruzain, and TLCK for trypsin. The Z-FR-MCA concentrations were 18.5 µM at cruzain (K_M = 1.8 µM), 7.4 µM at papain (K_M = 0.7 µM) and 10.9 µM at trypsin (K_M = 1.1 µM). The substrate concentrations were almost 10-fold over the K_M for all enzymes in the study.

Computational docking

In the docking studies, the 1.8 Å resolution structure files for trypsin, papain and cruzain were downloaded from the Brookhaven Protein Data Bank (PDB code 2RA3, 1PE6 and 1F2C, respectively) (Assis et al., 2012). This structure was prepared using the Protein Preparation Wizard protocol implemented in the Schrödinger Suite (2009a). After the protein preparation step, the docking procedure was carried out using the Induced Fit Protocol (IFD) Suite (Schrödinger Suite, 2009b).

The top 20 poses for each test ligand (with regard to the GlideScore) from the initial softened-potential docking step, were retained to sample the protein plasticity using the prime program from the Schrödinger, LLC suite. In stage 2, a conformational search and minimization were carried out on residues having at least one atom within 5.0 Å of distance to the ligand for each of the 20 ligand-protein complexes. In stage 3, the 20 induced conformations created for each inhibitor were ranked by the total prime energy (molecular mechanics plus solvation) of the complexes. Those within 30 kcal mol^{-1} of the minimum energy structure were subjected to re-docking with the ligands and scored using glide. Glide XP (extra precision) was used for all re-docking calculations. The binding affinity of each complex was established through the GlideScore, which approximates the Gibbs free energy. The more negative the value of the GlideScore, the more favorable the binding (Jacobson et al., 2004). Maestro 9.0 was used as the graphical user interface (GUI) for all of Schrödinger's computational programs (Maestro, 2009).

Data analysis

Statistical analysis was performed using Grafit 5.0 software. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± standard deviation (SD) unless otherwise specified.

RESULTS AND DISCUSSION

The natural biflavonoid compounds 1 to 3, semisynthetic compounds 4 to 6 and the reference compounds TLCK and E64 were assayed against the cysteine proteases papain and cruzain and the serine protease trypsin to determine the potential inhibitory effects as quantified in terms of IC_{50} values (Table 1). The results show that all compounds tested inhibit the assayed proteases with different degrees of selectivity. The derivative compounds 4 and 5 were the most potent inhibitors to papain (IC_{50} = 0.60 ± 0.02 µM) and trypsin (IC_{50} = 1.64 ± 0.11 µM), respectively.

From the results (Table 1), compound 4 has a 35-fold higher affinity to papain than to trypsin. On the other hand, compound 5 has a 9-fold higher affinity to trypsin compared to papain. The results therefore showed that only compounds 4 and 5 exhibit a substantial degree of selectivity between the cysteine and serine proteases. However, these same compounds (4 and 5) did not efficiently inhibit the cysteine protease cruzain, where we

Table 1. Quantitative *in vitro* inhibitory effects of natural and semisynthetic biflavonoid derivatives on the cysteine proteases cruzain and papain and the serine peptidase trypsin.

Compound	IC_{50} value[a] (μM)		
	Papain	Trypsin	Cruzain
1	11.00 ± 3.00	0.86 ± 0.15	50 ± 2
2	23.00 ± 4.00	106 ± 7.00	119 ± 5
3	10.5 ± 0.3	3.8 ± 0.1	9.6 ± 1
4	0.60 ± 0.02	21.1 ± 1.3	537 ± 63
5	15.4 ± 0.7	1.64 ± 0.11	235 ± 38
6	15.7 ± 0.5	19.8 ± 1.3	8.1 ± 0.6
TLCK[c]	-	11.8 ± 0.6	-
E64[c]	0.0013 ± 0.0001	-	0.0068 ± 0.0002

[a]Each IC_{50} value represents the mean ± standard deviation of triplicate determined by the software Grafit 5.0. [b]ND: IC_{50} value not established by the sensitive method employed. [c]The compounds E-64 and TLCK were used as reference inhibitors of the corresponding proteases where they have been assayed.

found the IC_{50} values of 537 ± 93 and 235 ± 38 μM, respectively. Comparing the inhibitory effects of both natural and semisynthetic biflavonoid compounds against the trypanossomatid cysteine proteases rCPB2.8 and rCPB3 from *L. mexicana* (Gontijo et al., 2012b) and cruzain from *T. cruzi*, it is clear that the biflavonoids studied here also showed species selectivity. The derivative compounds 4 and 5 are 801- and 335-fold more potent inhibitors for rCPB2.8 and rCPB3, respectively, than for cruzain (Data not shown, Table 1) (Gontijo et al., 2012b).

As shown in Table 1, the inhibitory potential (IC_{50}) of compound 1 was approximately 123 and 4.4-fold higher than compounds 2 and 3, respectively, against trypsin. Compound 1 also displayed 2.1 and 2.4-fold higher inhibition against papain and cruzain than compound 2, respectively. Compared to compound 1, compound 3 showed a similar inhibitory effect against papain, but was more active against cruzain (5.2-fold). Excluding the natural glycoside compound 2, compounds 1 and 3 and the semisynthetic compounds 4, 5 and 6 showed IC_{50} values ranging from 0.6 to 15.7 μM against papain, and from 0.86 to 21.1 μM against trypsin.

As observed in Table 1, cruzain was poorly inhibited by both the natural and semisynthetic compounds. However, the biflavonoid compounds 3 (natural) and 6 (semisynthetic) were significantly effective in the inhibition of cruzain with IC_{50} values of 9.6 ± 1.0 and 8.1 ± 0.6 μM, respectively. The derivative compounds 4 and 5 showed different degrees of inhibition of proteases, although the compound 4 bound preferentially to papain (IC_{50} = 0.60 ± 0.02 μM) making it a potential antiproteolytic. It is important to highlight that the classical reference irreversible inhibitor of serine proteases, TLCK, was 7-fold less effective at trypsin inhibition (IC_{50} = 11.8 ± 1.3 μM) than compound 5. On the other hand, the cysteine protease irreversible inhibitor E64 is 4614-fold and 78970-fold more effective in the inhibition of papain and cruzain, respectively. All three tested enzymes are known as trypsin-like hydrolases due to S_1 specificity for both Arg and Lys basic residues, cleaving the C–N bond of substrate Z-FR-MCA between the Arg amino acid and MCA group (Assis et al., 2012).

At this point, the following interesting structure–activity relationship could be proposed: the capacity to inhibit the enzymatic activity of papain decreases according to the number of carbons in the R_1=R_2=R_3=R_4=R_5 ligands (acetyl, methyl or butanoyl ligands) attached at the biflavonoid core; the absence of carbonyl group in compound 5 increases the potential inhibitory effect over trypsin by almost 13-fold.

The docking simulations of compounds with enzymes (Figure 2) showed very similar modes of binding. The amino acid sequences of papain and cruzain are 38% homologous, and their active sites are nearly identical (Assis et al., 2012; Melo et al., 2001; PDB ID, 1996). Papain and trypsin were selected because they are commercially available in large quantities, and their structures are known. The hydrophobic interactions and hydrogen bonds for compound 3 in papain, trypsin and cruzain were related previously (Martins et al., 2007, 2009; Assis et al., 2012; Sasaki et al., 1986, 1990).

According to the molecular docking simulations, papain establishes hydrophobic interactions with ring A and with rings A/B by the residues of the S_2 subsite with compound 1 (Figure 2A). One the other hand, hydrophobic interactions are established with ring C and ring A with residues of the S_2 subsite with compound 2 (Figure 2B).

Compounds 4, 5 and 6 are semisynthetic molecules derived from natural compound 3 (Figures 2C, 3C and 4C) with modifications in the five hydroxyl groups as shown in Figure 1. The acetyl, methyl and butanoyl groups of ring C from compounds 4 (Figure 2D), 5

Table 2. Values of established intramolecular hydrogen bonds of natural and semisynthetic biflavonoid derivatives on cysteine proteases cruzain and papain and serine peptidase trypsin.

Enzyme	H-Bonds														
	# 1	Amino acid	Å	# 2	Amino acid	Å	# 4	Amino acid	Å	# 5	Amino acid	Å	# 6	Amino acid	Å
	2A(O34)	VAL133(H)	2.21	2B(H73)	GLY66(O)	2.33	2D(O47)	TRP69(HE1)	1.76	2E(O34)	TYR67(HH)	1.81	2F(H93)	TRP69(HE1)	2.15
	2A(H82)	GLY66(O)	2.30	-	-	-	2D(O52)	LYS156(HZ1)	2.15	2E(O36)	ALA160(H)	2.23	2F(O52)	LYS156(HZ1)	2.03
Papain	2A(H72)	SER205(O)	2.14	-	-	-	2D(O54)	LYS156(HZ1)	2.06	-	-	-	-	-	-
	2A(H59)	SER131(O)	2.57	-	-	-	2D(H70)	GLY23(O)	1.99	-	-	-	-	-	-
	2A(H79)	ASP158(O)	1.91	-	-	-	2D(O56)	ALA160(H)	2.11	-	-	-	-	-	-
	3A(H74)	TRP151(O1P)	1.67	3B(H80)	SER190(HG)	1.84	3D(O52)	HIS57(HD1)	2.13	3E(H61)	SER214(O)	1.79	3F(H80)	SER214(O)	1.96
	3A(H79)	TRP215(O)	1.70	3B(O55)	CYS191(N)	2.86	3D(O54)	SER195(HG)	2.13	3E(O40)	GLY216(H)	2.12	-	-	-
	3A(H78)	ASP189(OD2)	2.06	3B(H79)	CYS191(O)	1.96	3D(H65)	CYS220(H)	2.09	-	-	-	-	-	-
Cruzain	3A(H77)	GLY216(O)	1.88	3B(H76)	GLN192(OE1)	2.11	-	-	-	-	-	-	-	-	-
	3A(O36)	ARG96(HH21)	1.97	3B(H66)	GLN192(OE1)	2.32	-	-	-	-	-	-	-	-	-
	-	-	-	3B(H82)	TRP215(O)	1.94	-	-	-	-	-	-	-	-	-
	-	-	-	3B(H75)	LYS97(O)	1.79	-	-	-	-	-	-	-	-	-
	4A(O37)	GLN19(HE22)	2.00	4B(H49)	GLY66(O)	1.99	4D(H70)	GLY23(O)	1.98	4E(O37)	GLN19(HE22)	2.23	4F(O35)	GLN19(HE22)	2.04
	4A(H72)	GLN21(O)	1.85	4B(H74)	LEU157(O)	1.87	4D(O54)	GLN156(HE21)	1.99	-	-	-	4F(H108)	ALA136(HB3)	2.13
	4A(H74)	GLY20(O)	1.88	4B(O48)	ASP158(O)	2.96	4D(O52)	GLN156(HE22)	2.27	-	-	-	-	-	-
Trypsin	4A(H78)	GLY66(H)	1.77	4B(H73)	ASP158(O)	2.05	-	-	-	-	-	-	-	-	-
	4A(H82)	LEU157(O)	1.96	4B(H79)	GLU205(OE1)	2.29	-	-	-	-	-	-	-	-	-
	4A(H71)	ASP158(O)	1.74	4B(H82)	GLU205(OE1)	1.62	-	-	-	-	-	-	-	-	-
	4A(O47)	ASP158(HA)	2.55	-	-	-	-	-	-	-	-	-	-	-	-

[#]Compound number; Å is extension of hydrogen bond in Angström.

(Figure 2E) and 6 (Figure 2F), respectively, are establishing hydrophobic interactions with the S_2 subsite of papain.

The hydroxyl groups from ring A of compound 1 are responsible for forming three hydrogen bounds (Table 2) with papain, and the OH- from the glycosyl group are forming another two H-bonds, and ring C is establishing a π-π stacking interaction between Trp^{69} from the S_2 subsite (Figure 2A). Compound 2 are forming H-bonds between OH- from ring A with Glu^{66}, and π-π stacking interaction between S_2 subsite with rings A`/B` (Figure 2A). Hydrogen bonds (Table 2) are established between compound 4 (Figure 2D) with Trp^{69}, Ala^{160}, Lys^{156}, and Gly^{23}; compound 5 (Figure 2E) with Tyr^{67} and Ala^{160}; and compound 6 (Figure 2F) with Lys^{156} and Trp^{69}.

Observing the molecular modeling of trypsin (Figure 3), we verified hydrophobic interactions between compound 1 (Figure 3A) at rings A`/B` with residues of S_1`, ring C` with residues of the S_2 subsite, and the glycosyl group with residues of amino acids that stabilize the S_1 subsite; on the other hand, compound 2 (Figure 3B) established hydrophobic interactions of the glycosyl group with amino acids of the S_2 subsite and Gly^{193}/Ser^{195}, which are responsible for forming the oxyanion hole of trypsin.

For interaction of compounds 4, 5 and 6 (derivatives from compound 3) with the serine protease trypsin, we verified hydrophobic interactions of acetyl groups of compound 4 (Figure 3D) at residues of S_1, S_1´, and S_3/S_4 subsites; a methyl group of compound 5 (Figure3E) with residues of S_1 subsite; and butanoyl groups of compound 6 (Figure 3F) with residues of S_1 and S_3/S_4

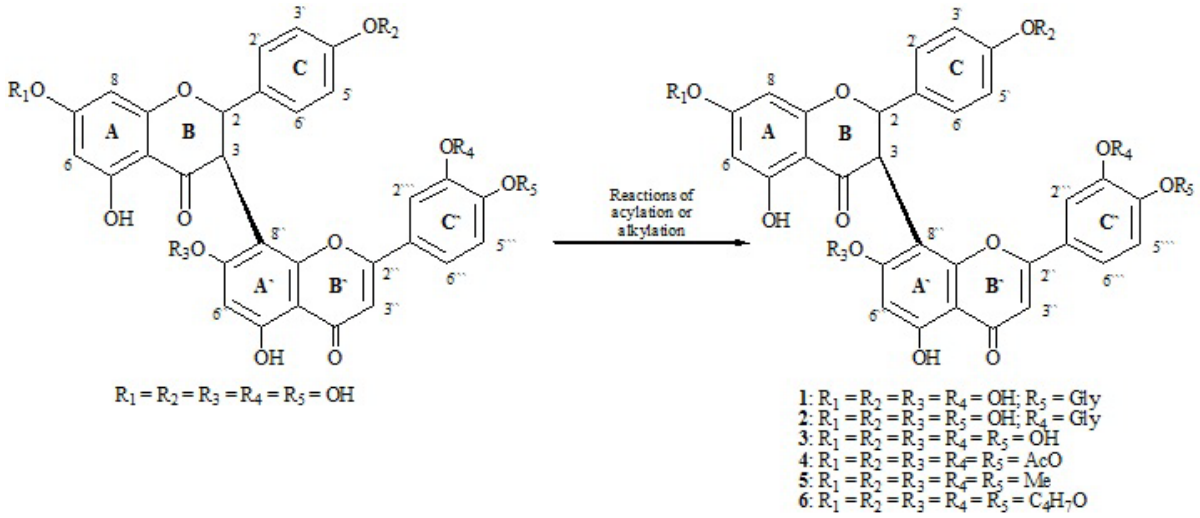

Figure 1. Structures of the natural and semisynthetic biflavonoid compounds **1 to 6**.

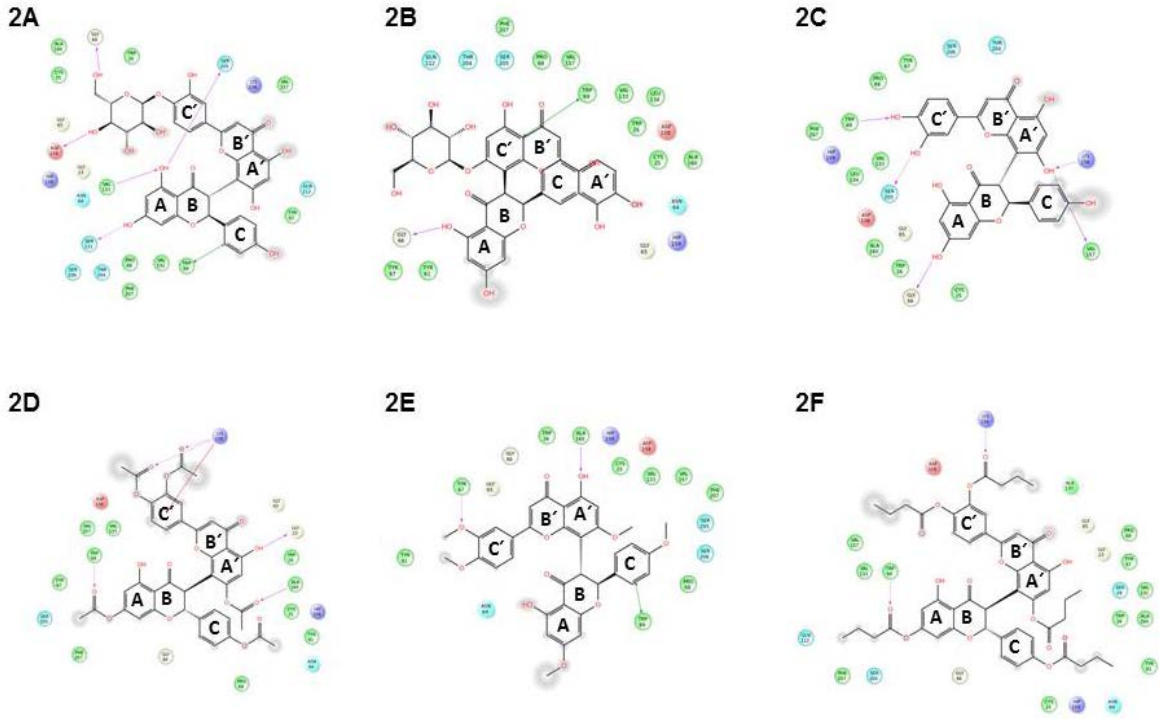

Figure 2. Molecular docking of compounds **1 to 6** with papain. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 2RA3 for papain (PDB ID, 1996). A) Morelloflavone-4```-glicoside (**1**)/papain; B) (±)-Fukugiside (**2**)/papain; C) Morelloflavone (**3**)/papain; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/papain; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/papain; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/papain.

subsites.

It is important to note that some amino acid residues of trypsin can occupy two subsites, as can be observed for Gln[192] (member of S_1 and S_{1}` subsites) and for residues of S_3 and S_4 subsites like Leu[99], Trp[215], Val[213], Ser[217].

Moreover, residues of trypsin established hydrogen bonds (Table 2) between compound 1 (Figure 3A) and residues Arg[96], Trp[151], Asp[189], Trp[215], and Gly[216]. On the other hand, compound 2 is establishing OH-bonds (Figure 3B) with residues Lys[97], Ser[190], Cys[191], Gln[192],

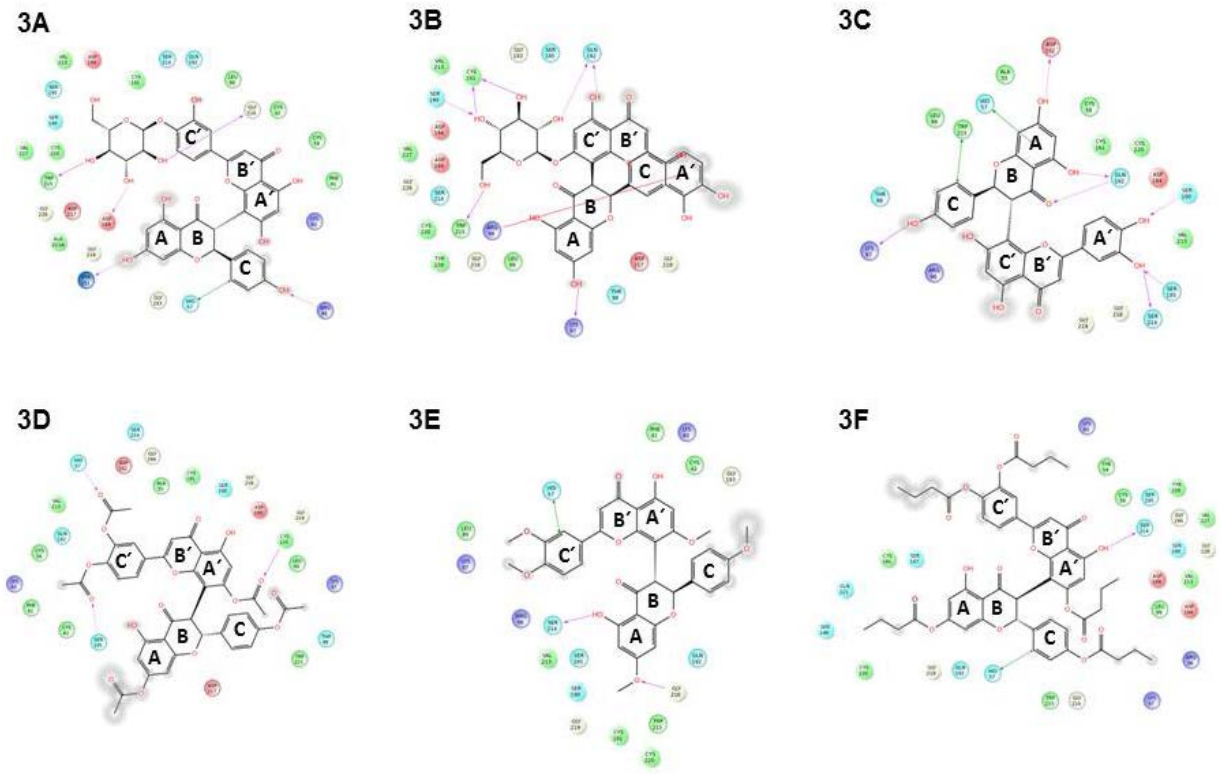

Figure 3. Molecular docking of compounds **1 to 6** with trypsin. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 1F2C for trypsin (PDB ID, 1996). A) Morelloflavone- 4```-glicoside (**1**)/trypsin; B) (±)-Fukugiside (**2**)/trypsin; C) Morelloflavone (**3**)/trypsin; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/trypsin; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/trypsin; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/trypsin.

and Trp[215]. Furthermore, the substitution of hydroxyl groups of compound 3 at the acetyl (Figure 3D→ compound 4), the methyl (Figure 3E→ compound 5) or the butanoyl (Figure 3F→ compound 6) promotes a decrease in the number of hydrogen bonds with trypsin when compared with compounds 1, 2, and 3. Compound 6 also performs a π-π stacking interaction between His[57] and ring C.

In the molecular docking of cruzain with compounds 1 (Figure 4A) and 2 (Figure 4B), the glycosyl groups are establishing hydrophobic interactions with residues of the S_2 subsite (Trp[26], Leu[67], Met[68], Ala[133], and Gly[160]); and compound 4 (Figure 4D) at the acetyl group with residues of the S_2 subsite; and compound 5 (Figure 4E) at the methyl group of C4` of ring C with Cys[22], Gly[23], Cys[25], Ala[136], Ser[139], Trp[141], and Asp[158]. Furthermore, it performs a π-π stacking interaction between ring A with Trp[177]. Compound 6 (Figure 4F) performs hydrophobic interactions at the butanoyl group of C3``` of ring C` with residues of the S_2 subsite.

The Trp[177] residue is widely conserved throughout the papain superfamily (McGrath 1999), and the quality of the aromatic–aromatic interactions is, in part, dependent upon the length of the inhibitor. The indole ring of this residue promotes the correct orientation of catalytic asparagine to interact with catalytic histidine by a π-NH interaction facilitating the formation of a thiolate-imidazolium ion pair during the catalysis (Brinen et al., 2000). Thus, blocking the formation of this ion pair prevents catalysis.

Cruzain protease is establishing hydrogen bonds (Table 2) with bioflavonoid compounds. Compound 1 (Figure 4A) performs H-bonds with Gln[19], Gly[20], Gln[21], Gly[66], Leu[157], and Asp[158]; compound 2 (Figure 4B), with residues Gly[66], Leu[157], Asp[158], and Glu[205]; compound 4 (Figure 4D), with Gly[23] and Gln[156A]; compound 5 (Figure 4E), one H-bond with Gln[19]; and compound 6 (Figure 4F), H-bonds with Gln[19] and Ala[136]. The increase of hydrophobicity of the compounds causes a reduction to the number of hydrogen bonds due to replacement of OH-groups by carbon chains.

Conclusion

Three semisynthetic biflavonoid derivatives have shown potential inhibitory effects on papain and trypsin proteases; the structural modifications of compounds are

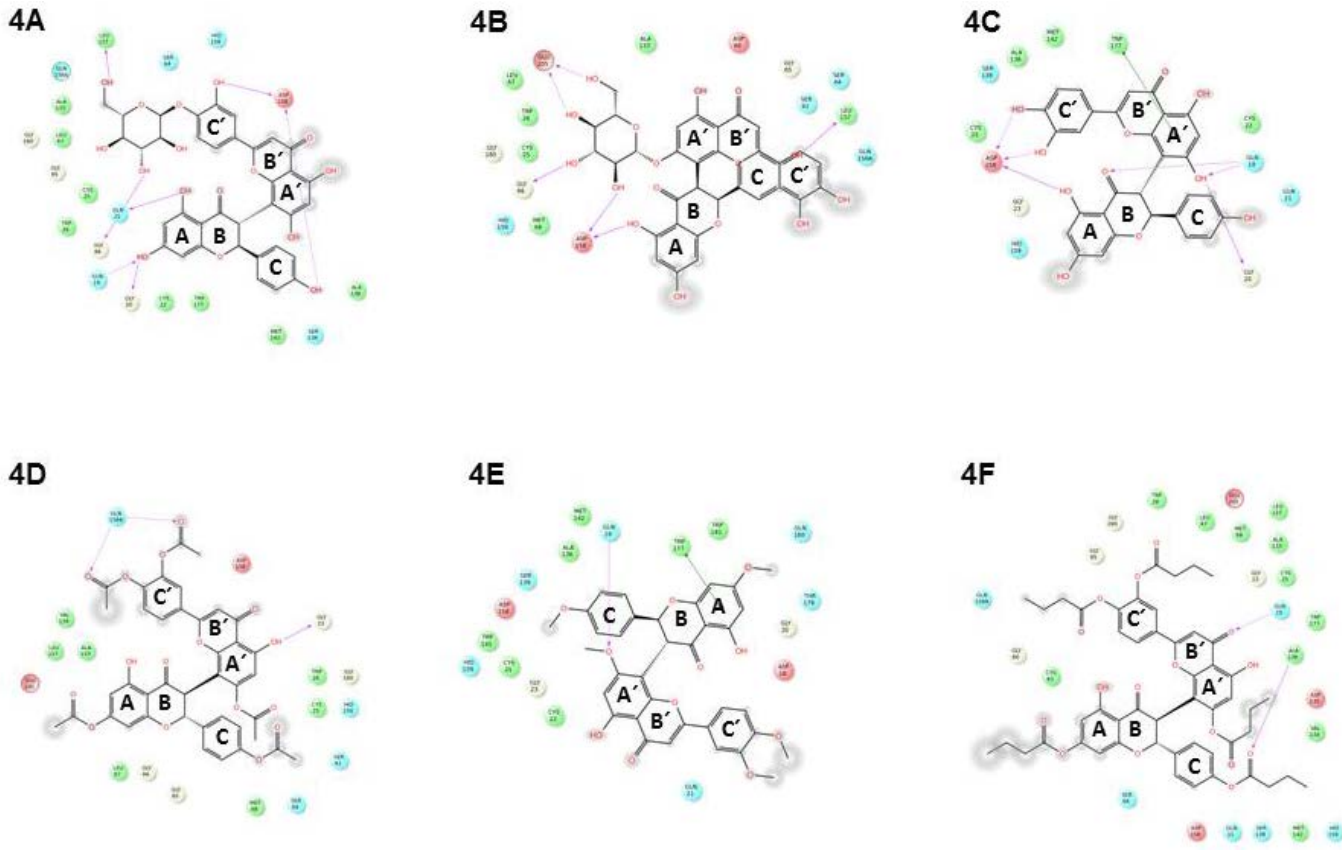

Figure 4. Molecular docking of compounds **1 to 6** with cruzain. The protease structures (1.8Å resolutions) were obtained from the Brookhaven Protein Data Bank. The PDB code was 1PE6 for cruzain (PDB ID, 1996). A) Morelloflavone-4```-glicoside (**1**)/cruzain; B) (±)-Fukugiside (**2**)/cruzain; C) Morelloflavone (**3**)/cruzain; D) Morelloflavone-7,4`,7``,3```,4```-penta–*O*–acetyl (**4**)/cruzain; E) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-methyl (**5**)/cruzain; F) Morelloflavone-7,4`,7``,3```,4```-penta-*O*-butanoyl (**6**)/cruzain.

important for inhibitory activity of enzymes. The concentration of morelloflavone-7,4`,7``,3```,4```-penta-O-butanoyl (compound 6) to selectively inhibit trypsin is only 1.6-fold to that of the peptide-based classical inhibitor of this enzyme, TLCK. TLCK can potentiate such derivative as antiproteolytic drug for treatment of diseases in which this protease is involved. Therefore, the structural modifications were significantly enhanced inhibition of protease enzymes by improving the activity of natural compound 3, as observed by compounds 4 and 5 over papain and trypsin, respectively. The molecular docking models showed that the compounds mainly interact with the cysteine proteases papain and cruzain at the S_2 subsite; on the other hand, the interactions occur mainly at the S_1 subsite of the serine protease trypsin. However, parts of amino acid residues can participate in more than one subsite, making characterization of residue interaction with a specific subsite difficult. The structural characteristics and the hydroxyl group substitutions in the natural compound 3 increased the potency of some compounds *in vitro*. These semisynthetic biflavonoid derivatives are therefore potential candidates to therapeutically inhibit proteases involved in parasitic infections caused by *T. cruzi*.

ACKNOWLEDGEMENTS

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Abbreviations:

r-CPB2.8, Recombinant cysteine protease type B; **EOAc,** ethyl acetate extract; **MeOH,** methanol; **Z-Phe-Arg-MCA,** carbobenzoxicarbonil-Phe-Arg-7-amino-4-methylcoumarin; **DTT,** dithiothreitol; **SARs,** structure–activity relationships; **FT-NMR,** Fourier transform nuclear magnetic resonance; **Me_4Si,** trimethylsilane; **TLCK,** N-alpha-tosyl-L-lysinyl-chloromethylketone; **E-64,** 1-[[(Ltrans-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane.

Conflict of interest

Authors have not declared any conflict of interest.

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One-pot synthesis of potential antioxidant agents, 3-carboxylate coumarin derivatives

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A simple and efficient route to synthesize 3-carboxylate coumarin derivatives through three-component one-pot reaction in a single step has been recommended. This method provides a direct and rapid access to get 3-carboxylate coumarin derivatives. The structures of these synthetic products were identified and the antioxidant activities were tested by inhibiting DPPH· and ·OH radicals capacities. The data suggested that 2H-1-penzopyran-3-carboxylicacid, 7,8-dihydroxy-2-oxo-, ethyl ester showed excellent activity in reducing both DPPH· and ·OH radicals in concentration-dependent manners with IC_{50} value of 83.1 µg/ml in DPPH· radicals and less than 0.12 mg/ml in the ·OH scavenging activity.

Key words: 3-Carboxylate coumarin, synthesis, antioxidant activity.

INTRODUCTION

Coumarins (2*H*-1-benzopyran-2-ones) are one family of naturally occurring compounds that are widely distributed in plants (Vazquez-Rodriguez et al., 2013). Coumarins could also be synthesized through chemical processes. In fact, more than 1,300 coumarins have been identified from natural sources, especially green plants (Hoult et al., 1996). Clinical and experimental studies have found that coumarin derivatives are known to possess a wide range of biological activities (Gabriele et al., 2008). These compounds possess anticancer (Gabriele et al., 2008; Paul et al., 2013), antioxidant, trypanocidal, anti-inflammatory (Vazquez-Rodriguez et al., 2013; Melagraki et al., 2009; Ćavar et al., 2012), antibacterial and cytotoxic properties (Canning et al., 2013). Coumarins are therefore used in treating metastatic malignant melanoma, renal cell carcinoma (Marshall et al., 1991; Thati et al., 2007; Thornes et al., 1994), and many other diseases. Moreover, coumarins have been extensively used in diverse sectors, such as pharmaceuticals, fragrances, agrochemicals, additives in food, cosmetics and insecticides (Kostova and Momekov, 2006).

Coumarin-3-carboxylate is one of the important coumarin derivatives, and an important intermediate that could be used in the synthesis of coumarins (Song et al., 2003). Coumarin-3-acyl derivatives were tested *in vitro* for their distinct human monoamine oxidase A and B (hMAO-A and hMAO-B) inhibitory activity, especially the 3-ethyl ester coumarin ring. They are considered as highly potent and selective hMAO-B inhibitors with IC_{50} values in the nanomolar range (Secci et al., 2011). 7,8-dihydroxy-coumarin and TGF-β1 have a synergistic effect on strongly induced, rat adipose-derived mesenchymal

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stem cells (ADMSCs) that are differentiated from the cartilage (Liu et al., 2013).

A one-pot reaction, one simple and rapid method, consists of a combination of three components that are used to generate new products in a single step. This resource-effective method is also used in the emerged multicomponent reactions (MCRs) (Karami et al., 2012) without isolating the intermediate. The economical use of material in this reaction along with post-processing is associated with a less tedious process of recycling and regenerating the catalyst. Compared with multistep reactions, it would be a convenient and greener way to get series coumarins.

In this paper, we gave the simple and rapid way, one-pot, to get hydroxy 3-carboxylate coumarin derivatives, and elaborated the process in detail (Scheme 1). The procedures and results were introduced and analyzed comprehensively. We found out that the compounds 2*H*-1-benzopyran-3-carboxylic acid, 7,8-dihydroxy-2-oxo-, ethyl ester showed definite activity in reducing both DPPH· and ·OH radicals. In addition, 2*H*-1- benzopyran-3-carboxylic acid, 7,8-dihydroxy-2-oxo-, ethyl ester, along with 2*H*-1-benzopyran-3-carboxylic acid, 8-hydroxy-2-oxo-, ethyl ester and 2*H*-1-benzopyran-3-carboxylic acid, 7-hydroxy-2-oxo-, ethyl ester showed more activity than ascorbic acid when there was low concentration in the ·OH scavenging. These experimental results showed that three of the synthesized 3-carboxylate coumarin derivatives were potential antioxidant agents.

EXPERIMENTAL

General

With the exception of piperidine (technically pure), all reagents used in this study were analytically pure. The products were synthesized using DF-101D solar collector heating thermostat magnetic stirrer. The melting points were measured using melting point apparatus. The synthesized compounds were purified by recrystallization and analyzed by thin-layer chromatography (TLC), melting point determination (X-6), and HPLC (5 µm, 250 × 4.60 mm Gemini, C_{18} preparation column, Phenomenex). Infrared spectra was recorded in KBr and determined on a Perkin Elmer fourier transform infrared (FT-IR) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz Nuclear Magnetic Resonance Spectrometer.

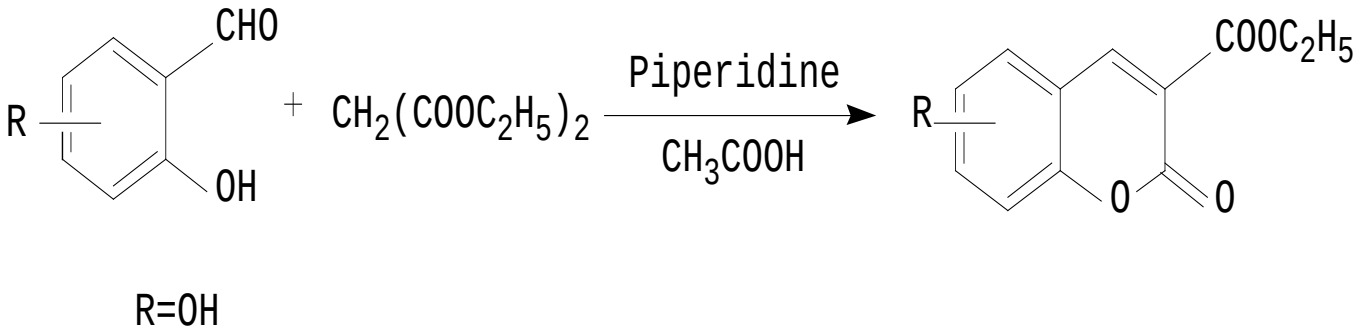
Synthesis procedure

2 *H-1-benzopyran-3-carboxylic acids*, 7-hydroxy-2-oxo-, ethyl ester. 2,4-Dihydroxybenzaldehyde (5.522 g, 0.040 mol), diethyl malonate (6.8 ml, 0.045 mol) with ethyl alcohol (25 ml), piperidine (0.5 ml), were added to a round-bottom flask(250 ml) that was equipped with a magnetic stirrer and spherical condenser. A small amount of glacial acetic acid was added to this flask to dissolve these chemicals. This mixture was heated to about 85°C for 3 h. The completion of this reaction was monitored by thin layer chromatography (TLC) using EtOAc: petroleum (3:1) as eluent. After completion of this reaction, the mixture was transferred to a beaker containing 35 ml of water. Then, the beaker was cooled and leached. The filter cake was washed thrice using 50% ethyl alcohol. Thereafter, the filter cake was washed using 95% ethyl alcohol. Finally, it was dried to obtain high purity product (faint yellow crystal, 60.5% yield), m.p. 171.4 to 173.5°C (otherwise the references 172 to 173°C (Valizadeh and Vaghefi, 2009). ^{1}H NMR (acetone-d_6, 500 MHz), *δ:* 8.58(s, 1H), 7.71 (d, *J*=7.0 Hz, 1H), 6.90(dd, *J*=2.0 Hz, *J*=5.5 Hz, 1H), 6.76(s, 1H), 4.29(d, *J*=7.0 Hz, 2H), 1.32(t, *J*=7.0 Hz, 3H). ^{13}C NMR (Acetone-d_6, 125 MHz), *δ*: 163.6, 163.1, 157.6, 156.1, 148.7, 131.8, 113.8, 113.6, 111.1, 102.1, 60.8, 13.6. IR(KBr), $\tilde{\nu}$ /cm^{-1}: 3550, 3471, 3056, 1739, 1680, 1606, 1466, 1447, 1245, 1145, 1087.

2H-1-Benzopyran-3-carboxylicacid, 8-hydroxy-2-oxo-, ethyl ester: 2,3-dihydroxybenzaldehyde (4.143 g, 0.030 mol), diethyl malonate (6.0 ml, 0.040 mol), ethyl alcohol (20 ml), and piperidine (0.5 ml) were added to a round-bottom flask (250 ml) that was equipped with a magnetic stirrer and spherical condenser. A small amount of glacial acetic acid was added to this flask to dissolve these chemicals. The mixture was heated to about 85°C for 4 h. Use the TLC monitor condition with EtOAc: petroleum (3:1) as an eluent to test the completion of this reaction. Then, the mixture was transferred to a beaker and 50 ml of water was added for cooling purposes. Finally, the cooled mixture was leached. The filter cake was washed twice using 50% ethyl alcohol. Then, it was dissolved using 25% ethyl alcohol and recrystallized. Finally, it was washed twice with 50% ethyl alcohol and dried to obtain high purity product (yellow crystal, 68.6% yield), mp 178.9 to 180.5°C (otherwise the references 174 to 175°C (Alvim et al., 2005)). ^{1}H NMR (acetone-d_6, 500 MHz), *δ:* 8.60(s, 1H), 7.26(m, 3H), 4.32(q, *J*=7.0 Hz, 2H), 1.33(t, *J*=7.0 Hz, 3H). ^{13}C NMR (Acetone-d_6, 125 MHz), *δ*: 162.8, 155.5, 148.4, 144.4, 143.5, 124.8, 120.5, 120.4, 118.8, 118.5, 61.1, 13.3. IR(KBr), $\tilde{\nu}$ /cm^{-1}: 3306, 3047, 1748, 1697, 1612, 1584, 1473,1265, 1231, 1032.

2*H*-1-benzopyran-3-carboxylicacid, 7,8-dihydroxy-2-oxo-, ethyl ester. 2,3,4-Trihydroxybenzaldehyde (3.084 g, 0.020 mol), diethyl malonate (4.0 ml, 0.026 mol), ethyl alcohol (20 ml), piperidine (0.5 ml) were added to a round-bottom flask (250 ml) that was equipped with a magnetic stirrer and spherical condenser. A small amount of glacial acetic acid was used as a solvent to dissolve these chemicals in the flask. The mixture was heated to about 85°C for 4 h. The same procedure was replicated and analyzed with TLC. After completion of this reaction, the mixture was transferred to a beaker and 50 ml water was added for cooling purposes. Finally, the cooled reaction mixture was leached. The filter cake was washed twice using 50% ethyl alcohol and then this filtered cake was dissolved using 95% ethyl alcohol. Thereafter, 50 ml water was added and the mixture was leached. The mixture was washed again with 95% ethyl alcohol and 50 ml water was added. Then, the mixture was leached and washed twice with 50% ethyl alcohol. Finally, the leached mixture was dried to obtain the product (faint yellow crystal, 39.9% yield), mp 238.4 to 240.0°C (otherwise the references 233 to 234°C (Alvim et al., 2005)). ^{1}H NMR (Acetone-d_6, 500 MHz), *δ:* 8.56 (s, 1H), 7.27 (d, *J*=8.5 Hz, 1H), 6.93(d, *J*=9.0 Hz, 1H), 4.29(q, *J*=7.0 Hz, 2H), 1.32(t, *J*=7.0 Hz, 3H). ^{13}C NMR (Acetone-d_6, 125 MHz), *δ*: 163.0, 155.6, 151.7, 149.3, 144.6, 131.5, 121.5, 113.4, 113.1, 111.7, 60.7, 13.6. IR(KBr), $\tilde{\nu}$ /cm^{-1}: 3482, 3218, 3055, 1694, 1611, 1588, 1514, 1263, 1193, 1083.

2 H-1-Benzopyran-3-carboxylic acids, 6-hydroxy-2-oxo-, ethyl ester. 2,5-Dihydroxybenzaldehyde (2.762 g, 0.020 mol), diethyl malonate (4.0 ml, 0.026 mol), ethyl alcohol (20 ml), piperidine (0.5 ml) and a small amount of glacial acetic acid were added to a round-bottom flask (250 ml) equipped with a magnetic stirrer and spherical condenser. This mixture was heated to about 85°C for 4 h. The same conditions were monitored by TLC. After completion of the reaction, the mixture was transferred to a beaker and 40 ml water was added for cooling purposes. Thereafter, this reaction mixture was leached. The filter cake was washed twice using a small amount of 50% ethyl alcohol. Subsequently, the filter cake



Scheme 1. The synthesis route for 3-carboxylate coumarin derivatives.

was dissolved in 95% ethyl alcohol and recrystallized. Thereafter, 30 ml water was used for washing the filter cake. A small amount of 50% ethyl alcohol was used for washing the filter cake again. The crude mixture was heated to dissolve with 25% $_{ethyl}$ alcohol (40 ml) and recrystallized. Finally, it was dried to obtain this purity product (primrose yellow crystal, 70.2% yield), mp 188.8 to 190.4°C (otherwise the references 182 to 184°C (Kraus and Pezzanite, 1979)). ^{1}H NMR (Acetone-d_6, 500 MHz), δ: 8.83(s, 1H), 8.53(s, 1H), 7.23 (m, 3H), 4.31 (q, J=7.5 Hz, J=7.0 Hz 2H), 1.33(t, J=7.5 Hz, J=7.0 Hz, 3H). ^{13}C NMR (Acetone-d_6, 125 MHz), δ: 163.0, 156.0, 154.0, 148.9, 147.5, 122.3, 119.0, 118.6, 117.2, 114.6, 61.1, 13.6. IR (KBr), $\tilde{\nu}$ /cm^{-1}: 3333, 3090, 1748, 1605, 1574, 1502, 1245, 1191, 1044.

The antioxidant activity test

Assay for the DPPH· radical-scavenging activity: a series of sample solutions was prepared in dimethyl sulfoxide (0.5 ml). The concentrations of these solutions ranged between 10 to 200 μg ml^{-1}. 3.0 ml of DPPH· solution in 95% alcohol was added to each of these solutions. The reaction mixtures were protected from light and incubated at room temperature for 30 min. The absorption was read at 517 nm and the mean value was measured for three duplicated readings. The ascorbic acid was used as a positive control. The scavenging activity was determined from the following equation (Lin et al., 2008; Tyagi et al., 2005; Tantry et al., 2012).

DPPH· scavenging activity (%) = 100 × [1 – (A_1 - A_2) / A_0]

A_0: absorbance of 3 ml DPPH· solution containing 0.5 ml DMSO;
A_1: absorbance of 3 ml DPPH· solution containing 0.5 ml sample;
A_2: absorbance of 3 ml 95% alcohol added 0.5 ml sample.

Assay for the scavenging effect on hydroxyl radicals: The scavenging effect was evaluated using the hydroxyl radical system that was generated by the Fenton reaction (Heo et al., 2005) with a minor modification. Briefly, samples were dissolved in dimethyl sulfoxide at 0 (control), 0.04, 0.08, 0.16, 0.24, 0.40 and 0.80 mg/ml. The reaction mixture consisted of the following reagents: 2 ml of salicylic acid and absolute ethanol solution (9 mM), 2 ml of $FeSO_4$ (9 mM), 2 ml of H_2O_2 (9 mM), and 2 ml samples of varying concentrations. The absorbance of this mixture was measured at 510 nm after incubating it at 37°C for 30 min. The hydroxyl radical-scavenging rate was calculated with the following equation (Sun et al., 2010).

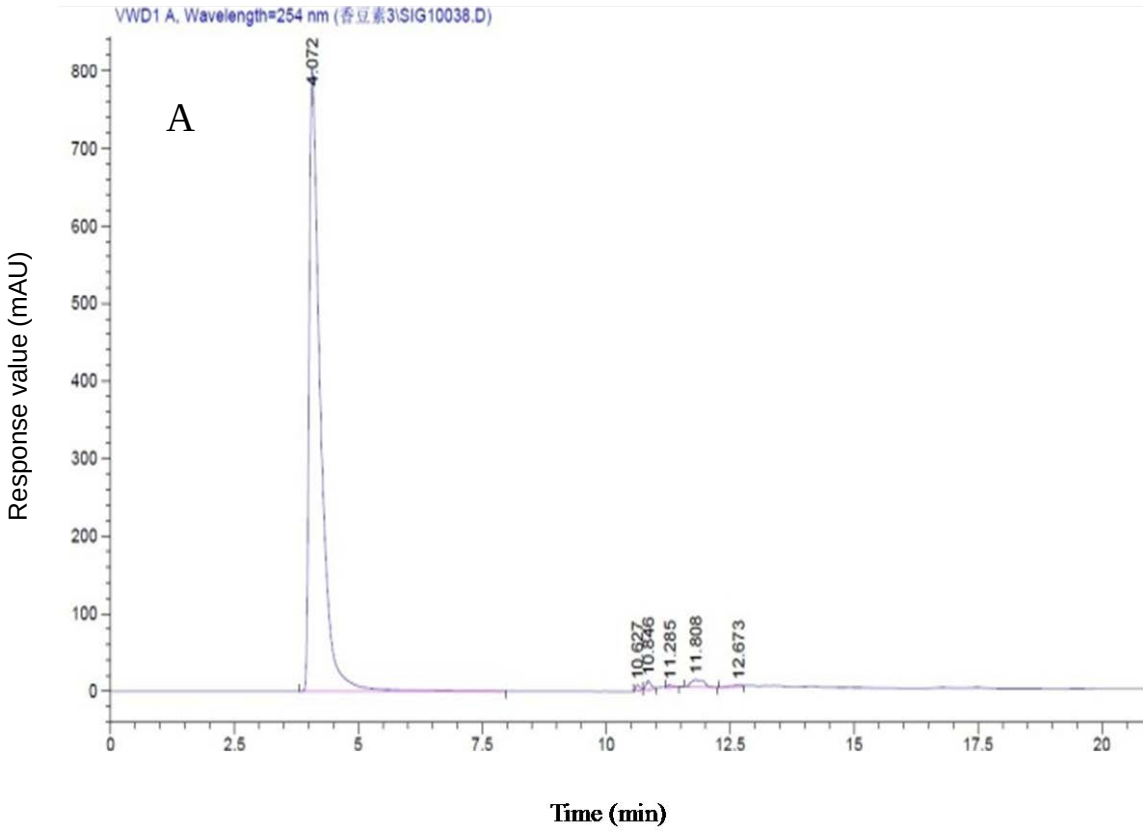
Hydroxyl radical-scavenging rate (%) = 100 × [1 - (A_s - A_w) / A_c]
A_s: absorbance of the mixture solution containing 2 ml sample;
A_w: absorbance of the mixture solution in which 2 ml water was replaced with 2 ml H_2O_2;
A_c: absorbance of the mixture solution in which 2 mL water was replaced with either 2 ml sample or vitamin C.

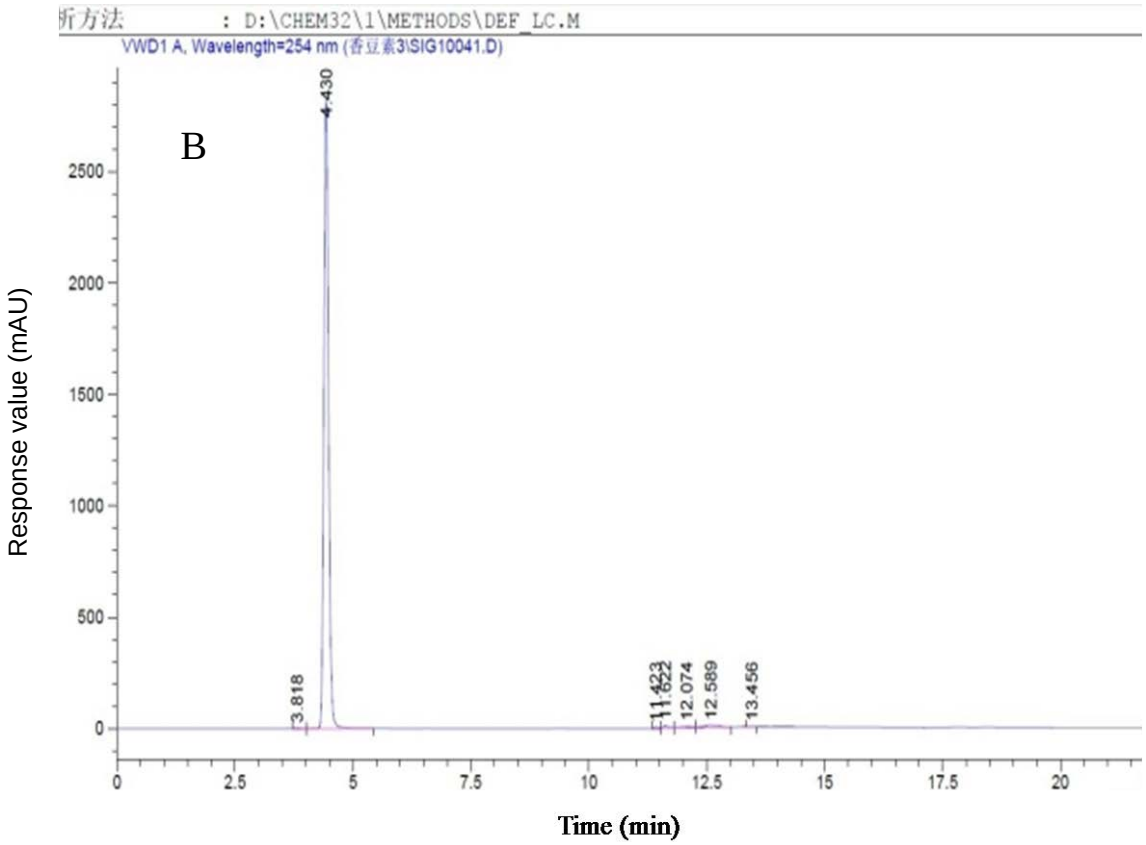
RESULTS AND DISCUSSION

Herein we gave detailed description of the synthesized compounds. In accordance with the references described in the procedure, the melting points of compounds differed by about 5 to 6°C. This could be attributed to the difference in the usage of the dissolved solvent and the measurement condition effects. The products were found to be highly purified as the melting point of every compound altered by only 1 to 2°C. The crystals of all compounds were yellow in appearance (Valizadeh and Azimi, 2011), except for the crystals of the compound 6-hydroxycoumarin-3-carboxylic acid ethyl ester. The crystals of this compound appeared yellowish green in color. The high performance liquid chromatography (HPLC) data is summarized and illustrated through Figure 1. The mean peak area of the samples was over 97%, and this indicated the high purity of products. While performing the experiments, the sample size was set in accordance with the concentration of every sample. In this case, a good, symmetrical, mean peak shape was obtained, which benefited from effective separation, high purity products, and medium sample size.

The reported data was identical with the standard spectra data (Lin et al., 2008; Horváth et al., 2005; Gong and Ding, 2006). Figure 2 displays the infrared (IR) spectra of 7,8-hydroxycoumarin-3-carboxylic acid ethyl ester. On the other hand, Table 1 illustrates those of other coumarins. They could also be recognized by ^{1}H NMR and ^{13}C NMR analysis (Table 2). Figure 3 displayed the partial nuclear magnetic resonance (NMR) spectra of compounds.

The abilities of inhibiting DPPH· and ·OH radicals of test compounds were assessed, and the findings were given as visualized in Figure 4. This examination of radical scavenging effect reflected the antioxidant activities of examined compounds to a certain extent. 2*H*-


VWD1 A, Wavelength=254 nm (香豆素3\SIG10038.D)
A
4.072
10.627
10.846
11.285
11.808
12.673
Response value (mAU)
Time (min)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B
4.430
3.818
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11.622
12.074
12.589
13.456
Response value (mAU)
Time (min)

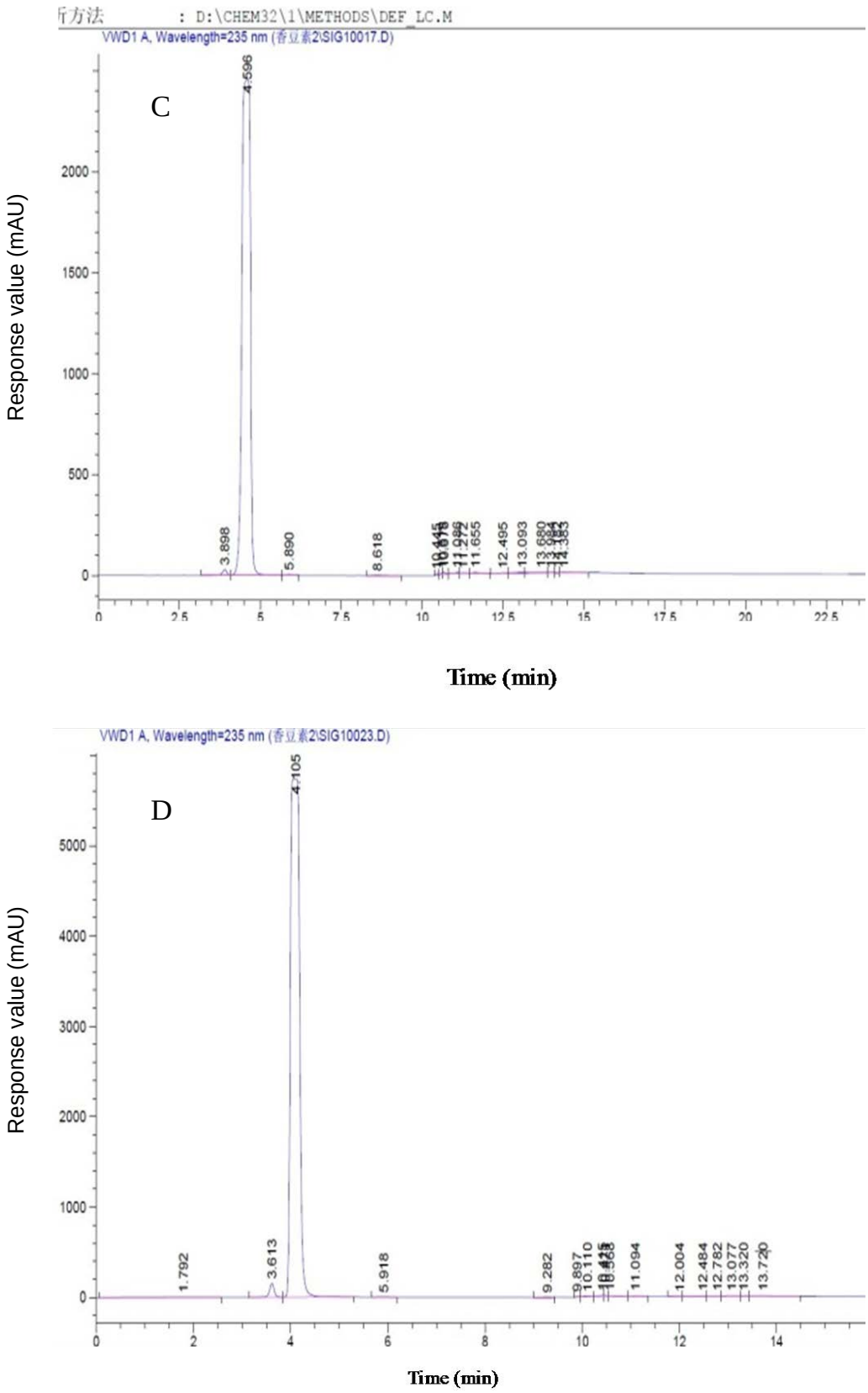


Figure 1. The HPLC spectra of 3-carboxylate coumarins. A, B, C and D (A) 7, 8-dihydroxycoumarin-3-carboxylicacid ethyl ester; (B) 8-hydroxycoumarin-3-carboxylicacid ethyl ester (C) 7-hydroxycoumarin-3-carboxylicacid ethyl ester (D) 6-hydroxycoumarin-3-carboxylicacid ethyl ester.

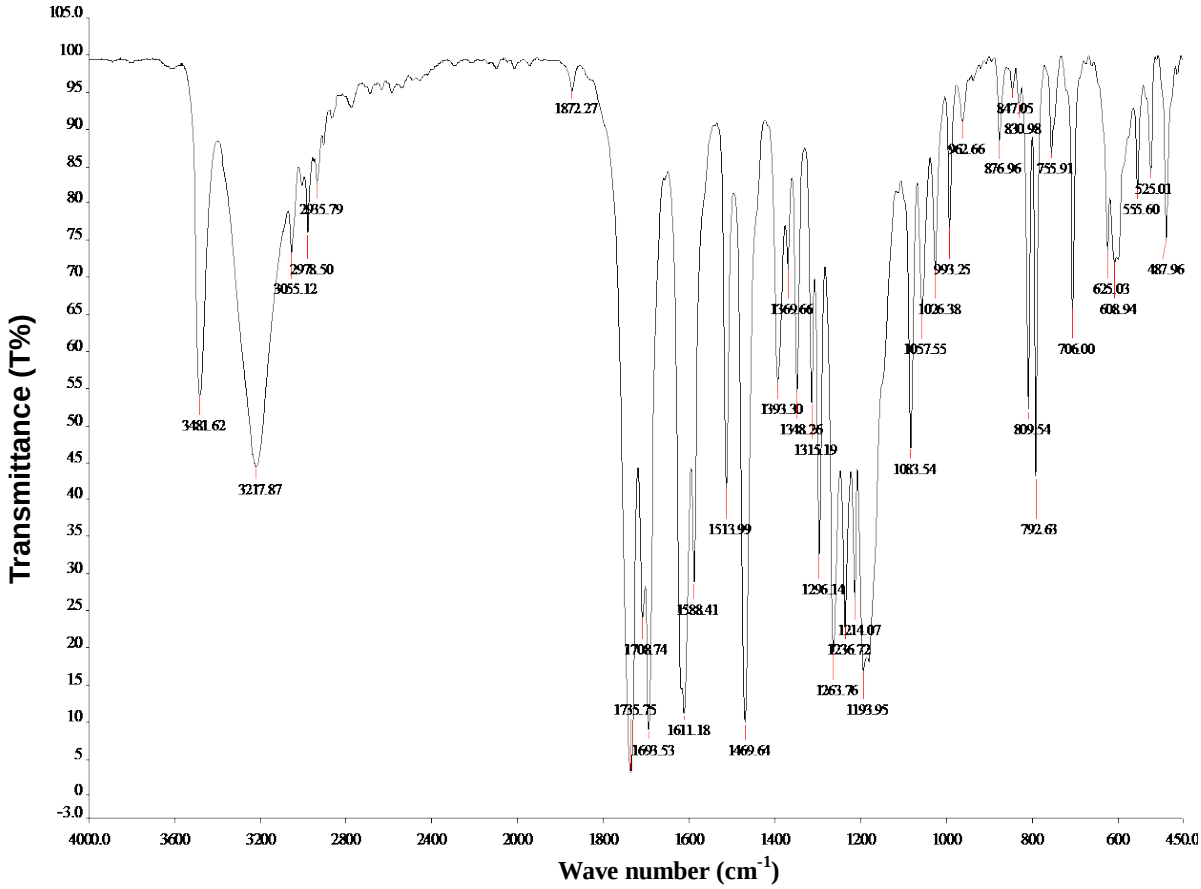


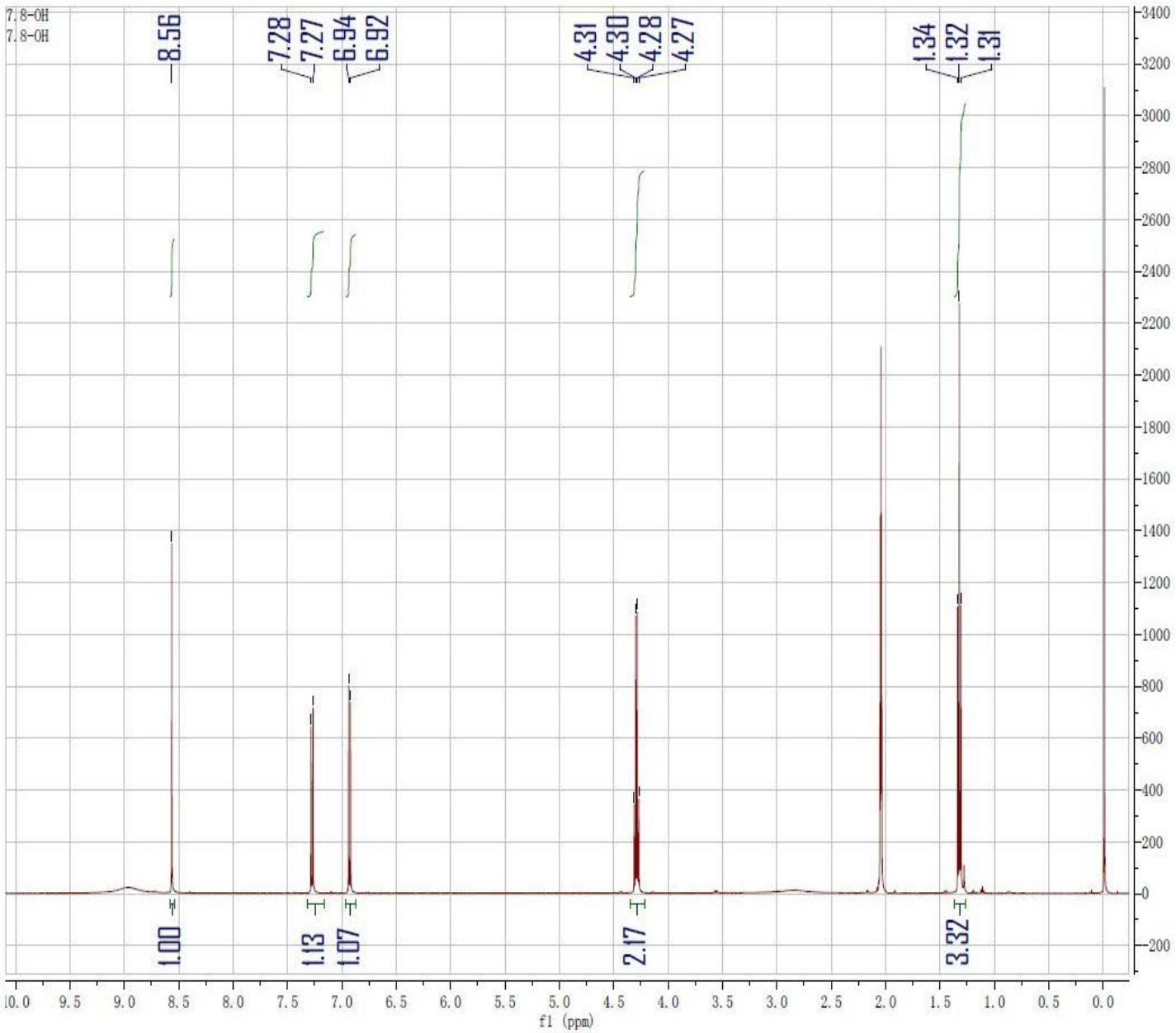
Figure 2. IR spectrum of 2H-1-Benzopyran-3-carboxylicacid, 7,8-dihydroxy-2-oxo-, ethyl ester

Table 1. IR spectra data of the objective products (KBr, cm^{-1}).

Product	ν_{O-H}	ν_{Ar-H}	$\nu_{C=O}$	$\nu_{C=C}$	$\nu_{Ar-C=C}$	ν_{C-O}
2H-1-Benzopyran-3-carboxylic acid, 7,8-dihydroxy-2-oxo-, ethyl ester	3482, 3218	3055	1694	1611	1611, 1588, 1514	1263, 1193, 1083
2H-1-Benzopyran-3-carboxylicacid, 8-hydroxy-2-oxo-, ethyl ester	3306	3047	1748, 1697	1612	1612, 1584, 1473	1265, 1231, 1032
2H-1-Benzopyran-3-carboxylicacid, 7-hydroxy-2-oxo-, ethyl ester	3550, 3471	3056	1739, 1680	1606	1606, 1466, 1447	1245, 1145, 1087
2H-1-Benzopyran-3-carboxylicacid, 6-hydroxy-2-oxo-, ethyl ester	3333	3090	1748	1605	1605, 1574, 1502	1245, 1191, 1044

Table 2. The objective products chemical shifts $\delta^{(ppm)}$.

Carbon spectra	2H-1-Benzopyran-3-carboxylicacid, 7,8-dihydroxy-2-oxo-, ethyl ester	2H-1-Benzopyran-3-carboxylicacid, 8-hydroxy-2-oxo-, ethyl ester	2H-1-Benzopyran-3-carboxylicacid, 7-hydroxy-2-oxo-, ethyl ester	2H-1-Benzopyran-3-carboxylicacid, 6-hydroxy-2-oxo-, ethyl ester
C=O	155.6	155.5	163.1	156.0
C-2	163.0	162.8	163.6	163.0
C-3	121.5	124.8	131.8	122.3
C-4	149.3	144.4	148.7	147.5
C-5	113.4	118.5	113.8	114.6
C-6	113.1	120.5	113.6	154.0
C-7	151.7	118.8	157.6	119.0
C-8	131.5	148.4	102.1	117.2
C-9	144.6	143.5	156.1	148.9
C-10	111.7	120.4	111.1	118.6
$-CH_3$	60.7	61.1	60.8	61.1
$-OCH_2-$	13.6	13.3	13.6	13.6

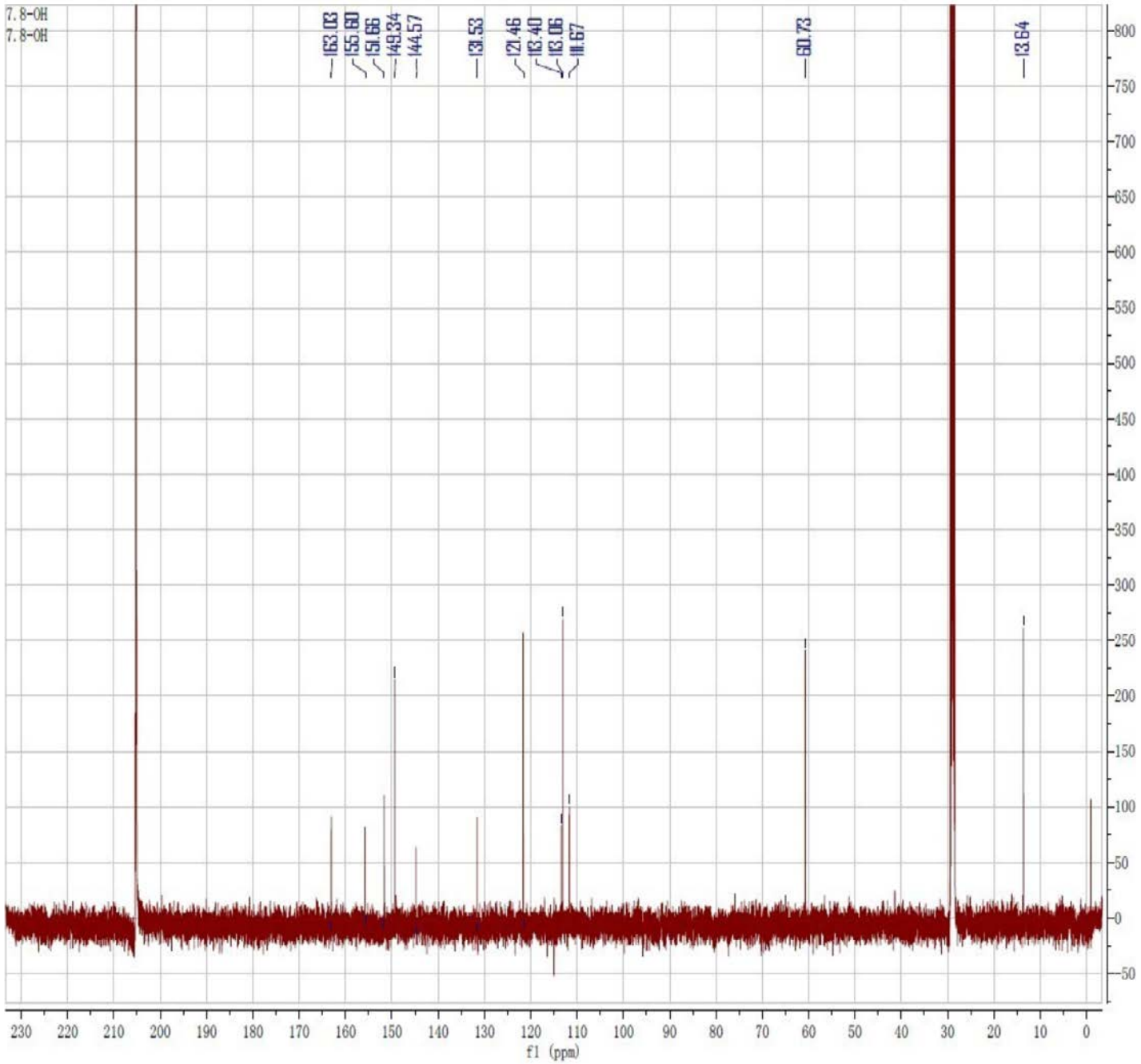


(A)

Figure 3A. 1H-NMR spectrum of 2H-1-Benzopyran-3- carboxylicacid, 7, 8-dihydroxy -2-oxo-, ethyl ester.

1-benzopyran-3-carboxylic acid, 7,8-dihydroxy-2-oxo-, ethyl ester was found to show excellent activity in reducing both DPPH• and •OH radicals in concentration-dependent manners. Figure 4a illustrates that 7,8-dihydroxyl coumarin inhibited DPPH· radicals with IC_{50} value of 83.1 µg ml^{-1}, compared to ascorbic acid with an IC_{50} value of 45.3 µg/ml. 7-hydroxyl coumarin displayed a less-potent effect with an IC_{50} value of 0.89 mg/ml. The other two selected coumarins had definite effect in scavenging DPPH· radicals. Figure 4b illustrates that similar activity was detected in the scavenging of ·OH radicals, whereas 7,8-dihydroxyl coumarin exhibited higher activity than ascorbic acid when the concentration was less than 0.12 mg/ml.

In addition, 7-hydroxyl coumarin and 8-hydroxyl coumarin were found to be more productive than ascorbic acid at a dose of below 0.04 mg/ml. In contrast, 6-hydroxyl coumarin showed little activity. Overall, under the experimental conditions, 7,8-dihydroxyl coumarin exhibited the strongest activity followed by 7-hydroxyl coumarin and 8-hydroxyl coumarin, with 6-hydroxyl coumarin displaying the weakest activity.



(B)

Figure 3B. ^{13}C-NMR spectrum of 2H-1-Benzopyran-3- carboxylicacid, 7, 8-dihydroxy -2-oxo-, ethyl ester.

Conclusion

In summary, we applied a simple way to get a series of ethyl coumarin-3-carboxylate containing hydroxyl group using the one-pot and multicomponent synthesis methods. These synthesized compounds have been elaborately elucidated, with the confirmation of their organic structures. This illustrates that workup reaction condition could be used in the synthesis of these products. Among the synthesized coumarins,7,8-dihydroxyl coumarin displayed excellent activities in reducing both DPPH· and ·OH radicals. 7,8-dihydroxyl coumarin was found to be more active than ascorbic acid under the concentration of 0.12 mg/ml. 7-hydroxyl coumarin and 8-hydroxyl coumarin were found to be more active than ascorbic acid within some limits

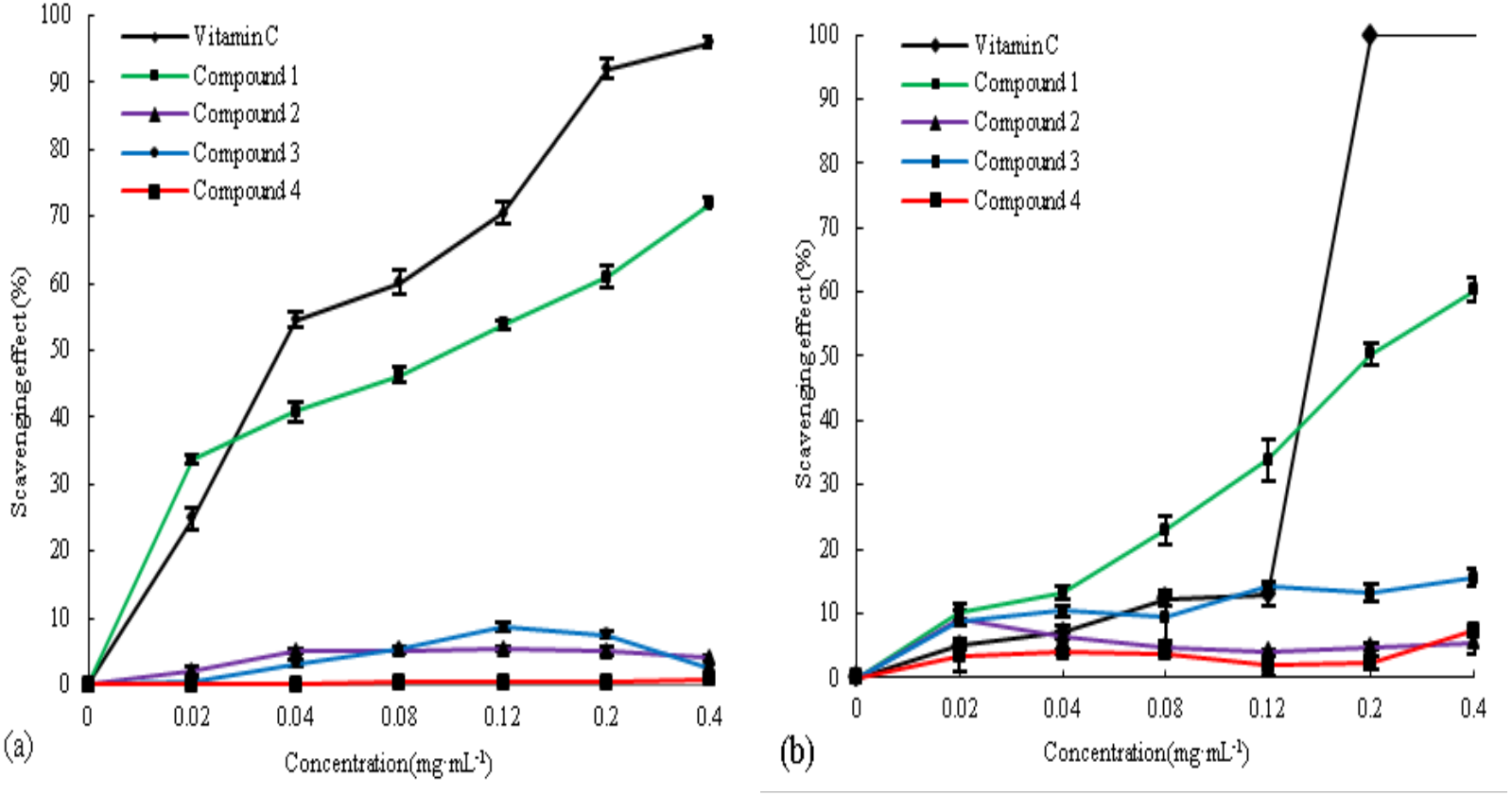


Figure 4. DPPH· scavenging effect (a) and ·OH scavenging effect (b) of the obtained coumarins. Compounds 1-4 are separated as follows: 2H-1-Benzopyran-3-carboxylicacid, 7,8-dihydroxy-2-oxo-, ethyl ester; 2H-1-Benzopyran-3-carboxylicacid, 8-hydroxy-2-oxo-, ethyl ester; 2H-1-Benzopyran-3-carboxylicacid, 7-hydroxy-2-oxo-, ethyl ester; 2H-1-Benzopyran-3-carboxylicacid, 6-hydroxy-2-oxo-, ethyl ester

concentration in the •OH scavenging. 6-hydroxylcoumarin exhibited little activity. The results of these experiments showed that three of the synthesized 3-carboxylate coumarin derivatives were potential antioxidant agents. New testing methods need to be devised to determine the other pharmacological activities of these compounds.

Conflict of Interest

Authors have declared no conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Antioxidant and cytotoxic activity of black and green tea from V*accinium meridionale* Swartz leaves

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Tea is a beverage made from leaves with high contents of polyphenolic substances that vary based on the process they are subjected to. In this study, the apical and young leaves from *Vaccinium meridionale* (named mortiño) were processed to obtain two kinds of tea: green and black tea. This was done in order to compare their antioxidant activity, content of secondary metabolites at different temperatures of extraction and their antiproliferative effect against SW480 colon cancer cells. Results showed that at 40°C, the green tea infusion presented higher antioxidant activity than the black tea infusion, based on their evaluation using Trolox equivalent antioxidant capacity (TEAC)-diphenylpicrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) techniques. The green tea also had maximum contents of epicatechin, caffeine, ferulic, chlorogenic and ascorbic acid than the black tea. The total contents of phenols, including hydroxycinnamic acids (caffeic and p-coumaric acid) presented similar results, in both types of tea at the same temperature, as well as the total contents of flavonoids and catechin. When temperatures increased, the extraction of bioactive compounds was more efficient in the black tea infusion than the green tea. This situation led to the increased growth rate per the temperature of the total content of phenols, among which chlorogenic, caffeic and p-coumaric acid were prominent, as well as the corresponding non-polyphenolic substances such as ascorbic acid. The latter may be responsible for the increased antioxidant activity as the temperature increased in the extraction. This antioxidant activity was observed in the black tea from mortiño leaves, using TEAC-DPPH, fluorescence recovery after photobleaching (FRAP) and ORAC assays. Both types of teas had a dose-dependent antiproliferative effect against SW480 colon adenocarcinoma cells. The IC_{50} of the green and black tea was 26.3 and 36 µg/ml, respectively. These findings suggest that a tea prepared from mortiño leaves may be a promising source of antioxidant and bioactive compounds against colon cancer cells.

Key words: Antioxidant, antiproliferative, mortiño, polyphenols.

INTRODUCTION

Vaccinium meridionale Swartz is a native Colombian plant belonging to the family of Ericaceae. The fruit is commonly known as mortiño or agraz; it is a dark purple globose berry when ripe. This fruit has a high potential for domestic consumption and has been included in the list of species with outward market, called "potential new

berry", "Andean blueberry" or "Colombian blueberry". There is a growing interest in this fruit that has been as considered a functional food due to its content of anthocyanins and other polyphenols. Garzón et al. (2010) evaluated the chemical composition, anthocyanin, non-anthocyanin phenolics and phenolic composition of mortiño. Cyanidin 3-galactoside was the major anthocyanin, while the most abundant non-anthocyanin phenolic was chlorogenic acid.

Gaviria et al. (2009) evaluated the content of phenols, anthocyanins and antioxidant activity by different methods. They found similar or higher values than those reported for other species of *Vaccinium*. Moreover, non-ethanolic extracts of *V. meridionale* Swartz rich in anthocyanins showed cardioprotective activity in rats during an ischemia-reperfusion process mediated by reactive oxygen species (ROS) (Lopera et al., 2013). Maldonado et al. (2014) studied the antioxidant activity of mortiño berry in aqueous extracts using fluorescence methods. They found it has the ability to trap total ROS and reactive nitrogen species (RNS), peroxyl, hydroxyl radicals; it has effects on the viability and growth of primary tumor cells of colon cancer (SW480) and their metastatic-derived cells (SW620), which are considered as *in vitro* model representing colon cancer progression to metastatic disease. However, there are no reports on the nutraceutical potential of the leaves, specifically in aqueous tea infusions.

Tea is a beverage known worldwide, prepared from hot water infusion of the leaves of *Camellia sinensis* L. (Cabrera et al., 2006). Tea consumption is similar to water and greater than coffee, beer, wine and beverage (Rietveld and Wiseman, 2003). This product is classified according to the type of process the leaves undergo to get the infusion as: green or unfermented tea, oolong or semi-fermented tea and black or fermented tea.

The production of green tea is characterized by a stage of fresh leaf wilting, followed by a coiled and short heating process at 300°C, designed to inactivate the polyphenol oxidase enzyme and generating native microflora of catalysis and aerobic oxidation. But, the processes of black and oolong tea are characterized by the development of four stages: first, leaf wilting where humidity is decreased and proteins are broken down by the action of the protease enzyme that can generate an increase in amino acid. Also, oxidation of lipids can occur, causing the level of unsaturated fatty acids to decrease by oxidative cleavage to form aromatics, contributing to the development of the aroma, color and flavor (Chen et al., 2011; TomLins and Mashingaidze, 1997). At this stage, the chlorophylls are degraded by 15%. In this regard, our green tea infusions showed a bottle-green colors, fresh flavors and herbaceous tastes. In addition, the black tea infusions showed a darker green colors than the previous ones, close to dark bole, and intense tastes and flavors.

The second stage is the leaf roll in which the polyphenols in vacuoles are mixed with polyphenol oxidase enzyme in the cytoplasm. This leads to the third step, fermentation, where polyphenoloxidases and other enzymes of the indigenous microflora are transformed in flavanols and polyphenol theaflavins and thearubigins, which also contribute to the dark color of black tea (Kuhnert et al., 2010) and reduce the astringency and flavor taste characteristic of this vegetal species (Wang et al., 2000). This stage is controlled and stopped according to the requirements of loss of green color (Cabrera et al., 2006). The last stage that is presented is drying, which helps to transform chlorophyll to pheophytin (Ramasamy et al., 2013).

Tea is recognized as an essential source of bioactive compounds such as (+) catechin, (-) epicatechin, (-) epigallocatechin and (-) epicatechin gallate, among others. They contribute to the antioxidant activity of this drink and the organoleptic properties (Fernández et al., 2000; Kim et al., 2011). Also, this type of drink is known for its action against diseases such as cancer, in addition to its pharmaceutical activity due to its antihypertensive, anti-atherosclerotic and hypolipidemic properties.

These properties may be attributed to antioxidant activity from polyphenols such as flavonoids (Chen et al., 2001). In this regard, a freshly prepared infusion of green tea contains 30 to 42% of catechin in weight; in black tea, the aqueous extract of the dried material contains between 3 and 10% of catechin, 2 and 6% of theaflavin and over 20% of thearubigin (Lambert and Yang, 2003). Additionally, natural sources contain other compounds such as tannins, vitamins and terpenoids which have similar characteristics with those in the phenol compounds (Exarchou et al., 2002) biological properties. It is necessary to note that the variety, weight, presentation and technical processing affect the estimation of these flavonoids.

The mode of action of these bioactive substances is based on redox mechanism, which these compounds exert as reducing agents; therefore, they can scavenge or quench reactive oxygen and nitrogen species, including free radicals such as superoxide anion (O_2), hydroxyl (OH) and nitric oxide (NO), as well as other species such as hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2) (Oh et al., 2013; Zhu et al., 2002). Tea polyphenols have antiproliferative activity and induce apoptosis against cancer cell lines and animal studies (Yang et al., 2011; Cordero-Herrera et al., 2013). Based on these considerations, the aim of the research was to determine and compare the content of bioactive

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metabolites, antioxidant activity and antiproliferative effect of two infusions of leaves from mortiño prepared as green and black tea extracted at different temperatures. Taking together the results obtained, let us suggest the nutraceutical potential of these beverages.

MATERIALS AND METHODS

Chemicals and equipment

Diphenyl - 1 - 2.2 picrylhydrazyl (DPPH), 6 - hydroxy - 2,5,7,8 - tetramethyl chromane - 2 - carboxylic acid (trolox), 2,4,6 - tris (2 - pyridyl - s - triazine) (TPTZ), 3,4,5 - trihydroxybenzoic acid (gallic acid), folin ciocalteu, sodium carbonate, L-ascorbic acid, methanol, formic acid, iron chloride, 3.6 buffer, pH 1 buffer, pH 4.5 buffer, pH 7.4 buffer, fluorescein and 2,2 '-azobis (2-amidino-propane) (AAPH) were obtained from Merck (Germany). The water used in the experiments was bi-distillated type. Vis Jenway 6405, a spectrofluorimeter brand Perkin Elmer LS 55 precisely and Shimadzu brand high-resolution chromatography high performance liquid chromatography (HPLC)-ultra violet (UV) spectrophotometer were used.

Plant sample collection

Leaves were harvested at the Catholic University in Rionegro, Antioquia, Colombia. The leaves were collected manually and selected on the basis of youthfulness and position in the tree. Younger leaves near the apical bud were selected. This material has a voucher number ILS 14050070. Samples were transported to laboratory in plastic bags sealed and washed with distilled water.

Green tea manufacturing

The elaboration process for obtaining both green tea and black tea from the leaves of *V. meridionale* S. was the same as cited by Gil (2010) from leaves of *C. sinensis* L., so there is no definite via for obtaining.

In the case of green tea, the process carried out on the leaves of *V. meridionale* S. consisted of several steps: first, leaves were subjected to a drying process in an oven at 48°C, for 1 h, in order to remove about 30% of their original moisture, which was previously determined on an average value of 62.7%; the obtained leaves were manually rolled and dried in an oven at a temperature between 90°C for 1 h, with the initial goal of reducing the moisture content that remained after step 1 to 4%. However, it resulted to only 13.2%. Subsequently, the plant material obtained was crushed and put into tea bags, after which the infusion was performed by dipping them in 50 ml of water at 40, 60, 80, 90 and 100°C for 10 min. Then, we proceeded to evaluate the antioxidant activity of the tea obtained via the Trolox equivalent antioxidant capacity (TEAC) techniques-DPPH, fluorescence recovery after photobleaching (FRAP) and oxygen radical absorbance capacity (ORAC), to determine the ascorbic acid, phenols and total flavonoids content, and to identify and quantify the amount of catechin, epicatechin and caffeine, hydroxycinnamic acids; they were chlorogenic, caffeic, ferulic and p-coumaric acid and sugars, among which glucose, fructose and sucrose were considered.

Black tea manufacturing

In the case of black tea, the process carried out on the *V. meridionale* S. leaves consisted of several stages: first, leaves were subjected to a drying process in an oven at 48°C, for 1 h, in order to remove about 30% of their original moisture which was previously determined (57%). Subsequently, the leaves obtained were manually rolled, and subjected to a process of "fermentation", in which the leaves were placed in contact inside a sealed chamber, with oxygen in an atmosphere characterized by 95% moisture, generated by a potassium nitrate solution at 27°C. The leaves were weighed at 2 and 5 h after the start of the “fermentation" process. They were hydrated until approximately 54% humidity. With these features, the leaves were subjected to a drying oven at 90°C for a certain time, for two purposes: firstly, to inactivate the activity of the polyphenol oxidase enzyme and secondly to reduce the moisture. The moisture content was approximately 1%. Subsequently, the plant material obtained was crushed and put into tea bags, after which the infusion was performed by dipping them in 50 ml of water at 40, 60, 80, 90 and 100°C for 10 min. Then proceeded to evaluate the antioxidant activity of the tea obtained via the TEAC techniques-DPPH, FRAP and ORAC, to determine the content of ascorbic acid, phenols and total flavonoids, and to identify and quantify the content of catechin, epicatechin and caffeine, hydroxycinnamic acids; they were chlorogenic acid, caffeic, ferulic and p-coumaric acid, and sugars, among which glucose, fructose and sucrose were considered.

DPPH method

Radical scavenging activity against the stable radical DPPH was measured using the methods of Brand-Williams et al. (1995), with certain modifications. The method is based on the reaction of 10 ml of sample with 990 ml of DPPH solution for 30 min at room temperature, followed by determining the decrease in absorbance at 517 nm associated with a reduction in the DPPH concentration. The results were expressed in units, TEAC.

FRAP assay

The antioxidant capacity of tea was estimated according to the procedure described by Benzie and Strain (1996), with some modifications. This method is based on the increase in absorbance due to the formation of 2,4,6-tripyridil-s-triazine (TPTZ)-Fe (II) in the presence of reducing agents. The FRAP reagent contained 2.5 ml of 10 µM TPTZ in 40 mM HCl. $FeCl_3$ (2.5 ml of 20 µM) and acetate buffer (25 ml of 0.3 µM, pH 3.6) were freshly prepared and warmed to 37°C. A volume of 50 µl of extract was mixed with 950 µl FRAP reagent already dissolved in acetate buffer (pH 3.6). The absorbance increase was measured at 590 nm. The FRAP values were expressed as AEAC (ascorbic acid equivalent antioxidant capacity: mg ascorbic acid per g dry powder) using an ascorbic acid standard curve.

ORAC assay

The ORAC assay was determined using the following methodology: 3 ml was prepared from the following solution: 21 µl of a 10 µM solution of fluorescein, 2899 µl of 75 mM phosphate buffer (pH 7.4), 50 µl of 600 mM 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 30 µl of extract. Fluorescence was recorded on a Perkin Elmer LS45 spectrofluorometer with a thermostated multicell. The results were expressed as micromoles of Trolox® equivalents per 100 g lyophilized according to Equation 1.

$$ORAC = \frac{(AUC - AUC^0)}{(AUC_{Trolox} - AUC^0)} f[Trolox] \quad (1)$$

where AUC is the area under the curve of the sample, AUC° is the area under the curve for the control, AUC_{Trolox} is the area under the curve for Trolox® and *f* is the dilution factor extracts (Romero et al., 2010).

Total phenols

The total phenolic content was determined according to the adapted Folin–Ciocalteu method (Singleton and Rossi, 1965). The extracts (50 µl) were mixed with 125 µl of Folin–Ciocalteu reagent and 400 µl of sodium carbonate solution (7.1% p/v), and the resulting solution was brought to a final volume of 1000 µl. The mixture was stirred and stored at room temperature for 30 min in the dark. The absorbance was measured at 760 nm against a blank. Aqueous solutions of gallic acid were used for calibration. The results are expressed as gallic acid equivalents (GAE)/g dry powder.

Total flavonoids

The flavonoids were determined by colorimetric method (Marinova, 2005). The extracts (100 µl) were mixed with 30 µl of $NaNO_2$ (5% p/v), 30 µl of $AlCl_3$ (10% p/v), 200 µl of NaOH (1M) and the resulting solution was brought to a final volume of 1000 µl with distillated water. The absorbance was measured at 510 nm. The results are expressed as catechin equivalents/g dry powder.

Hydroxycinnamic acids determination by HPLC- diode array detector (DAD)

Hydroxycinnamic acids were analyzed by direct injection of the samples, previously filtered through a 0.45 Rm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C_{18} column (5 Rm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). Chlorogenic, caffeic, ferulic and p-coumaric acids were adopted as the standard for identification and quantification of hydroxycinnamic acids at 320 nm. The mobile phase was a sample of 10 RL of a mixture of acetonitrile, acidified water (phosphoric acid at pH = 2.5) (40:60) v/v, supplied at a rate of 0.8 ml/min (Kelebek et al., 2009).

Catechin and epicatechin determination by HPLC-DAD

(+) - Catechin and (-) - epicatechin were analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C_{18} column (5 µm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). (+) - Catechin and (-) – epicatechin were adopted as the standard for identification and quantification at 280 nm. The mobile phase was methanol (A) acidified water (0.1% formic acid) (B) with gradient elution of 0.01 min 60% A was used; 5 to 12 min 80% A; 13 to 14 min 60% A. Flow rate of mobile phase was 1.0 ml/min (Oliveiro et al., 2009).

Caffeine determination by HPLC–DAD

Caffeine was analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C_{18} column (5 µm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). Caffeine was adopted as the standard for identification and quantification at 280 nm. The mobile phase was methanol. It was used in isocratic mode working at a flow of 1.0 ml/min (Brunetto et al., 2007).

Cell culture

SW480 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured according to a previously described procedure (Maldonado et al., 2014). Cells were cultured in 75 cm^2 Falcon flasks with Dulbecco's modified eagle's medium supplemented with 25 mM glucose, 2 mM L-glutamine, 10% heat (56°C)-inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acids. Incubations were carried out at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 48 h. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 10 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS defined medium). Cells were exposed to different extracts for 24 h after seeding.

Sulforhodamina B (SRB) assay

The effect of extracts on growth cells was studied by using the SRB assay according to Gossé et al. (2005), a colorimetric assay based on staining of total cellular protein from cells with SRB dye. In brief, 3000 viable cells from each cell line were exposed to extracts for 24 h after seeding and incubated for different times. Control cells were treated with 0.1% dimethyl sulphoxide (DMSO). Culture media were replaced every 48 h. The cell culture was stopped by the addition of trichloroacetic acid (50% v/v), and cell proteins were determined by staining with 0.4% (w/v) SRB (Sigma-Aldrich, United States). The relationship between cell number (protein content/well) and absorbance is linear from 0 to 2 × 10^5 cells per well. All experiments were performed in triplicate. The concentration able to kill 50% of cells (IC_{50}) was calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The absorbance of control group (non-treated cells) was considered as 100% viability. The percent inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (OD_t / OD_c)] \times 100$$

Where OD_t is the optical density (OD) of treated cells, and OD_c for control (non-treated cells).

Statistical analysis

The variables were characterized in terms of the extraction temperature, using the Statgraphics Centurion XVI statistical program. Analysis of variance (ANOVA) was applied to each variable depending on the stages of development, with a significance level of 5%.

RESULTS

Antioxidant activity and secondary metabolites

Figure 1A and B show the effect of temperature on the peroxyl radical scavenging capacity (ORAC) and the content of total phenols, respectively, both aqueous extracts of green tea and black tea. In green tea, the

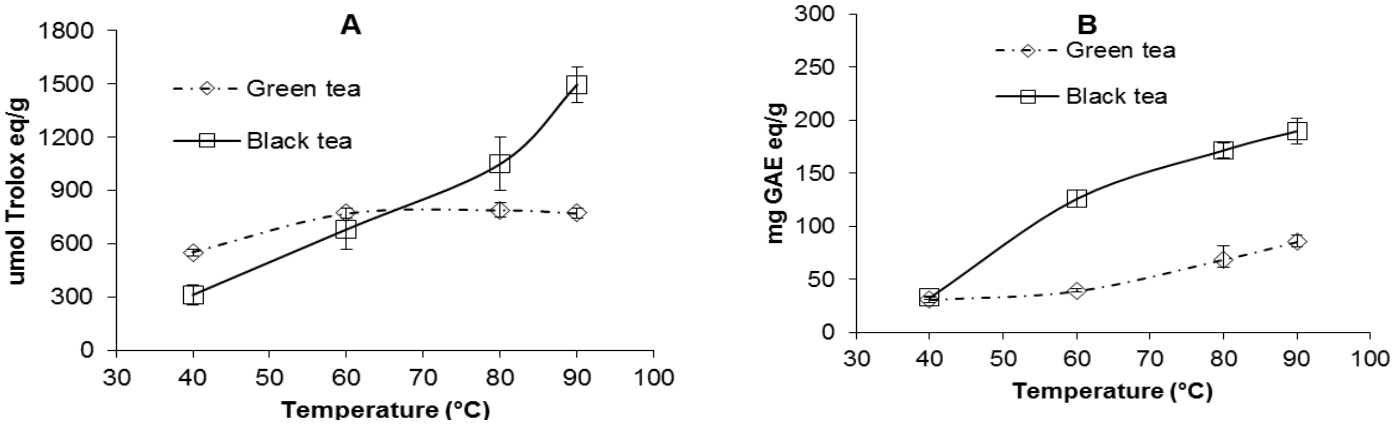

Figure 1. Effect of temperature on ORAC values (A) and total phenol content (B) in green and black tea.

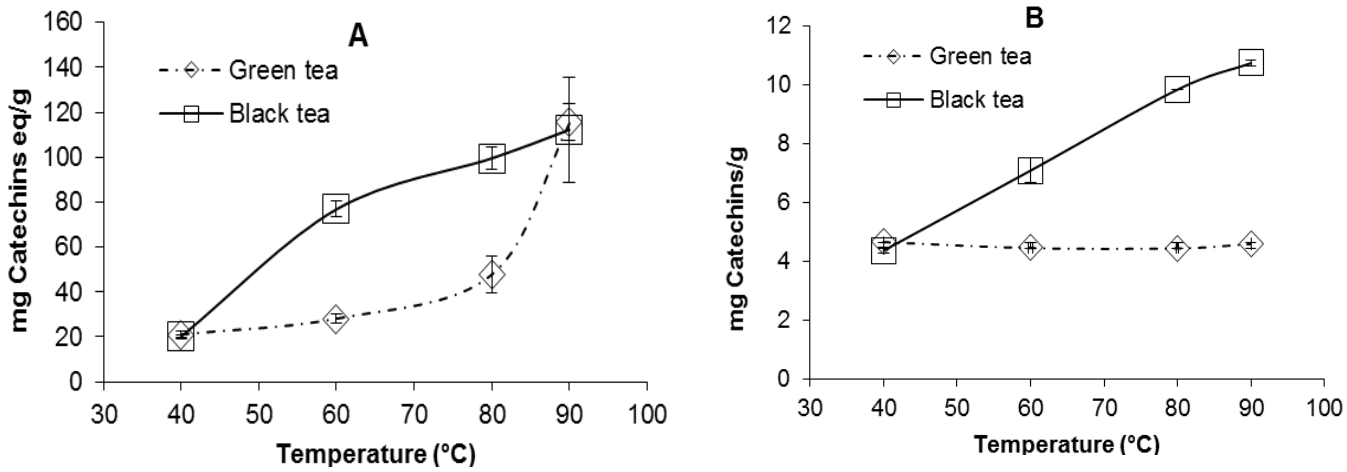

Figure 2. Effect of temperature on total flavonoids (A) and catechin content quantified by HPLC (B).

results obtained from the first technique varied significantly between 40 and 60°C, with values of 542.04 ± 18.02 and 778.8 ± 27.22 µmol Trolox/g of dry leaf, respectively. Subsequently, the values remained nearly constant in the temperature range between 60 and 90°C. However, the values respective to black tea aqueous extracts increased significantly throughout the whole period of temperature, obtaining values of 313.65 ± 37.06, 682.16 ± 40.34, 1050.63 ± 100.45 and 1496.82 ± 101.7 µmol Trolox/g of dry leaf, for temperatures 40, 60, 80 and 90°C, respectively. The speed rates found for both green and black tea aqueous extracts were 4.3 and 22.4 µmol Trolox/°C (Table 1), respectively.

According to Figure 1B, the total phenols content in green tea aqueous extracts increased significantly between 60 and 90°C, with values ranging between 39.28 ± 1.45 and 85.62 ± 4.9 mg GAE/g of dry leaf. By contrast, black tea aqueous extracts increased their total phenols content significantly over the extraction temperatures period, yielding values between 32.66 and 189.83 ± 11.9 mg GAE/g dry leaf, for temperatures of 40 and 90°C. Moreover, the speed rate was obviously higher in black tea aqueous extracts, compared with the green tea, obtaining values of 16.8 and 90.9 mg GAE/°C, respectively.

Meanwhile, Figure 2A and B show the extraction temperature effect on the total flavonoids content, expressed as mg catechin equivalents per gram of dried leaf, and mg of catechin per gram of dried leaf but quantified by HPLC technique. In both cases it was observed that content of catechin, corresponding to black tea aqueous extracts increased significantly during the extraction period, with values ranging from 20.23 ± 0.70 to 112.20 ± 23.23 mg catechin equivalents/g dried leaf and from 4.37 ± 0.10 to 9.72 ± 0.10 mg of catechin/g of dry leaf (HPLC). In addition, as shown in Table 1, the speed rate of catechin content in the black tea aqueous extracts was significantly greater than for green tea.

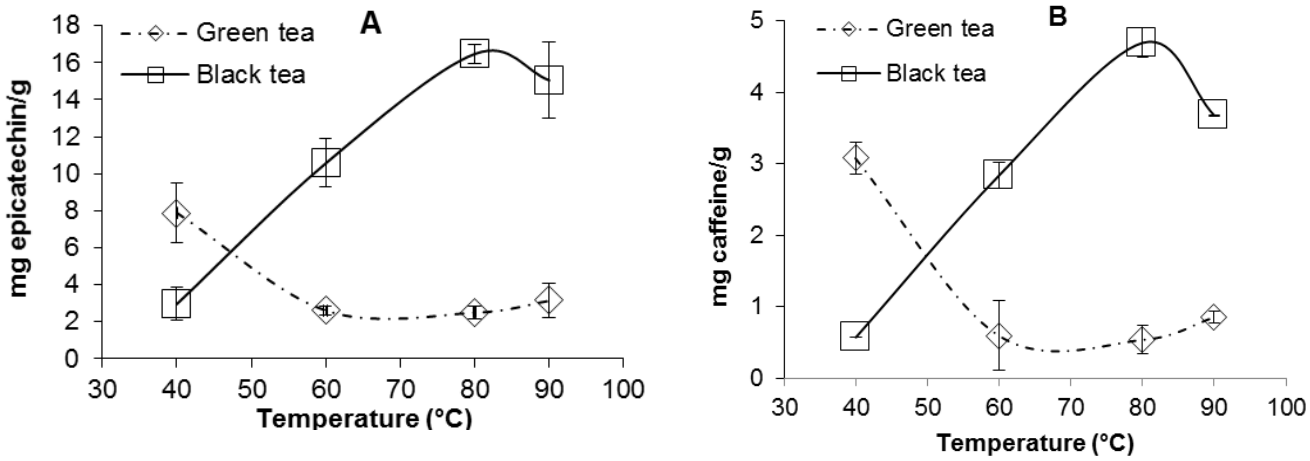

Figure 3. Effect of temperature on the content of epicatechin (A) and caffeine (B) in green and black tea. Data are the mean

Figures 3A and B show the extraction temperature effect on the caffeine and epicatechin concentration in aqueous extracts for both kinds of tea. The green tea epicatechin values decreased for extraction temperatures of 40 and 60°C (10.14 and 4.7 mg/g of dry leaf, respectively) and remain constant afterwards. Conversely, black tea epicatechin values increased significantly for each extraction temperatures, with values between 2.96 ± 0.88 and 5.15 mg of epicatechin/g of dry leaf from 40 to 80°C. Both green and black tea aqueous extracts showed opposite behavior respect to the caffeine content; in this case, the black tea caffeine values increased significantly for each extraction temperatures, with values between 0.57 ± 0.02 and 3.68 ± 0.08 mg caffeine/g of dry leaf from 40 to 80°C. By contrast, the caffeine content of green tea aqueous extracts decreased significantly for extraction temperatures 40 and 60°C, with values of 3.08 ± 0.00 and 0.85 ± 0.01 mg of caffeine/g of dry leaf, and remain constant afterwards.

Finally, chlorogenic, caffeic, p-coumaric and ferulic acids were determined by HPLC technique. The green tea aqueous extract values obtained at 90°C were 2.9 ± 0.13, 3.5 ± 0.08, 6.2 ± 1.2 and 3.03 ± 0.02 mg/g of dry leaf, respectively; the black tea aqueous extract values of at 90°C were 3.4 ± 0.04, 4.2 ± 0.05, 7.75 ± 1.1 and 3.7 ± 0.02 mg/g of dry leaf, respectively. Likewise, the antioxidant activities of green and black tea at 90°C were determined by FRAP and DPPH assays obtaining values of 78.8 ± 6.5 and 157.8 ± 18.2 mg ascorbic acid equivalent, respectively, and 491.58 ± 46.56 and 1118.15 ± 171.97 µmoles Trolox/g dry leaf, respectively.

Effect of mortiño black tea and green tea on SW480 cell growth

The effect of mortiño black and mortiño green tea on the SW480 cell growth is as shown in Figure 4 where optical density at 490 nm (OD) corresponds to the proteins of the cells untreated or after treatment with mortiño green or black tea. As shown in Figure 4A, after treatment with mortiño black tea for 72 h, 76.3% of SW480 cell growth (20 µg/ml) was reduced by 58.2% compared to non-treated cells (DMSO 0.1%). While, the effect of green tea on SW480 cell growth at the same conditions was 82.2% at 20 µg/ml and reduced by 49.6% at 200 µg/ml (Figure 4B) compared to non-treated cells.

The black tea reduced SW480 cell from 23.7% (20 µg/ml) to 41.9% (200 µg/ml) after 72 h of treatment, while green tea reduced cell viability between 19.5 and 50.4% under the same conditions.

DISCUSSION

Green and black tea from V*. meridionale* S. leaves contain a large variety of polyphenolic compounds such as catequin, epicatequin and chlorogenic, caffeic, ferulic and p-coumaric acids, and alcaloids such as caffeine. All these compounds determine the antioxidant activity of different aqueous infusions obtained from leaves of this species. To evaluate the antioxidant potential and stability of some metabolites in the processing of green and black tea, monitoring was carried out at different temperatures. Multivariate analysis was made to correlate the extraction temperature effect with the secondary metabolites content in both types of tea. Majority of the test showed statistically significant differences.

Initially, in ORAC technic, green tea showed higher activity than black tea; however, with increasing extraction temperature black tea also increased peroxyl radical scavenging capacity. This increased at a rate of 22.4 Trolox umol/°C, whereas the green tea made a speed of 4.3 µmol Trolox/°C.

Antioxidant activity can be explained by the content of phenolic compounds. The total phenolic contents are similar to the ORAC assay, which shows that phenolic

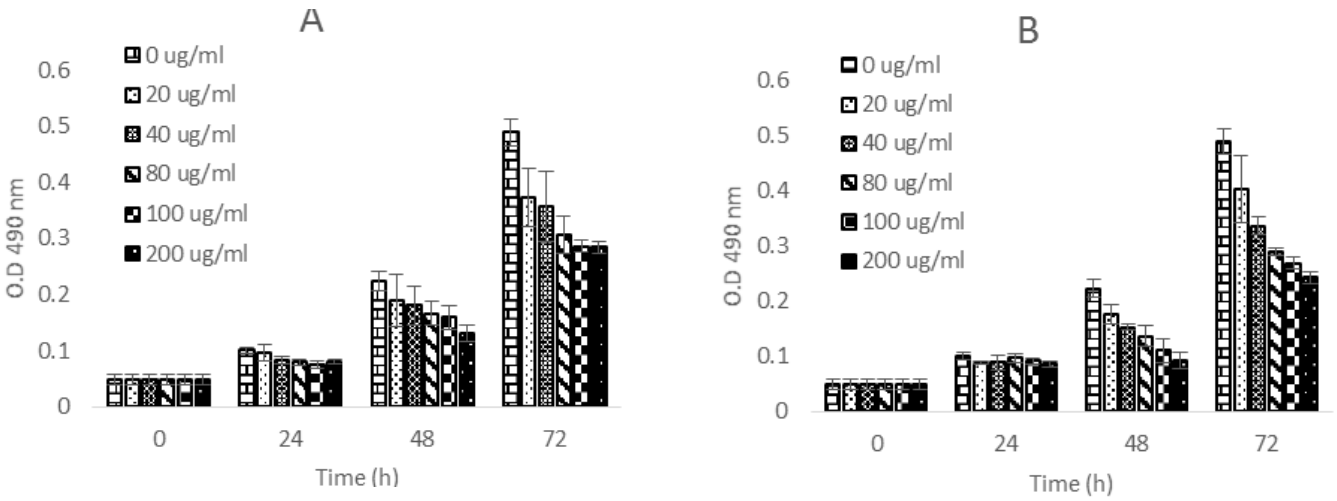

Figure 4. Effect of mortiño black tea (A) and mortiño green tea (B) on the growth of SW480 cells. The cells (3000 cells/well in 96-well plate) were exposed to concentrations of 0 to 200 µg/ml for 24, 48 and 72 h. Control cells were treated with the extract vehicle (dimethylsulfoxide, 0.1% DMSO in culture medium). Data are mean ± standard error of the mean (n = 3).

Table 1. Rate of increase and decrease per temperature.

Technique	Black tea	Green tea
ORAC (umol Trolox)	22.4	4.3
Phenols (mg GAE)	90.9	16.8
Flavonoids (mg catechins)	29.9	30.3
Catechin (mg catechins)	0.1	0.002
Epicatechin (mg epicatechin)	0.3	-0.09
Caffeine (mg caffeine)	0.07	-0.04

compounds from black tea were better extracted than green tea at 90°C. Oh et al. (2013) reported values of 82.2 ± 1.8 and 82.9 ± 3.2 mg GAE/g for green and black tea, respectively at 80°C. These tea values are comparable with matte aqueous infusions that contain 136.8 ± 24.8 mg GAE/g (Mejía et al., 2010; Oh et al., 2013). The values of black tea have a higher growth rate than the corresponding green tea rate.

As expected, the higher the water temperature, the higher the phenolic compounds in tea solution. This is due to the effect of temperature on the solubility and diffusion rate of compounds in the bulk solution. Generally, the solubility and the diffusion coefficient of a substance increase with an increase in the temperature of the solvent (Atkins, 2001). The dissolution and diffusion of caffeine in the aqueous solution, therefore, increased with an increase in water temperature.

At 40°C the two types of tea have similar content of flavonoids; but with gradual increase in extraction temperature; these values grow at different rates. The values obtained are better than those reported in the literature, which shows values of 16.4 ± 0.2 units in green tea and 14.9 ± 0.6 mg catechin equivalents/g of dry leaf at 80°C (Oh et al., 2013).

Catechin was measured by HPLC and showed at 40°C, similar values of 4.7 ± 0.05 and 4.4 ± 0.10 mg catechin/g dry leaf, respectively for green and black tea. However, at 90°C, black tea was significantly greater than green tea. This behavior is similar to those reported by Carloni et al. (2013), who obtained 0.3 ± 0.06 values for green tea units and 0.2 ± 0.03 units for black tea. The values found for black tea may be due to the enzymatic oxidation of catechin during the process of developing the product generating phenolic pigments such as theaflavins; therefore, there is little effect of the temperature on the increase of this metabolite (Kerio et al., 2013).

For the highest content of epicatechin found in green tea, values decrease with increasing extraction temperature; however, in the black tea epicatechin increased. A similar behavior was observed with the caffeine measured by HPLC, where green tea showed at 40°C a higher value than black tea, decreasing afterwards with the extraction temperature. These results are comparable with the data reported by Carloni et al. (2013), who obtained 0.6 ± 0.04 results for green tea units and 0.4 ± 0.05 units for black tea. In general, the

content of phenolic compounds is dependent on the temperature and time of extraction.

Cytotoxic effect indicated that the green tea was better than black tea on this colon adenocarcinoma cell line. This is consistent with the IC_{50} value in which for mortiño black tea it was 36.0 µg/ml and for the mortiño green tea it was 26.3 µg/ml. These results indicate that both mortiño black and green tea have a better cytotoxic and antiproliferative effect than other infusions made from green tea (*C. sinensis)* against colon adenocarcinoma cells HT29 (IC_{50} = 90.8 ± 8.3 µg/ml), from yerba mate tea (IC_{50} = 17.7 ± 8.3 µg/ml (Mejía et al., 2010) and white tea (*C. sinensis*; IC_{50} = 86.68 ± 0.73 µg/ml) (Hajiaghaalipour et al., 2015). In addition, results obtained here were better than that observed against colon adenocarcinoma $CaCo^{-2}$ cells treated with green tea (IC_{50} = 161.0 ± 17.4 µg/ml) and yerba mate tea (IC_{50} = 220.0 ± 12.6 µg/ml) (Mejía et al., 2010). Other authors showed green tea extract (*C. sinensis)* inhibit the growth of two renal carcinoma cell lines with IC_{50} values of 54 ± 10 and 129 ± 28 87 µg/ml for A-498 and 769-P cells, respectively (Carvalho et al., 2010).

According to the criteria of the National Cancer Institute of the United States, an extract is considered active if the IC_{50} is less than 30 µg/ml on cancer cells (Suffness and Pezzutto, 1990). In this study, the antiproliferative activity of both mortiño black and green tea against SW480 cells was considered to have medium (IC_{50} = 36.0 µg/ml) and high (IC_{50} = 26.3 µg/ml) cytotoxic and antiproliferative activity, respectively. These results are similar to the evidence obtained with green tea that possess greater efficacy against colon cancer cell lines than black tea. This is attributed to Epigallocatechin-3-gallate (EGCG), the major cathechin found in green tea (Li et al., 2013).

Although structure-activity relationship of the compounds analyzed on mortiño green and black infusions was not explored on SW480 cells, Du et al. (2012) reported that flavonoids, such as catechin and epicathechin, did not show antiproliferative effect on SW480 cells. The gallic acid, a phenolic acid, showed some antiproliferative effect. On the other hand, galloylated catechins (cathechin gallate, epigallocathechin, epicathechin gallate and EGCG) increased significantly the inhibition of SW480 cell growth compared to catechin and epicathechin. This fact suggests that the two close parallel aromatic rings in galloylated catechins and a third aromatic ring vertical to the two parallel rings may play a key role in their biological activities. However, in this study, Mortiño green and black tea infusions presented a similar content of flavonoids, catechin and hydroxycinnamic acids like caffeic and p-coumaric acid, but green tea infusion contained more epicatechin, caffeine, ferulic, chlorogenic acid than black tea infusion. Thus, the higher cytotoxic and antiproliferative activity of green tea related to black tea may be the result of the synergistic action of these and other unknown compounds present in green tea.

Finally, many mechanisms have been proposed for the inhibition of colon cancer cell growth by tea compounds like the ones obtained here in mortiño green and black tea. The mechanisms proposed include inhibition of MAP-kinases and PI3K/AKT pathways, NF-κB and AP-1 mediated transcription, growth factor-mediated signaling, aberrant arachidonic acid metabolism and proteinase activities (Yang et al., 2011).

Conclusion

The results suggest that green tea and black tea made from Mortiño leaves can be used as regular drink and they are a rich source of polyphenols, whose concentrations varied depending on the process and extraction temperature. They exhibited greater free radical scavenging capacity at higher temperatures. Also, both types of tea showed cytotoxic and antiproliferative activity with the growth of SW480 cells from colon carcinoma, so that the continuation of this study for evaluation of safety *in vivo* is suggested. The fact that both types of tea inhibit the growth of colon cancer cells *in vitro* makes these kinds of infusions yet unknown potential source of chemopreventive agents that could be taken into account in future research.

Conflict of Interest

Authors have not declared any conflict of interest.

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Antioxidant capacity of different African seeds and vegetables and correlation with the contents of ascorbic acid, phenolics and flavonoids

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The antioxidant capacity of 2 African seeds and 8 vegetables were analyzed using ferric reducing antioxidant power assay (FRAP assay) after water and acetone extractions. The contents of ascorbic acid, phenolics and flavonoids were determined and their correlations with FRAP value were investigated. The results showed that *Vernonia amygdalina* was stronger (4.84 mM/100 g dry weight (DW)) than other vegetables analyzed in antioxidant capacity based on total FRAP values. *Baphia nitida* was the lowest in total FRAP value (1.26 mM/100 g DW). *Treculia africana* seed was higher than *Telfairia occidentalis* seed in total FRAP value. *T. occidentalis* leaf had antioxidant capacity than its corresponding seed. All water extracts were higher in FRAP value than the acetone extracts. FRAP value was significantly correlated with the contents of ascorbic acid, phenolics, or flavonoids in water extracts and with flavonoids in acetone extract, in which ascorbic acid and flavonoids contributed most in the water extracts based on multivariate regression analysis. In conclusion, the different African seeds and vegetables were remarkably different in antioxidant capacity.

Key words: Antioxidant capacity, ascorbic acid, flavonoids, phenolics, African seeds and vegetables.

INTRODUCTION

Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer (Temple, 2000). These antioxidants are a class of compounds thought to prevent certain types of chemical damages caused by an excess of free radicals-charged molecules that are generated by a variety of sources including pesticides, smoking, radiation and exhaust fumes. The defensive effects of natural antioxidants in fruits and vegetables against these free radicals are related to three major groups: vitamins, phenolics and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants (Halliwell, 1996). These antioxidants are effective in scavenging various free radicals, inhibiting initiation of chained reactions by binding to metal ions (Peschel et al., 2006).

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Previously, a group of Spanish researchers discovered a novel and powerful natural antioxidant (Feruloylnoradenaline) which is 4.5 times more potent than vitamin E and 10 times more potent than vitamin C in tomato plants when under stressful condition (Lopez et al., 2011). The free radical theory of aging researched by Dr. Siegfried Hekimi and his student Dr. Wen Yong was tested by creating mutant worms that had increased production of free radicals, predicting they would be short lived even longer than regular worms! Moreover, their enhanced longevity was abolished when they were treated antioxidants such as vitamin C (Wen and Siegfried, 2010). Although non-antioxidant mechanisms are still undefined, flavonoids and other polyphenols may reduce the risk of cardiovascular disease and cancer (Arts and Hollman, 2005). Many epidemiological studies showed a significant inverse correlation between the intake of fruits and vegetables and the incidence of some chronic diseases (Dauchet et al., 2006). Therefore, increase in the consumption of fruits and vegetables have been frequently recommended to be one of the strategies in the prevention against oxidative stress related diseases (Kaliora et al., 2006; Garrido et al., 2010, 2014).

Several assays have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products and foods for clinical studies including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulforic acid) (ABTS) (Leong and Shui, 2002), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Gil et al., 2002), ferric reducing antioxidant power (FRAP) (Benzie and Strian, 1999), and the oxygen radical absorption capacity (ORAC) (Prior et al., 2003).

The vegetables under review in this research studies include: *Heinsia crinita*- a local shrub commonly known as “atama”, “*Vernonia amygdalina*” locally known as “bitter leaf” because of its bitter taste, “*Lasianthera africana*” called “editan” by the Efiks and Ibibios, “*Gongronema latifolium*” a tropical rainforest plant commonly known as “utasi” by the Efiks and Ibibios in the South-South states of Nigeria, “*Telfairia occidentalis*”, a tropical vine grown in West Africa, commonly known as “fluted pumpkin”, “*Acalypha torta*”, commonly known as “nettle”, *Alchornea cordifolia”,* a perennial shrub with a height of 4 m, commonly called “Christmas bush”, “*Baphia nitida*” a shrubby hard- wooded African tree commonly known as “camwood”. *T. africana* seed” known locally as “ukwa seed” by the Igbos (South-Eastern Part of Nigeria), “*T. occidentalis* seed”, a dark-red seed which bears fluted pumpkin leaves.

In the present study, the ferric reducing antioxidant power assay (FRAP assay) was used to determine the antioxidant capacity of different African seeds and vegetables. The correlations between the FRAP value and content of ascorbic acid, phenolics, or flavonoids were also analysed. The main objective of this study is to make a comprehensive comparison among different African seeds and vegetables and to identify the ones with high antioxidant capacity.

MATERIALS AND METHODS

Collection of plant material

A total of 8 different vegetables and 2 seeds were obtained from different plants at the Botanical Garden, which were identified by Botanists at the Department of Biological Sciences, University of Calabar.

The 2 seeds were (*T. occidentalis*, that is, fluted pumpkin seed and *T. africana* seed, that is, african breadfruit seed), while the 8 were vegetables were (*T. occidentalis*) (fluted pumpkin), *H. crinita* (atama), *A. torta* (nettle), *A. cordifolia* (Christmas bush), *B. nitida* (camwood), *G. latifolium* (utazi), *Lasianthera africana* (editan), *V. amygdalina* (bitter leaf). The vegetables and seeds were properly washed and dried in the laboratory under room temperature for 2 weeks to make them moisture free. Subsequently, they were ground into powdery form with a blender before extraction using water and acetone (Fatope et al., 1999).

Extraction with water and acetone

The extracts where prepared using standard procedures described by Fatope et al. (1999). This involves soaking 50 g of the powdered plant extract in both 95% water and 95% acetone for 48 h at room temperature to allow for maximum extraction. This was subsequently filtered to obtain the water fraction of the plant extracts. The filtrate obtained from the mixture of the powdered extract and acetone was evaporated using a rotary evaporator. The residues were retained as a crude extract for each of the test vegetables and stored in reagent bottle s. Both water and acetone extracts were used directly for Ferric Reducing Antioxidant Power (FRAP) assay on frozen at 20°C before the analysis of ascorbic acid, phenolics and flavonoids within two weeks (Fatope et al., 1999).

Ferric reducing antioxidant power assay (FRAP)

The procedure described by Benzie and Strain (1996) was followed. The principle of this method is based on the reduction of the ferric-tripyridyltriazine complex to its ferrous coloured form in the presence of antioxidants. The FRAP reagent contained 2.5 ml of 10 mM 2,4,6-tripyridy-s-triazine solution in 40 mM Hcl and 2.5 ml of 20 mM $Fecl_3$ and 25 ml of 0.3M acetate buffer, pH 3.6, and was prepared freshly and prewarmed at 37°C. Aliquots of 40 µl of extracts were mixed with 0.2 ml of distilled water and 1.8 ml of FRAP reagent. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$. Adequate dilution was performed if the FRAP value measured was over the linear range of the standard curve.

Determination of ascorbic acid, phenolics and flavonoids

Ascorbic acid

A colorimetric procedure for the measurement of ascorbic acid, including dehydroascorbic acid in fruits, vegetables and derived products was followed in the determination of ascorbic acid in water extracts. Briefly, ascorbic acid was oxidized by activated charcoals to yield dehydroascorbic acid, which was further reacted with 2,4-dinitrophenylhydrazine to form osazone, a light-absorbing substance. The absorbance was read at 490 nm spectrophotometrically and the content of ascorbic acid was calculated based on a standard curve.

Phenolics

The content of phenolics in both water and acetone was determined according to the method reported by Singleton et al. (1999). Aliquots of 0.5 ml of water extracts were mixed with 2.5 ml of 10-fold-diluted Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate (Na_2CO_3). The mixture was allowed to stand for 90 min at room temperature before the absorbance was measured at 760 nm spectrophotometrically. The contribution by ascorbic acid contained in water extract was calculated. The measurements were conducted in triplicate and the results reported as mean ± standard deviation (SD) values. The final results were calibrated to deduct the contribution from ascorbic acid and expressed as gallic acid equivalent.

Flavonoids

The content of flavonoids in both water and acetone extracts was measured using a modified colorimetric method described by Jia et al. (1999). A volume of 2.5 ml of water and acetone extract was transferred to a test tube, mixed with 0.15 ml of 5% sodium nitrite for 5 min. Then, 0.15 ml of 10% aluminium nitrate were added. After 6 min, the reaction was stopped by adding 1 ml of 1M sodium hydroxide. The mixture was further diluted with distilled water up to 5 ml. The absorbance of the mixture was immediately measured at 510 nm. The flavonoid content was calculated and expressed as rutin equivalent.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 20. Measurements were carried out in triplicate. Data were presented as mean ± standard error of mean (SEM). The correlation between the FRAP value and the content of ascorbic acid, phenolics or flavonoids were analyzed. Multivarate regression analysis was also applied to investigate the relative importance to the contribution of the FRAP value by ascorbic acid, phenolics or flavonoids. Significant levels were tested and accepted at value of $P<0.05$.

RESULTS

Antioxidant capacity of different African seeds and vegetables

The FRAP values of 2 seeds and 8 vegetables of African origin (Table 1) shows that out of the 8 vegetables analyzed, *V. amygdalina* ranked the highest in total FRAP value (4.84 mM/100 g dry weight (DW)). This value is consistent with that obtained by Kelly et al. (2013). *G. latifolium* ranked second in total FRAP value (4.26 mM/100 DW), while the remaining vegetables had FRAP value ranging from 1.26 to 3.49 mM/100 DW. *B. nitida* was the lowest (1.26 mM/100 g DW). Two seed fractions (*T. africana* and *T. occidentalis*) were also analyzed. *T. africana* seed was higher in total FRAP value than *T. occidentalis* seed.

All water extracts were higher in FRAP value than the acetone extracts among all the vegetables analyzed in this study. Similar report was given by Ji et al. (2011), though with their own local vegetables. It was also shown that the leaf fraction of *T. occidentalis* was stronger in antioxidant capacity as measured by FRAP assay than the corresponding seed fraction. This is in agreement with the report by Mohammed et al. (2012) whose work showed that *T. occidentalis* leaf had antioxidant activity.

It is time-consuming and also difficult technically to isolate the antioxidants one by one from the vegetables, because there are too many compounds displaying antioxidant activity in the vegetables, such as ascorbic acid, β-carotene, phenolics, flavonoids, and others (Hein et al., 2002; Seifried, 2007). In the current study, the contents of ascorbic acid, phenolics and flavonoids of different African seeds and vegetables were measured in an attempt to investigate the relative contribution to the antioxidant capacity by different antioxidants.

DISCUSSION

Ascorbic acid is a well-known antioxidant and plays an important role in collagen synthesis and iron absorption (Mandl et al., 2009). It was found that *V. amygdalina* contained more than 300mg of ascorbic acid per 100g DW of the leaf fraction analyzed (Table 2) indicating that this vegetable is a good source of ascorbic acid.

This value is in contrast with that obtained by Olajire et al. (2011). Odukoya et al. (2007) reported a lower value for *V. amygdalina*. *T. occidentalis* leaf contained more than 200 mg of ascorbic per 100 g DW. However, the ascorbic acid content of the remaining vegetables (*G. latifolium*, *A. torta*, *L. africana*, *B. nitida* and *A. cordifolia*) was below 194.0 mg/100 g DW. Between the two (2) seeds analyzed, it was shown that *T. occidentalis* seed had higher ascorbic acid content (193.33 mg/100 g DW) than *T. africana* seed (178.33 mg/100 g DW). Comparatively, it was found that the leaf fraction of *T. occidentalis* was higher (218.00 mg/100 g DW) in ascorbic acid content than the corresponding seed fraction (193.33 mg/100 g DW) in the water extract.

Phenolics are small molecules containing one antioxidant activity *in vitro*. They play an important role in the protection of plants against ultraviolet radiation, or pathogens and predators (Strack, 1997). Most phenolics present in vegetables are water soluble in nature. For example, Toor and Savage (2005) reported that the hydrophilic phenolics contributed 78 to 87% of the total phenolics presents in the tomato pulps. In this study, the phenolics content of different vegetable fractions were determined in water extracts. The result showed that *G. latifolium* had the highest phenolic content (327.67±1.15 mg/100 g DW), followed by *T. occidentalis* leaf and *H. crinita*, in which more than 100 mg of phenolics per 100 g DW was detected. The other vegetables contained less than 100 mg of phenolics per 100 g DW. The seed (*T. africana* seed and *T. occidentalis* seed) contained less hydrophilic phenolics than the vegetable leaf fraction. The leaf fraction of *T. occidentalis* had high phenolic content (137.67 mM/100 g) than the corresponding seed.

Table 1. FRAP values of 2 different seeds and 8 vegetables of African origin (mg/100 g DW).

Vegetable	Fraction			
	Water	Acetone	Total	Rank
Vernonia amygydalina	4.31±0.07	0.54±0.01	4.84	1
Gongronema latifolium	3.85±0.04	0.41±0.01	4.25	2
Acalypha torta	1.84±0.04	0.19±0.00	2.04	9
Lasianthera africana	2.03±0.05	0.20±0.01	2.23	8
Baphia nitida	1.09±0.07	0.17±0.01	1.26	10
Alchornea cordifolia	2.08±0.06	0.20±0.01	2.28	6
Heinsia crinita	3.33±0.11	0.35±0.02	3.68	3
Teculia africana seed	3.18±0.03	0.31±0.00	3.49	4
Telfairia occidentalis leaf	2.93±0.03	0.27±0.03	3.20	5
Telfairia occidentalis seed	2.04±0.04	0.22±0.01	2.26	7

Data are expressed as mean±SEM. Each seed and vegetable was analyzed 3 times.

Table 2. The contents of ascorbic acid, phenolics and flavonoids of 2 different African seeds and 8 vegetables.

Vegetable	Ascorbic acid (mg/100 g DW)	Phenolics in water extract (mg/100 g DW)	Flavonoids (mg/100 g DW)		
			Water	Acetone	Total
Vernonia amygydalina	311.00±6.08	97.33±0.67	10.40±0.46	0.50±0.03	10.90
Gongronema latifolium	193.33±2.40	327.67±1.45	18.00±0.58	0.52±0.04	18.52
Acalypha torta	57.33±0.88	88.00±0.58	5.00±0.15	0.35±0.01	5.35
Lasianthera africana	122.00±1.15	96.33±0.88	4.57±0.15	0.32±0.01	4.88
Baphia nitida	70.00±2.52	79.33±2.60	3.50±0.06	0.09±0.01	3.59
Alchornea cordifolia	91.67±2.03	67.00±2.08	1.71±0.03	0.20±0.01	1.91
Heinsia crinita	46.33±1.0.88	115.33±2.60	9.17±0.20	0.48±0.02	9.64
Teculia africana seed	178.33±0.88	33.67±2.40	0.70±0.02	-[b]	0.70
Telfairia occidentalis leaf	218.00±1.53	137.67±1.45	11.00±0.58	0.52±0.03	11.52
Telfairia occidentalis seed	193.33±2.40	46.33±0.88	0.47±0.03	-[b]	0.47

Data are expressed as mean±SEM. Each seed and vegetable was analyzed 3 times. -[b]Means not detected.

Odukoya et al. (2007) reported that the phenolic content of twelve Nigerian vegetables including *T. occidentalis*, *V. amygdalina*, *L. africana*, and *G. latifolium* ranged from 21.83 mg/100 g DW to 546.97 mg/100 g DW, while a range between 33.67 mg/100 g DW to 327 mg/100 g DW was obtained in this work.

Flavonoids are a class of plant secondary metabolites and possess many biological actions, such as anti-allergic, anti-inflammatory, anti-microbial, and anti-cancer activities (Middleton et al., 2000). *G. latifolium* had the highest (18.00 mg/100 g DW) content of flavonoids among all the vegetable in water extract. The seeds in the water extract were poor in flavonoids. The vegetables in acetone extracts were also poor in content of flavonoids (Values <0.60 mg/100 g DW). No detectable flavonoids were found in the seed fractions (*T. africana* seed and *T. occidentalis* seed) in acetone extract. The leaf fractions contained more flavonoids than the seeds. More flavonoids were distributed in the water extract than the acetone extracts. Ji et al. (2011) reported that the water extracts of vegetable fractions was higher in FRAP value than the acetone extracts.

Correlations between the FRAP value and content of ascorbic acid, phenolics, or flavonoids in different African seeds and vegetables

The individual correlation graph is indicated (Figures 1 to 4). For the water extracts, the FRAP value of different African seeds and vegetables was significantly correlated with the content of ascorbic acid, phenolics or flavonoids (R^2=0.4285, 0.2132, 0.4331). For the acetone extracts, the FRAP value was also significantly correlated with the content of flavonoids (R^2=0.28). Based on multivariate regression analysis (Table 3), it was indicated that among ascorbic acid, phenolics, and flavonoids, ascorbic acid and flavonoids are the most important factor in

Table 3. Multivariate regression analysis among the FRAP value and contents of ascorbic acid, phenolics, and flavonoids in water extracts.

Variable	Unstandardized coefficient		Standard coefficient		
	B	S.E	Beta	T	Sig (p)
Constant	1.234	0.240	-	5.130	0.000
Asorbic acid	0.006	0.001	0.488	3.008	0.000
Phenolics	-0.005	0.003	-0.407	-1.501	0.145
Flavonoids	0.89	0.22	0.494	4.052	0.000

Significant difference at p>0.05.

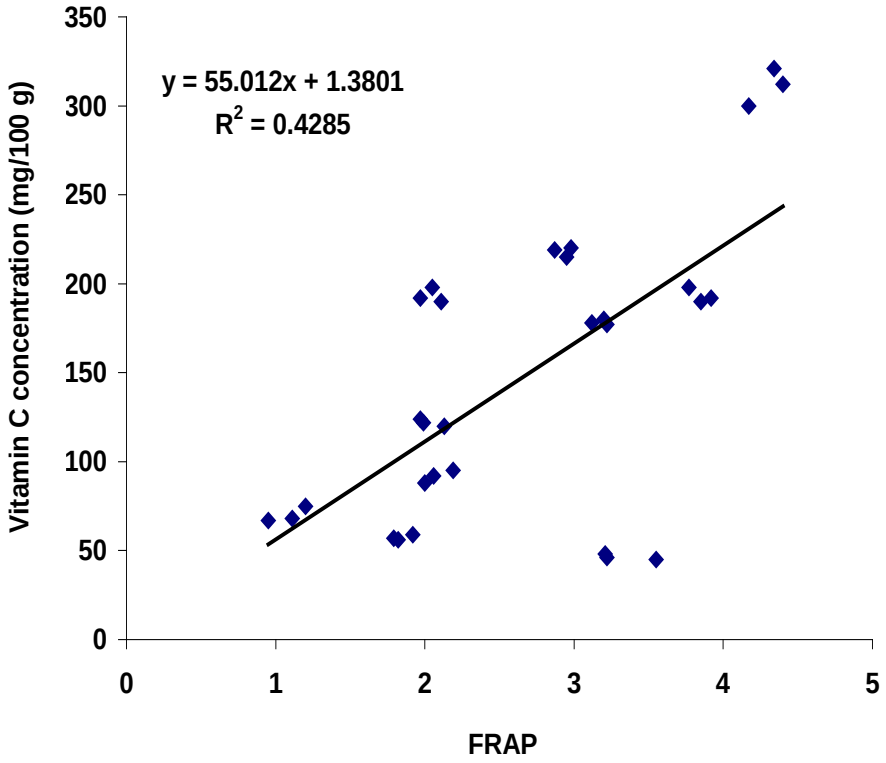

Figure 1. Relationship between FRAP and vitamin C content of water extract.

contributing to the FRAP value of the different African seeds and vegetables in water extracts (Table 3). This is in contrast with the result obtained by Oboh et al. (2004). They reported that phenol contributed more to the antioxidant properties of vegetable than ascorbic acid. This is because the content of ascorbic acid reduces when the vegetables are sun dried. Comparatively, the vegetables used for this research work were not sun dried.

Conclusion

It was found that the different seeds and vegetables were remarkably different in antioxidant capacity. *V. amygdalina* was the highest in total FRAP value among all the vegetables analyzed. *T. africana* seed was higher than *T. occidentalis* seed fraction in total FRAP value. *T. occidentalis* leaf had high antioxidant capacity than its corresponding seed. All water extracts were higher in FRAP value than the acetone extract. Ascorbic acid and flavonoid contributed most significantly to the antioxidant capacity of the vegetables and seeds in the water extracts.

This study is a systematic comparison of antioxidant capacity among different African seeds and vegetables. Further studies should be carried out on other fractions of these vegetables to investigate the specific components

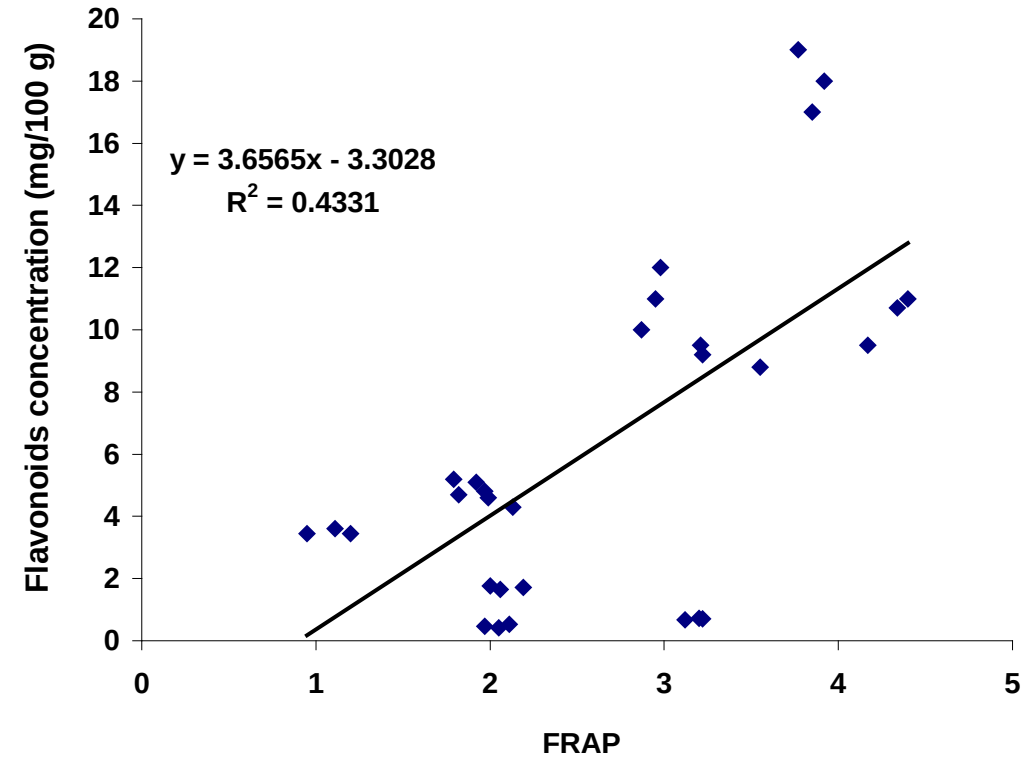

Figure 2. Relationship between FRAP and flavonoids content of water extract.

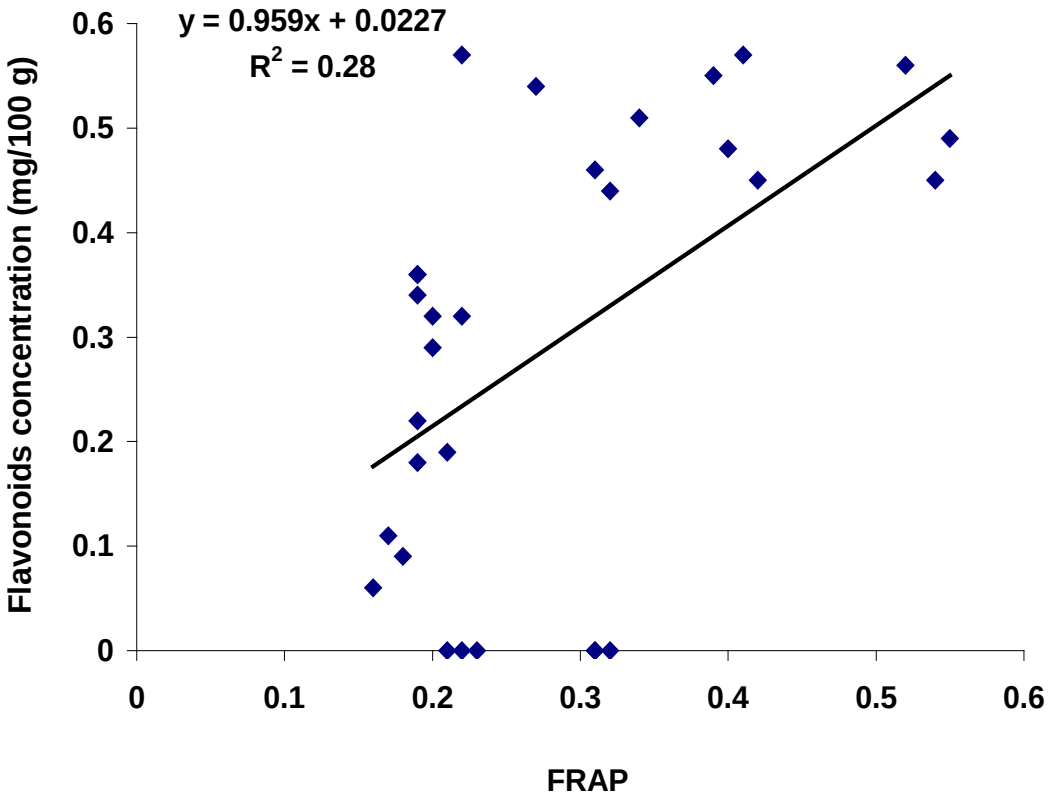

Figure 3. Relationship between FRAP and flavonoids content of acetone extract.

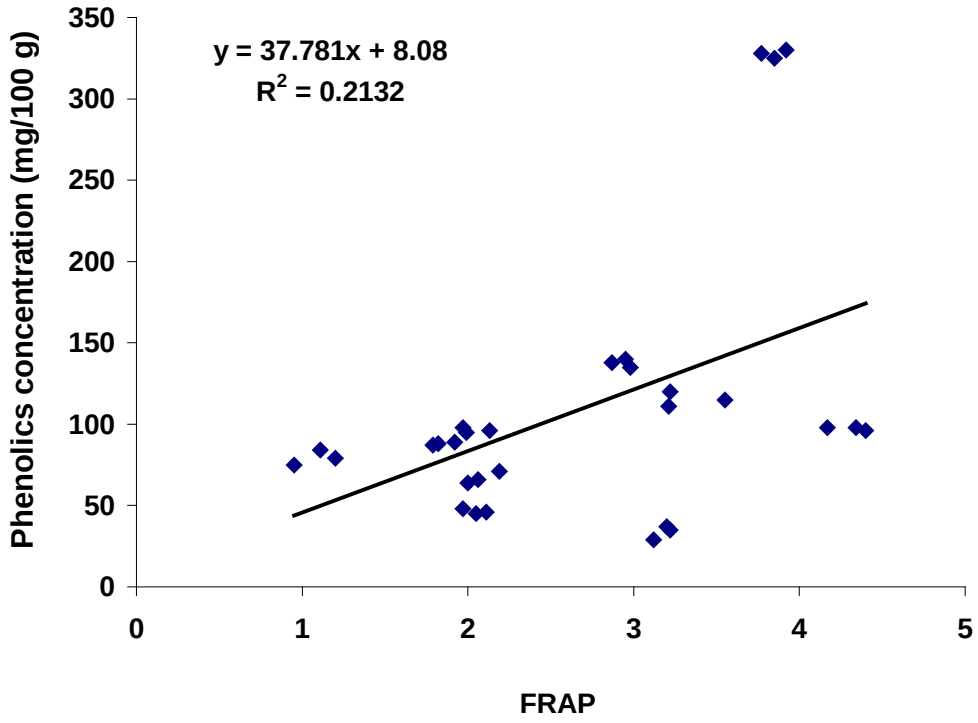

Figure 4. Relationship between FRAP and phenolics content of water extract.

responsible for their high antioxidant capacity and their possible health effects.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Effect of *Commiphora swynnertonii* resin extract on various physiological parameters in chickens

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In the current study, various haematological and biochemical parameters of chickens were evaluated following an oral administration of resin extract from *Commiphora swynnertonii*. Sixty chickens (8 months old) were randomly assigned into 5 groups (n=12): G1 served as a negative control, that is, chickens received normal saline only, while G2 to G5 chickens were given different doses of the resin extract orally for 14 consecutive days. Results revealed no signs of sickness or death in groups G1, G2 and G3 throughout the experimental period of 28 days. The body weights of chickens in G2, G3, G4 and G5 decreased significantly in a dose dependent manner from day 3 of treatment with the resin. The resin administration did not affected packed cell volume (PCV), total white blood cell (WBC) count, differential WBC count (heterophils and eosinophils). However, haemoglobin (Hb), total red blood cell (RBC) count, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) decreased significantly ($P < 0.05$) with increasing concentration of the resin. Significant dose dependent reduction ($P < 0.01$) in plasma glucose and total cholesterol was observed. Levels of total protein, albumin and globulin in the resin-treated groups were not significantly affected. In the current study, liver transaminases (alanine transaminase (ALT) and aspartate aminotransferase (AST)) levels increased slightly to a maximum peak by day 21-post treatment. It is concluded that administration of the resin extract in chickens caused no undesirable effects at lower doses, but with increased doses signs of toxicity were evident. It is therefore suggested that, before the resin extract can be used for veterinary or medical purposes, safety margin must be established for each animal species in question.

Key words: *Commiphora swynnertonii*, haematological parameters, biochemical parameters, resin extract, treatment, chicken.

INTRODUCTION

Plants have been recognized as indispensable sources of both preventive and curative traditional medicine preparations for human beings and livestock since time immemorial (Dharmananda, 2003; Adnan et al., 2010). According to World Health Organization, approximately 80% of the world's inhabitants use traditional medicines, particularly herbal preparations (WHO, 2007). In some countries, the use of herbal preparations is being

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gradually integrated into the primary and secondary health care systems (El-Mahmood and Ameh, 2007). As a result of this increased interest, research on traditional medicines is on the rise as manifested by plenty of scientific literature on antimicrobial activity of plants and their secondary metabolites (Adnan et al., 2010). *Commiphora swynnertonii* which belong to the family Burseraceae is among plants that claimed to have a broad spectrum of medicinal activities. In Tanzania, various parts of this plant are used for treatment of health related problems including wounds, diarrhoea, respiratory aliments and intestinal parasites (Kaoneka et al., 2002). Recently, resin from *C. swynnertonii* was shown to have strong activity against New Castle disease virus, bacteria and coccidian parasites in chickens (Bakari et al., 2012a, b, 2013). Despite of these promising results and the fact that C. *swynnertonii* resin is widely used in Tanzania, there is no scientific documentation on safety and/or any adverse effects associated with its use both in humans and animals. This study was therefore designed and carried out to investigate any side effects that could be associated with administration of the resin in chickens. In particular, selected internal organs, biochemical and haematological parameters were examined following administration of varying loads of the resin in growing chickens.

MATERIALS AND METHODS

C. swynnertonii resin extract

Resinous material from *C. swynnertonii* tree was collected, extracted and preserved as described by Parekh and Chanda (2006). Briefly, 500 g of the resinous material were soaked in 1,000 ml of ethanol (99.8% v/v) in a conical flask plugged with aluminium foil and kept for 2 h in a dark place at room temperature. After soaking, the suspension was filtered using Whatmann® filter paper No. 1. The filtrate was concentrated on water bath at 50°C using a rotary evaporator (BUCHI, Switzerland). The resulting crude resin extract was stored at 4°C in airtight bottles until used.

Experimental animals and housing

Healthy chickens (Black Australorp) of 8 months old were purchased from commercial farmers in the township of Morogoro. The chickens were caged in pairs and maintained on a basal feed of growers mash, with mineral supplements (Amintotal®) and *ad libitum* access to drinking water. All chickens were dewormed and vaccinated against Newcastle (ND) and Infectious Bursal disease (IBD); they were also assessed for any signs of diseases. The chickens were left for three weeks to acclimatize with experimental environment. Following acclimatization, they were weighed, wing tagged and randomly assigned into five experimental groups of 12 chickens each.

Experimental design

Treatment allocations are shown in Table 1. Groups 2 to 5 chickens were given different doses of aqueous resin extract orally by means of a 5-ml plastic syringe once a day for 14 consecutive days. Group 1 remained as negative control, that is, chickens that received normal saline only. Immediate and extended signs of toxicity and changes in body weight were observed. Blood samples were collected for evaluation of haematological and biochemical parameters.

Collection of blood for haematological and biochemical parameters

Blood samples (approximately 3 ml) were collected from wing veins using syringe and 23G needle at regular interval from day 0, 3, 7, 14, 21 and 28. About 1 ml blood was then transferred into blood sample bottles containing EDTA for haematological parameters analysis, while the remaining 2 ml were immediately centrifuged at 3000 rpm for 10 min to obtain fresh plasma, which was used for analysis of biochemical parameters. To avoid diurnal variability, sampling was carried out in the morning between 7 and 9 a.m.

Determination haematological parameters

These parameters were determined as described by Fudge (2000). Briefly, packed cell volume (PCV) was determined using a Hawksley haematocrit reader. Enumeration of erythrocytes and leucocytes was carried out using an improved Neubauer haematocytometer. Haemoglobin (Hb) was determined by spectrophotometer at 540 nm. Cellular characterization involved staining of blood smears with Giemsa followed by microscopic examination of not less than 50 fields.

Determination of biochemical parameters

Determination of blood glucose and cholesterol levels was done as explained by Trinders et al. (1969). Total protein was determined by Biuret method as described by WHO (2006). Albumin was measured by bromocresol green based on principles described by Bush (1991). Liver enzymes (alanine transaminase (ALT) and aspartate aminotransferase (AST)) were determined by the kinetic methods described by International Federation of Clinical Chemistry without pyridoxal phosphate. Creatinine was determined by colorimetric method using Jaffe Elitech® kits.

Post mortem examination

Chickens were humanly killed and tissue sections of muscles, liver, kidney and intestines were preserved for histopathological examination. The preserved tissues were processed as described by Drury and Wallington (1976).

Data analysis

Haematological results were presented as mean ± standard error of mean (SEM). One way analysis of variance (ANOVA) was used to determine significance between tests and controls. P-values less than 0.05 were considered significant.

RESULTS

Clinical signs

No signs of sickness or death were observed in the G1,

Table 1. Grouping and treatments allocation.

Group (n =12)	Treatment given once daily for 14 days
G1	Control (not given resin extract)
G2	250 mg resin/kg
G3	500 mg resin/kg
G4	750 mg resin/kg
G5	1000 mg resin/kg

G2 and G3 group throughout the experimental period of 28 days. Twenty one percent (5/24) of chickens from G4 and G5 showed signs of dullness and voided loose whitish faeces. These signs were evidently seen from day 7 of resin treatment.

Mean body weights

Mean body weights of chickens in G1 increased gradually ($P < 0.001$) throughout the experimental period compared to resin treated groups. From day 3 of the treatment, the body weights of chickens in G2, G3, G4 and G5 decreased significantly in a dose dependent manner ($R^2 = 0.85$; $P = 0.02$). From day 14 post treatment, the mean body weights were almost constantly decreased till the end of experimental period.

Haematological parameters

Haematological parameters are shown in Table 2. PCV values of chickens in the negative control group (G1) were significantly higher ($P < 0.05$) compared to those of resin-treated groups (G2, G3, and G5). There was no significant difference in the levels of PCV among the resin treated groups. Hb levels for all treated groups were significantly ($p < 0.001$) lower than that of the negative control group (G1). Similar trend was observed with total red bllod cell count (RBC) count whereby the levels for all groups treated with aqueous resin extract were lower than that of G1.

The values for the calculated MCH were observed to be significantly higher in G_2 and G5 as compared other treated groups. While the MCHC was observed to decrease in all groups but more the decrease was highly noted in G2 and G5. Total white blood cell (WBC) count of chickens in G1 and G2 was significantly lower ($p< 0.001$) as compared to G3, G4 and G5. This significant difference was attributed to increased levels of monocytes and lymphocytes compared to other cells such as heterophils and eosinophils. The levels of heterophils and eosinophils were similar in all groups regardless of the treatment given (Figures 1 and 2).

Biochemical parameters

Plasma glucose levels of chickens treated with aqueous resin extract are shown in Figure 3. Daily treatment with resin extract at dosages of 250, 500, 750 and 1000 mg/kg produced significant ($P < 0.01$) decrease in the plasma glucose levels. This decrease in plasma glucose level was dose and time dependent ($R^2 = 0.83$; $p= 0.01$). For instance, the glucose levels on day 7 after the last resin dose was 228.3 to 110.0 mg/dl and 222.4 to 138.0 mg/dl for G4 and G5, respectively.

The total plasma cholesterol decreased in a dose dependent manner as shown in Figure 4. By day 3 of treatment, levels in the treated groups were significantly ($p < 0.01$) lower than that of the negative control group (G1). There was a negative correlation between the increase in doses of resin with that of plasma total cholesterol level (correlation coefficient = - 0.84).

The resin extract did not induce any significant variations in the levels of total plasma protein including those of albumin and globulin. That is, similar patterns were observed across the groups regardless of resin treatment. Mostly, the total protein values ranged from 4.4 ± 0.3 to 5.9 ± 0.5 g/dl. For instance, levels of all the three parameters in all groups were the lowest around day 15 of treatment and increased steadily towards the end of observation period.

The effect of the extract on liver function markers (ALT and AST) was not significant although between days 14 and 21 levels in G3, G4 and G5 were slightly higher ($P = 0.08$) than in the control group. For instance, the trends of ALT in G3 ranged from 30.4 ± 3.0, 48.6 ± 4.6 and 50.3 ± 6.5 IU/L from day 0, 7 and 14, consecutively. Similarly, AST in the same group had the same trend which ranged from 113.5 ± 13.2, 122.9 ±4 9.8, 128.2 ± 11.0 and 141.6 ± 12.3 IU/L from day 0, 7 and 14, consecutively, though the levels in all groups were within the normal range.

Plasma creatinine levels increased in a dose dependent manner for all treated groups as compared to the negative control groups. The levels were significantly ($P < 0.01$) increased from day 7 of treatment. Uric acid levels did not differ significantly ($P > 0.05$) among treated groups, although the increased level was seen from last day of treatment with resin extract. For plasma creatinine and uric acid, the levels resumed immediately to the normal level following cessation of resin administration.

Pathological and histopathological findings

Two chickens from each group were sacrificed and examined for visual and histopathological lesions in selected organs and tissues. Liver of chickens in G4 showed marked enlargement with numerous yellowish patches of different sizes (mottling appearance).

No visual changes were observed in the intestines, kidneys, lungs, spleen, muscles in normal and chickens treated with resin extract at doses lower than 500 mg/kg. Histopathological results showed structural damage to the liver and kidney tissues of chickens treated with 250 and 500 mg extract per kg bodyweight. However, at higher

Table 2. Mean haematological parameters of chickens following oral administration of *C. swynnertonii* resin extract.

Parameter	Day	Group				
		G1	G2	G3	G4	G5
PCV (%)	0	23.0 ± 1.1	24.2 ± 0.7	24.9 ± 0.8	24.2 ± 0.8	26.2 ± 1.0
	14	26.8 ± 1.5*	24.8 ± 0.7	23.9 ± 1.4	21.9 ± 1.4	24.2 ± 1.4
Hb (g/dl)	0	8.1 ± 0.4	7.9 ± 0.8	8.2 ± 0.4	7.9 ± 0.4	9.1 ± 0.4
	14	8.5 ± 0.3	6.3 ± 0.5*	5.6 ± 0.5**	5.5 ± 0.5**	5.4 ± 0.5***
RBC count ($\times 10^6$ µL)	0	3.9 ± 0.4	3.7 ± 0.2	3.8 ± 0.2	3.6 ± 0.2	3.9 ± 3.0
	14	3.9 ± 0.1	2.8 ± 0.3*	2.8 ± 0.1**	2.3 ± 0.2**	1.9 ± 0.3***
MCH (pg/cell)	0	21.9 ± 1.7	21.4 ± 1.8	21.4 ± 1.6	22.2 ± 1.8	25.3 ± 2.5
	14	21.7 ± 1.2	26.4 ± 4.2*	22.7 ± 1.7	24.7 ± 2.4	37.4 ± 6.9*
MCHC (%)	0	36.8 ± 3.4	33.1 ± 3.1	33.0 ± 2.7	32.7 ± 1.4	35.4 ± 2.4
	14	32.7 ± 2.3	23.5 ± 2.1*	28.9 ± 4.3	28.7 ± 2.8	23.0 ± 2.2**
Total WBC ($\times 10^3$ µl)	0	1.8 ± 0.2	1.5 ± 0.2	1.1 ± 0.2	1.7 ± 0.1	2.1 ± 0.3
	14	1.7 ± 0.2	1.5 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.2 ± 0.3
Lymphocytes (%)	0	57.2 ± 1.9	57.5 ± 1.7	57.5 ± 1.7	54.9 ± 2.1	56.6 ± 2.*1*
	14	57.1 ± 1.9	61.3 ± 2.4*	60.5 ± 2.7*	63.0 ± 2.1**	62.5 ± 1.8**
Heterophils (%)	0	32.1 ± 1.7	29.6 ± 1.4	30.5 ± 2.1	29.6 ± 1.4	29.6 ± 1.4
	14	26.8 ± 1.6	30.2 ± 1.9	30.3 ± 1.6	31.2 ± 1.8	32.8 ± 1.6
Monocytes (%)	0	7.0 ± 0.5	6.0 ± 0.4	5.9 ± 0.7	6.0 ± 0.5	5.3 ± 0.4
	14	6.8 ± 0.4	7.6 ± 0.4*	8.3 ± 0.3***	7.8 ± 0.3**	7.5 ± 0.3***
Eosinophils (%)	0	3.7 ± 0.5	4.3 ± 0.5	3.3 ± 0.4	4.3 ± 0.5	4.3 ± 0.5
	14	3.8 ± 0.6	4.4 ± 0.4	3.7 ± 0.4	4.4 ± 0.4	4.4 ± 0.4

Tabulated values are the mean ± standards error of the mean for 12 determinations; *P< 0.05; ** P<0.01; ***P<0.00.

doses of 750 and 1000 mg/kg, mild congestion, fatty degeneration and infiltration of mononuclear inflammatory cells around blood vessels (perivascular cuffing) was observed at subcapsular and around portal triad of the liver. In addition, cortical hemorrhages, medullary congestion, hydropic degeneration of the cortical - tubular epithelium and glomerulus were seen in the kidneys from G4. The group treated with 1000 mg/kg (G5) also showed acute glomerulonephritis, passive pulmonary congestion with mild atelectasis (collapse of alveolar tissues). Hyperplasia of lymphocytic white pulps was identified in their spleens.

DISCUSSION

This study has demonstrated the effect of aqueous crude resin extracts from *C. swynnertonii* on various haematological and biochemical parameters in chickens. Only chickens receiving higher resin doses (G4 and G5) showed signs of adverse effects including dullness and loose faeces. This was an indication that extended administration of high doses of the *C. swynnertonii* resin extract could be detrimental to the gastrointestinal tract (GIT) of chickens. Similar observations have been reported in rats (Scott, 2005) and humans (Olivier, 2009) who noted increased mucus production in the intestinal tract following treatment with resin from some *Commiphora* species. These findings suggest that the resin can stimulate production of mucus in the GIT. The negative effect of resin on body weight of chickens was clearly evident and was dose-dependent. The weight reduction effect has been associated with reduction in plasma cholesterol and glucose levels through

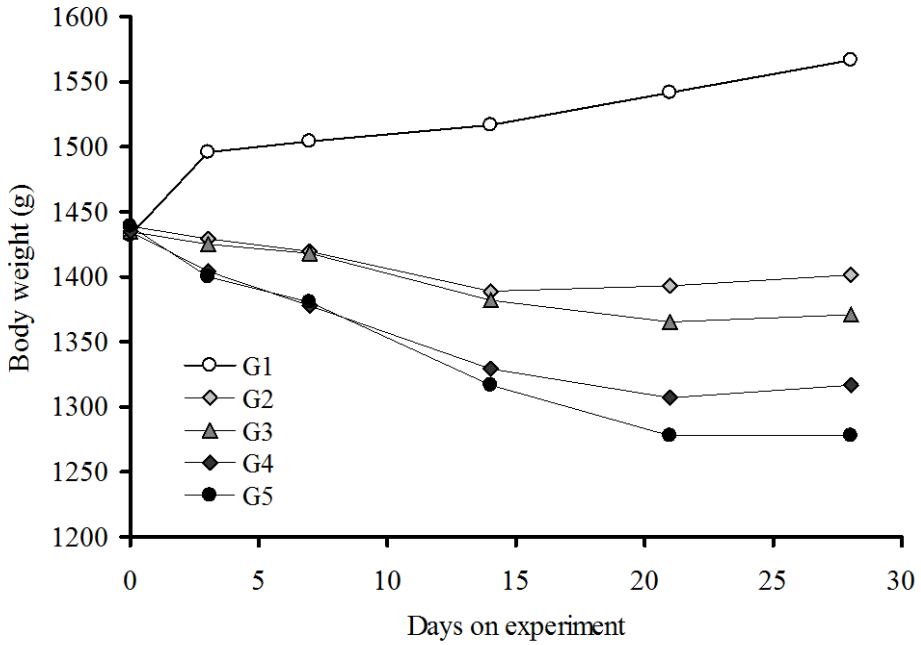

Figure 1. Changes in body weight following resin administration for 14 days with different levels of *C. swynnertonii* resin extract.

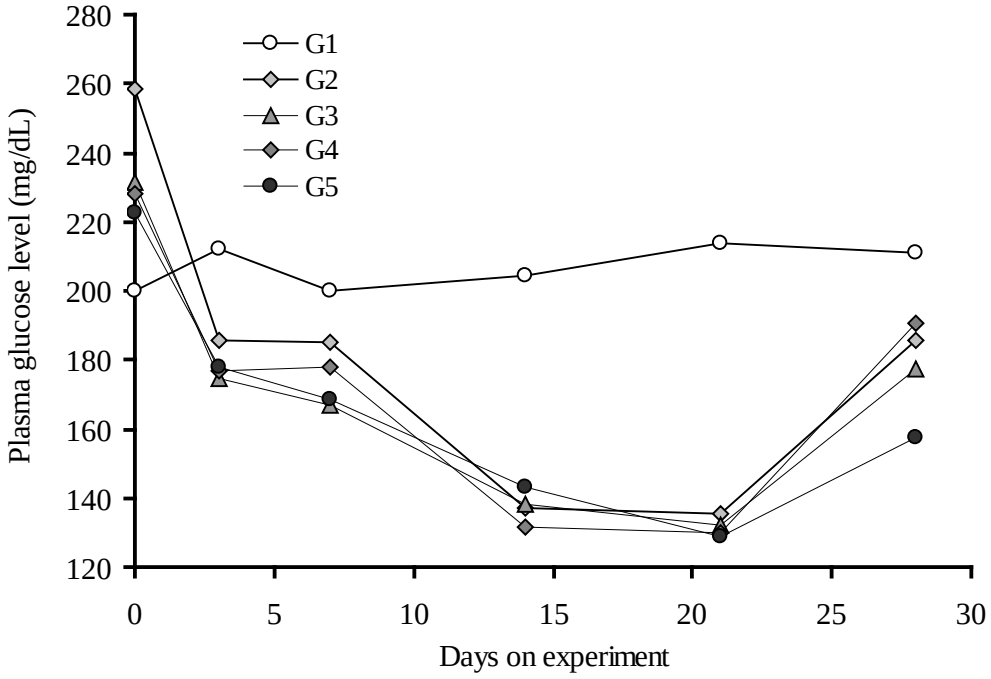

Figure 2. Total plasma glucose profiles of chickens following administration of *C. swynnertonii* resin extract.

stimulation of thyroid hormone (T3 and T4) function, thus interfering with basal metabolic rate leading to loss of body weight (Scott, 2005). Thyroid hormones (T3), stimulates the production of RNA polymerase I and II, and therefore, increases the rate of protein synthesis and potentiates the effects of the β-adrenergic receptors on

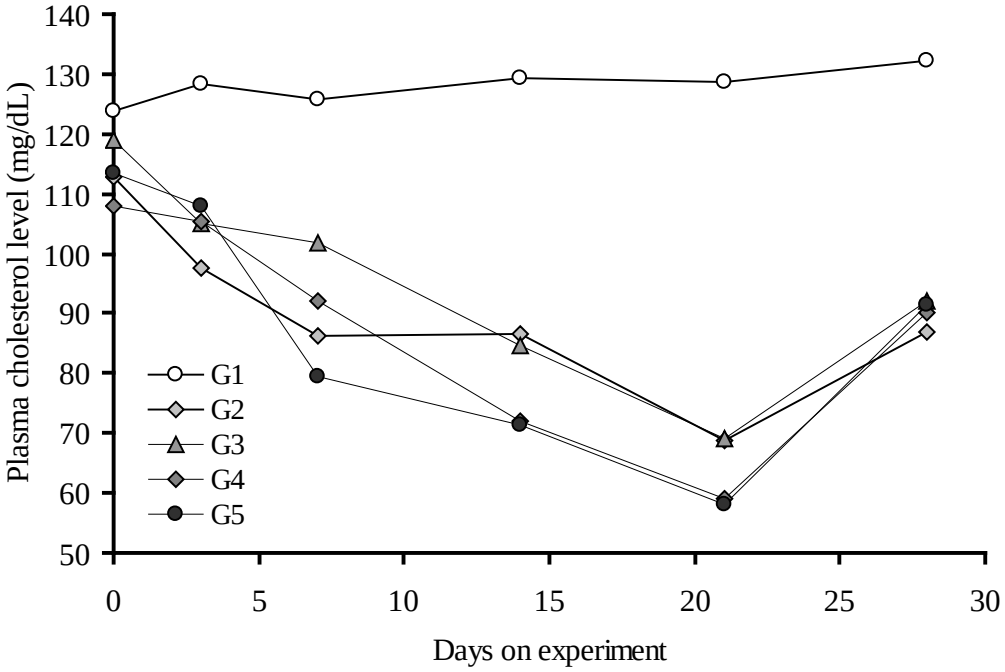

Figure 3. Total plasma cholesterol profiles of chickens following administration of *C. swynnertonii* resin extract.

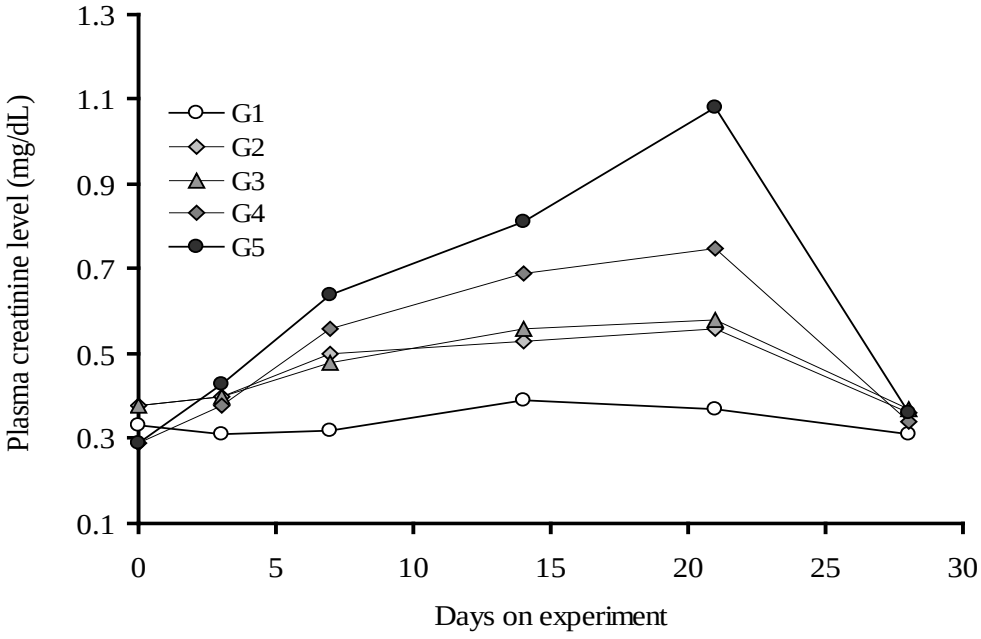

Figure 4. Plasma creatinine profiles of chickens following administration of *C. swynnertonii* resin extract.

the metabolism of glucose (Guyton and John, 2006). Also thyroid hormones stimulate the breakdown of cholesterol and increases the number of low-density lipoprotein (LDL) receptors, thereby increasing the rate of lipolysis (Guyton and John, 2006). In the current study, the metabolite reduction caused by the resin extract could be a probable cause of decreased body weight in chickens.

Administration of the resin to chickens affected some of

the haematological parameters in different ways. PCV, differential WBC count (neutrophils and eosinophils) were not affected. Hb, total RBC count, MCH and MCHC decreased with increasing concentration of the resin. This significant decrease could be as a result of the presence of saponins in the resin. Saponins are known to cause red blood cell breakdown by dissolving their membranes; hence, causing haemolytic crisis (Kayser et al., 2002) and suppression of growth and differentiation of RBC in the bone marrows (Elekofehinti et al., 2012). Similar studies with other *Commiphora* spp. reported no significant changes in PCV, Hb, MCH, MCHC and RBC counts in experimental animals (El-Naggar, 2011). The significant increase in lymphocytes and monocytes counts indicated that *C. swynnertonii* has the ability to activate the defence mechanism in chickens. In this study, increased mononuclear cell infiltration was observed in liver and kidney. A study by Haffor (2009) reported an increase in leucocytes proliferation in Wistar albino rats following an oral administration of resin from *Commiphora molmol*. The significant dose dependent reduction in plasma glucose and total cholesterol can be referred to as hypoglycemic and hypocholesteremic effect, respectively. Other studies using various *Commiphora* spp. also reported hypoglycemic effect in Wistar rats (Sheela and Augusti, 1992; Helal et al., 2006; Goji et al., 2009). This effect has been associated with increased glycogen intake by increasing insulin level. Helal et al. (2006) attributed the hypoglycemic effect of *Commiphora* with a decreased production of glucose precursors in the liver, suggesting the usefulness of this therapy in treating non-insulin dependent diabetes mellitus. Another interesting finding in the current study was the reduction in total plasma cholesterol following administration of the resin. Other studies involving differential cholesterol determination revealed reduction in total cholesterol, low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein (VLDL-c) cholesterol at the same time elevating the high density lipoprotein cholesterol (HDL-c) (Wang et al., 2004; Adebayo et al., 2006; Bellamkonda et al., 2011). The exact mechanism through which the *Commiphora* resin reduces plasma cholesterol levels is yet to be known. Some *Commiphora* spp. (e.g., *Commiphora mukul*) contains some compounds such as guggulsterone, which act by antagonizing the effect of the nuclear farnesoid × receptor (F×R) (Tu et al., 2000; Thrall et al., 2006). The F×R is a key transcriptional regulator for the maintenance of cholesterol and bile acid dynamics. F×R has been shown to regulate cholesterol metabolism by binding directly to the chenodeoxycholic acid (CDCA), a primary bile acid, which mediates the feedback suppression by bile acids of cholesterol 7-alpha-hydroxylase, thus limiting the enzyme in bile acid biosynthesis from cholesterol (Wu et al., 2002). Secondly, the F×R participates in the activation of intestinal bile acid binding protein, which is involved in the enterohepatic circulation of bile acids. Thus, according to Tu et al. (2000), F×R constitutes a potential therapeutic target that can be modulated to enhance the removal of cholesterol from the body. Another possible mechanism is through the presence of ketosteroid, an active compound of *C. mukul* which acts by stimulating the thyroid gland and has also been found to increase the activity of catecholamine and dopamine-p-decarboxylase that are involved in lowering plasma cholesterol (Wang et al., 2004; Wang et al., 2006). Some secondary plant metabolites such as coumarin, flavonoid, terpenoid, arginine and glutamic acids have been shown to confer glucose and cholesterol lowering effects in various experimental animal models (Akah and Okafor, 1992; Marles and Farnsworth, 1995). The significant hypoglycemic and hypocholesteremic effect observed in the current study can therefore be explained by the fact that *C. swynnertonii* contain remarkable amounts of terpenoids and flavanoids. Terpernoids and flavonoids appear to be involved in the stimulation of the ß-cells and the subsequent secretion of preformed insulin (Goji et al., 2009; Bellamkonda et al., 2011).

Liver enzymes transaminases (AST and ALT) are often used as specific markers of active hepatic injury and represent markers of hepatocellular necrosis (Davern and Scharschmidt, 2002; Thrall et al., 2006). Whereas ALT activity is primarily localised in the liver and largely specific for parenchymal diseases (Gatsing et al., 2005; Thrall et al., 2006), AST activity is present in a wide variety of tissues including heart, skeletal muscle, kidney, brain and the liver (Gatsing et al., 2005). In the current study, liver transaminases (ALT and AST) levels increased to the maximum peak by day 21-post treatment before decreasing significantly to levels similar to pre-treatment values. Similar studies reported no change in levels of AST and ALT activity after treating rats with *C. molmol* for 24, 48 and 72 h (Rao et al., 2001; Aliyu et al., 2007). In this study, it was observed that prolonged use of resin extract caused liver damage (mottling appearance, Plate 1) thus led to increased concentrations of liver enzymes in the blood.

The effect of the resin in kidneys was assessed through determination of plasma creatinine levels, which usually increase when there is significant renal impairment (Thrall et al., 2006). Thus, the significant increase in plasma creatinine levels observed in chickens in groups G4 and G5 after 14 days of treatment concur with the noticeable damage of the renal cortices and glomeruli as seen in histopathological sections (Plate 2). Similar findings were reported by Aliyu et al. (2007); that is, prolonged use of ethanolic leaf extracts from *Commiphora africana* caused noticeable damage to the cortex and glomerulus in rats. The significant increase in creatinine at high doses may possibly be due to some regenerative mechanisms by the kidney in response to the effect of resin extract. It is known that for any markers of kidney function (creatinine or uric acid) to significantly appear in blood, about 75% of the nephrons must have

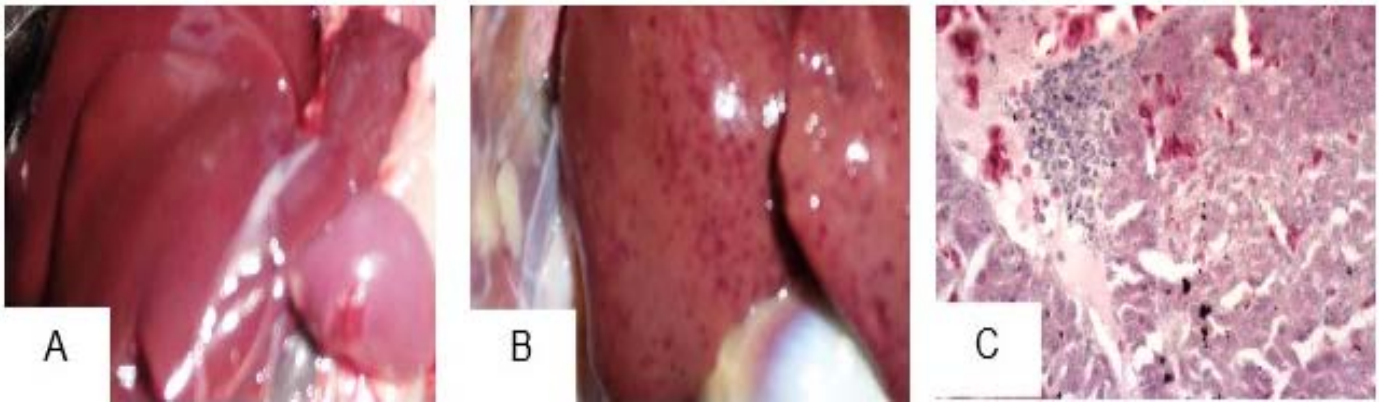

Plate 1. Liver: (A) normal liver (untreated group, G1); (B) motted appearance (G5); (C) nuclear cell infiltration at × 40 magnification, H&E stain (G5).

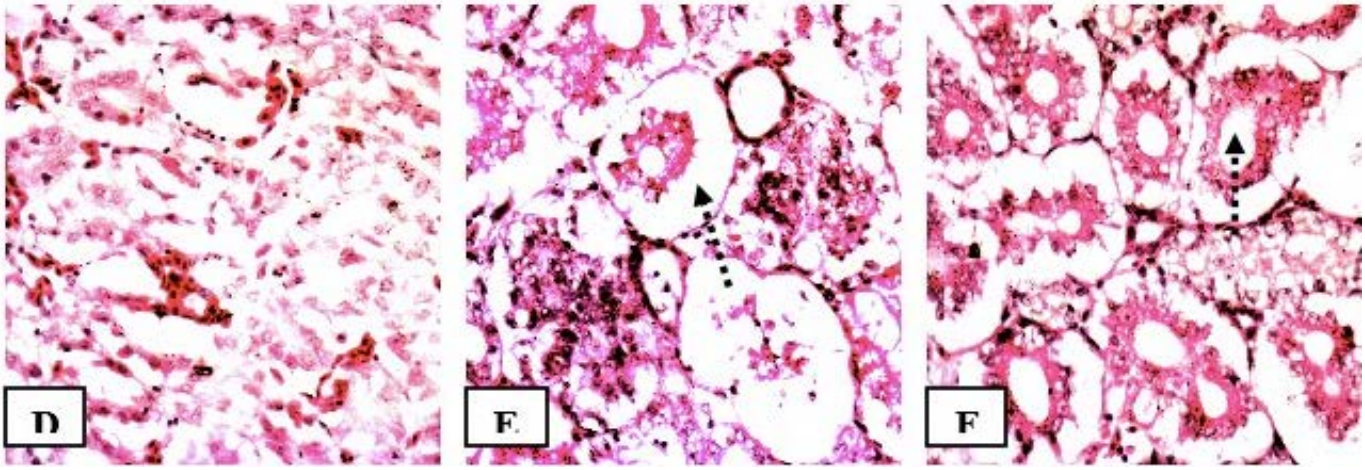

Plate 2. Kidney sections: normal kidney; (E) glomerular and hydropic tubular degeneration; (F) cystic luminal and cortical dilatation (× 40 magnification, H&E Stain).

been damaged (Boyd, 1983; Thrall et al., 2006). This suggests that administration of high doses of *C. swynnertonii* resin could be detrimental to kidneys.

In conclusion, this study has demonstrated that chickens can tolerate oral administration of *C. swynnertonii* resin at doses less than 750 mg/kg body weight whereby haematological parameters tested and liver functions were not significantly affected. Administration of higher doses had negative effects on liver, kidney and lung functions, which included acute glomerulonephritis and pulmonary congestion, respectively. The observed hypoglycemic, hypocholesteremic and body weight lowering effect were interesting findings, which can be used as a template for further research in humans. Further studies on the resin extract are needed to isolate the bioactive component(s), elucidate its exact mechanism(s) of action and validate its uses in the chickens and other animal species.

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Conflict of Interest

The authors declare that they have no conflict of interest. Experiments were carried out in accordance with ethical guidelines of the Sokoine University of Agriculture.

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