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Full Length Research Paper

Chemical Composition antimicrobial and free radical scavenging activity of essential oil from leaves of *Renealmia thyrsoidea* (Ruiz &Pav.) Poepp. & Endl.

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***Renealmia thryrsoidea* (Ruiz & Pav.) Poepp. & Endl is a plant used by the Amazonian indigenous people of Ecuador for its various medicinal properties. Its leaves exhibit a remarkable aroma with a hint of spiciness. The essential oil extracted from its leaves was analyzed by GM/MS, using two systems with columns of different polarity, in both was confirmed the presence of terpinolene (26.32%), α-felandrene (17.16%), γ-terpinene (6.55%), β-pinene (5.97%) and p-cymol (4.70%). Free radical scavenging activity was analyzed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) spectrophotometric methods; by applying ABTS method, the activity was comparable to that of the essential oil of *Thymus vulgaris*. The trials of antimicrobial activity show a strong inhibition against Gram negative bacteria as *Escherichia coli* and *Pseudomonas aeruginosa*.**

Key words: *Renealmia thryrsoidea*, essential oil, DPPH, ABTS, minimum inhibitory concentration (MIC).

INTRODUCTION

Essential oils are a group of secondary metabolites that currently have a productive significance (Schmidt, 2015). Many of them are valued for their qualities as potential medicinal products (Noriega, 2009) and cosmetics (Muñoz-Acevedo et al., 2015). Ecuador is a country with a very high plant diversity (Bendix et al., 2013) and many of these species are rich in essential oils that have various medicinal uses (Malagon et al., 2003; Sacchetti et al., 2005; Guerrini et al., 2009).

The *Renealmia* genus (Zingiberaceae family) is distributed throughout South America with 75 species (Maas, 1997). In Ecuador, the number of species reaches 25, 4 of which are endemic (Jorgensen and León-Yanez, 1999).

Renealmia thyrsoidea (Ruiz & Pav.) is a widely distributed species in tropical America, in countries such as Bolivia, Colombia, Ecuador, Guyana, Nicaragua, Panama, Peru, Suriname, Trinidad and Tobago and Venezuela. In Ecuador this spices is distributed in the three continental regions: the coast, the highlands, and Amazon; with a noted presence in the provinces of Cotopaxi, Imbabura, Morona Santiago, Napo, Pastaza,

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Pichincha, Sucumbios, Tungurahua and Zamora Chinchipe (Jorgensen and León-Yanez, 1999). An important reported use is that of acting as an antidote for snake bites (Davis and Yost, 1983). The antimalarial and antipyretic properties of *R. thyrsoidea* were reported by Schultes et al. (1990) and Céline et al. (2009), this plant possesses analgesic and anti-flu properties (de La Torre et al., 2008), and anti-leishmaniasis uses are reported in various regions (Yannick, 2009). The fruits are edible (de la Torre et al., 2008) and a dye is extracted from them to be used in ritual practices (Maas, 1997).

Several of the ethnic groups present in the Amazon region of Ecuador employ the species. The importance of this research lies in the assessment of the plant's essential oils for pharmacological and medicinal traits.

MATERIALS AND METHODS

Plant

The leaves of *R. thyrsoidea* were collected in the community of San Luis de Inimkis, Canton Macas, province of Morona Santiago, with the following geographic coordinates: 02° 26' South latitude and 78° 11' West longitude; at an altitude of 1070 m.o.s.l. The botanical identification took place in the herbarium Alfredo Paredes of the Universidad Central del Ecuador by the botanist Carlos Cerón. The fresh leaves were collected from mature plants and distilled in vapor stream, with an equipment of 250 liters capacity belonging to the Fundacion Chankuap recursos para el futuro, in the city of Macas.

Chemical composition

The essential oil composition was determined using gas chromatography and mass spectrometry equipped with two columns of different polarity systems. The sample was prepared by dissolving 25 µl of essential oil in 1 ml of acetone. For coupling 1, a gas chromatograph GC Agilent 7890 A was coupled to a mass spectrometer MS 5975. The column used was a DB1-MS, with a length of 60 m, 0.32 mm internal diameter and 0.25 um thickness. The analysis started at a temperature of 60°C with a rate of 4°C per min up to 280°C. The carrier gas was helium at a flow of 3.5 ml min^{-1}. The injection volume was 1 µl. For coupling 2, a gas chromatograph GC Agilent 6890 A was coupled to a mass spectrometer MS 5973. The column used was a DB-Wax, with a length of 30 m, 0.25 mm internal diameter and 0.25 um thick. The analysis started at a temperature of 60°C with a velocity gradient of 3°C per min up to 240°C. The carrier gas was helium at a flow of 3.3 ml min^{-1}. The injection volume was 1 µl. In both cases, electron ionization was used with energy of 70 eV and a range of m/z 35 to 400 Da. The component identification was performed by comparing the mass spectra to those present in the MASSLIB database and by determining the retention index for each component using as reference the standard series C8-C30 of hydrocarbons.

Free radical scavenging activity

Spectrophotometric methods DPPH (diphenyl-2-picrylhydrazyl) and ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] have been a permanent technique to evaluate the free radical scavenging activity in essential oils (Bardaweel et al., 2015; Ornano et al., 2015). Fort the DPPH assay, varying amounts of *R. thyrsoidea* essential oil were taken and dissolved in dimethyl sulfoxide (DMSO) to a volume of 100 µl. To each solution, 2.9 ml of DPPH (1×10^{-4} molar in ethanol) was added. The solution was stirred vigorously for 30 min in the dark at room temperature. The absorbance was measured at 517 nm in a Shimadzu UV 1240 mini. Similarly, the analysis proceeded with the ABTS test, to each solution dissolved in DMSO 0.9 ml of ABTS 40 mM (previously radicalized with a $K_2S_2O_8$ solution) was added. The absorbance was measured at 734 nm in a Shimadzu UV mini 1240. Antiradical activity for each mixture was calculated according to the following formula:

$$\text{DPPH or ABTS } \% = \frac{Ab - Aa}{Ab} \text{x } 100$$

Where Aa and Ab are the absorbance of blanks and samples, respectively after 30 min (DPPH) and 1 min ABTS. The activity of the essential oil was evaluated by calculating the IC_{50}, which is 50% inhibition of oxidation of DPPH and ABTS; this was calculated from the data of the calibration curves obtained from the data of the concentration versus percentage inhibition. As referents of activity, *Tymus vulgaris* essential oil and butylated hidroxianisole (BHA) were used.

Antimicrobial activity (MIC evaluation)

The antimicrobial activity was evaluated using the disc diffusion method often described as an applicable methodology to evaluate the antimicrobial activity of essential oils (Rivera et al., 2015; Pesavento et al., 2015). The strains tested were Grampositive bacteria: *Staphylococcus aureus* subsp. *aureus* ATCC 6538 and *Streptococcus mutans* ATCC 25175; Gramnegative bacteria: *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027. The yeasts *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 10231 were also tested. The antimicrobial activity is described as the minimum inhibitory concentration (MIC) in mg ml^{-1}. The essential oil of *T. vulgaris* was used as a reference for any appreciable activity (Rossi et al., 2011).

RESULTS AND DISCUSSION

Production of the essential oil

The essential oil yield was 0.047% (w/w). Essential oil density was 0.873 mg ml^{-1}.

Chemical composition

In both studies with both DB1 and DB-Wax column the most abundant compounds were: terpinolene (26.32%), α-felandrene (17.16%), γ-terpinene (6.55%), β-pinene (5.97%) and p- cymol (4.70%). The analysis with DB1 column reveals the presence of components 116; DB-Wax column revealed the presence of 107 compounds with the same equivalent percentages, as shown in Table 1.

Free radical scavenging activity

IC_{50} values indicate the ability to inhibit by 50%, the

Table 1. Components of essential oil of leaves of *R. thyrsoidea*.

CG/MS with column DB1-MS				CG/MS with a column DB-Wax			
No	**Compound**	**Retentionindex**	**Area (%)**	**No**	**Compound**	**Retentionindex**	**Area (%)**
1	α-thujene	923	0.20	1	6-p-menthen-2-on	765	0.02
2	α-pinene	930	2.38	2	α-thujene	1038	0.20
3	α-fenchene	941	0.04	3	α-pinene	1045	2.38
4	Camphene	943	0.05	4	1,4-cadinadien	1046	0.15
5	Verbenene	946	0.23	5	α-fenchene	1082	0.04
6	3-cyclohexeno, 4-methyl-	956	0.02	6	camphene	1091	0.05
7	Sabinene	965	0.02	7	β-pinene	1125	5.97
8	β-pinene	971	5.97	8	sabinene	1134	0.02
9	dehydrocineol/1,8-epoxy-2-p-menthen	978	0.02	9	verbenene	1139	0.23
10	β-myrcene	981	1.78	10	δ-2-carene	1149	0.03
11	δ-2-carene	991	0.03	11	β-myrcene	1164	1.78
12	α-phellandrene	998	17.16	12	δ-3-carene	1167	2.16
13	δ-3-carene	1005	2,16	13	α-phellandrene	1178	17,16
14	α-terpinene	1009	1.24	14	α-terpinene	1196	1.24
15	p-cymol	1012	4.70	15	dehydrocineol/1,8-epoxy-2-p-menthen	1206	0.02
16	β-phellandrene	1019	1.23	16	limonene	1213	3.40
17	Limonene	1021	3.40	17	β-phellandrene	1225	1.23
18	1,8 cineol	1025	Tr	18	1,8 cineol	1225	Tr
19	β-cisocimene	1025	0.08	19	β-cisocimene	1234	0.08
20	β-trans-ocimene	1036	1.89	20	γ-terpinene	1249	6.55
21	γ-terpinene	1048	6.55	21	β-trans-ocimene	1252	1.89
22	Fenchone	1066	0.01	22	p-cymol	1281	4.70
23	p- dimethylstyrol	1072	0.41	23	terpinolen	1287	26.32
24	Terpinolen	1079	26.32	24	menthatrien, 1,3,8-p-	1397	0.07
25	Linalool	1083	0.06	25	fenchone	1411	0.01
26	Fenchol	1097	0.07	26	p- dimethylstyrol	1443	0.41
27	menthatrien, 1,3,8-p-	1098	0.07	27	α-cubebe	1466	0.06
28	menthen-1-ol, trans-2-p-	1105	0.07	28	menthen, 4,8-epoxy-1-p-	1475	0.47
29	Kampfer	1119	0.02	29	α-copaene	1498	2.02
30	trans-pinocarveol	1122	1.05	30	pinocamphone	1519	0.05
31	menthen, 4,8-epoxy-1-p-	1126	0.47	31	α-ylangene	1521	0.03
32	methylcamphenilol	1131	0.03	32	kampfer	1529	0.02
33	pinocamphon	1135	0.05	33	iso-pinocamphon	1547	0.48
34	Pinocarvon	1136	0.02	34	linalool	1548	0.06
35	menthadien-8-ol, 1,5-p-	1137	0.02	35	menthen-1-ol, trans-2-p-	1563	0.07
36	iso-pinocamphon	1148	0.48	36	pinocarvon	1570	0.02
37	α-8hidroxy terpinen	1153	0.13	37	α-santalene	1577	1.12
38	menthadien-4-ol, 1,8-p-	1156	Tr	38	fenchol	1585	0.07
39	p- 8- cymenol	1157	0.34	39	α-bergamotene	1586	0.28
40	4-terpinenol	1160	0.29	40	bornylacetate	1588	0.03
41	Myrtenal	1167	0.03	41	methylcamphenilol	1596	0.03
42	α-terpineol	1170	0.70	42	γ-maaliene	1604	0.02
43	Myrtenol	1176	0.06	43	4-terpinenol	1607	0.29
44	Verbenon	1178	0.06	44	aromadendrene	1614	0.56
45	trans-piperitol	1187	0.04	45	myrtenal	1627	0.03
46	benzylaceton	1205	0.05	46	β-epi-santalene	1638	0.18
47	Citronellol	1208	0.03	47	trans-pinocarvylacetate	1647	0.06
48	menthen-2-on, 6-p-	1218	0.02	48	alloaromadendrene	1647	0.98
49	trans-myrtanol	1220	0.03	49	β-santalene	1652	Tr

Table 1. Contd.

50	perillaaldehyd	1243	0.01	50	trans-pinocarveol	1655	1.05
51	Phellandral	1246	0.03	51	trans-β- farnesene	1666	0.10
52	bornylacetate	1273	0.03	52	α-humulene	1670	0.07
53	Carvacrol	1275	0.05	53	menthadien-4-ol, 1,8-p-	1680	Tr
54	trans-pinocarvylacetate	1278	0.06	54	γ-muurolene	1688	0.38
55	benzalaceton	1316	0.02	55	ledene	1694	0.77
56	α-cubebene	1346	0.06	56	δ-selinene	1695	0.20
57	α –ylangene	1368	0.03	57	α-amorphene	1700	0.10
58	α-copaene	1373	2.02	58	α-terpineol	1701	0.70
59	dimethylanthranilat	1376	Tr	59	verbenone	1713	0.06
60	γ-guajene	1406	0.31	60	β-selinene	1720	0.06
61	b-ylangene	1412	Tr	61	menthadien-8-ol, 1,5-p-	1723	0.02
62	α-santalene	1415	1.12	62	α-muurolene	1724	0.19
63	γ-maaliene	1420	0.02	63	phellandral	1725	0.03
64	α-bergamotene	1429	0.28	64	8-hydroxy-α-terpinene	1728	0.13
65	aromadendrene	1433	0.56	65	β-bisabolene	1729	0.33
66	β-epi-santalene	1439	0.18	66	trans-piperitol	1742	0.04
67	trans-β-farnesene	1445	0.10	67	δ-cadinene	1756	1.30
68	α-humulene	1446	0.07	68	γ-cadinene	1757	0.24
69	β-santalene	1451	Tr	69	citronellol	1764	0.03
70	alloaromadendrene	1453	0.98	70	perillaaldehyde	1767	0.01
71	trans-isoeugenolmethylether	1458	0.02	71	myrtenol	1784	0.06
72	cadina-1(6),4-dien ii	1464	0.19	72	α-cadinene	1788	0.06
73	γ-muurolene	1467	0.38	73	trans-calamenene	1827	0.12
74	γ-amorphene	1470	0.10	74	8-p-cymenol	1834	0.34
75	trans-β-bergamotene	1475	0.07	75	benzylacetone	1852	0.05
76	β-selinene	1477	0.06	76	trans-myrtanol	1853	0.03
77	δ-selinene	1481	0.20	77	palustrol	1926	0.10
78	Ledene	1487	0.77	78	maaliol	1981	Tr
79	α-muurolene	1489	0.19	79	ledol	2027	0.36
80	β-bisabolene	1497	0.33	80	γ-guajene	2030	0.31
81	γ-cadinene	1502	0.24	81	trans-nerolidol	2034	0.49
82	trans-calamenene	1505	0.12	82	cubeban-11-ol	2046	0.23
83	δ-cadinene	1511	1.30	83	cubenol	2050	0.24
84	1,4-cadinadiene	1520	0.15	84	cubenol	2050	0.06
85	α-calacorene	1524	0.03	85	α-calacorene	2053	0.03
86	α-cadinene	1526	0.06	86	cubenol, 1,10-diepi-	2055	0.05
87	Euasaron	1533	0.02	87	globulol	2067	0.54
88	trans-nerolidol	1544	0.49	88	dimethylanthranilat	2068	Tr
89	Maaliol	1545	Tr	89	viridiflorol	2080	0.21
90	Palustrol	1555	0.10	90	guajol	2080	1.27
91	spathulenol p.1 (hm)	1558	0.10	91	propanol, 2-(4a,8-dimethyl-2,3,4,4a,5,6,7,8-octahydro-	2091	0.13
92	Globulol	1569	0.54	92	guaien-11-ol, 5	2104	0.55
93	Viridiflorol	1575	0.21	93	benzalacetone	2105	0.02
94	cubeban-11-ol	1578	0.23	94	spathulenol p.1 (hm)	2131	0.10
95	Guajol	1580	1.27	95	γ-eudesmol	2161	0.22
96	propanol, 2-(4a,8-dimethyl-2,3,4,4a,5,6,7,8-octahydro-	1585	0.13	96	a-santalal	2164	0.10
97	Ledol	1587	0.36	97	τ-cadinol	2167	0.27
98	cubenol, 1,10-diepi-	1597	0.05	98	trans-isoeugenolmethylether	2170	0.02
99	guaien-11-ol, 5	1605	0.55	99	carvacrol	2195	0.05
100	Cubenol	1610	0.24	100	δ-cadinol	2196	0.09

Table 1. Contd.

101	γ-eudesmol	1612	0.22	101	bulnesol	2203	0.77
102	τ-cadinol	1620	0.27	102	bisabolol, α- p.1	2213	0.03
103	δ-cadinol	1624	0.09	103	α-eudesmol	2217	0.25
104	Cubenol	1626	0.06	104	β-eudesmol	2229	0.29
105	β-eudesmol	1629	0.29	105	α-cadinol	2229	0.19
106	α-cadinol	1632	0.19	106	campherenol	2291	0.30
107	α-eudesmol	1634	0.25	107	γ-bicyclohomofarnesal	2366	0.03
112	tras-a-santalol	1676	0.09	Total 96.81%			
113	farnesylsaeuremethylester, e,e-	1758	0.04				
114	γ-bicyclohomofarnesal	1770	0.03				
115	prop-2-en-1-on, 1-(2,6-dihydroxy -4-methoxyphenyl)-3	2324	0.49				
116	2,4(8)-menthadienol-(1)		0.25				
Total		97.98%					

Table 2. Free radical scavenging activity of *R. thryrsoidea* leaf essential oil, by applying DPPH and ABTS methods.

Essential oils	DPPH IC_{50} mg ml^{-1}	ABTS IC_{50} mg ml^{-1}
R. thyrsoidea	20.86 ± 0.4	1.44 ± 0.1
T. vulgarys	2.42 ± 0.1	2.92 ± 0.2
BHA	0.059 ± 0.001	0.025 ± 0.001

Table 3. Antimicrobial activity of the essential oil of leaves of *R. thryrsoidea.*

Microorganismos	*R. thryrsoidea* MIC (mg ml^{-1})	*T. vulgaris* MIC (mg ml^{-1})
Gram negative bacteria		
Escherichiacoli ATCC 8739	0.35	0.1
Pseudomonas aeruginosa ATCC 9027	0.35	0.1
Gram positive bacteria		
Staphylococcus aureus subsp. *aureus* ATCC 6538	1.39	0.39
Streptococcus mutans ATCC 25175	5.56	0.1
Yeast		
Candida tropicalis ATCC 13803	1.39	0.1
Candida albicans ATCC 10231	0.35	0.1

concentration of free radicals present. For this test, the reference essential oil was that of *T. vulgaris* whose thymol and carvacrol components are widely known for this property (Mastelić et al., 2008; Lee et al., 2005). The results are shown in Table 2.

Antimicrobial activity

There are different results depending on the microbial strains evaluated. A good activity was observed in the Gram negative bacteria tested, close to the natural pattern registered with the essential oil of *T. vulgaris*, represented by the values in Table 3.

Conclusion

The study provides an insight into the chemical components present in the essential oil from the leaves of *R.*

thyrsoidea. Most notable was the presence of monoterpenes. As for the antioxidant activity appreciable activity highlighted in the evaluation of electron scavenging capacity ABTS methodology, whereas by the method DPPH oil is less active. Interestingly, anti-microbial activity evaluated in both Gram negative strains, whose values of minimum inhibitory concentration reaches 0.35 mg ml^{-1}.

Conflict of interests

The authors have none to declare.

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Bioactivity of *Platycerium angolense* Flavonoid Fraction on Biochemical Parameters of Acetaminophen-induced Rats

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This study evaluated the effect of flavonoid fraction (FF) of *Platycerium angolense* on acetaminophen-induced liver damage in rat with a view to considering the possibility of utilizing the extracts of the plant for the management and treatment of liver related ailments. Powdered leaves of *P. angolense* were extracted with methanol 70% (v/v) to yield methanolic extract of *P. angolense* (MEPA). The MEPA followed successive partitioning with n-hexane, chloroform and ethylacetate. The ethylacetate fraction was termed flavonoid fraction (FF). FF was phytochemically screened and total phenolic and flavonoid contents estimated. Biochemical effects of FF on liver marker enzymes (L-alanine and L-aspartate aminotransferases, gamma glutamyltransferase, acid and alkaline phosphatases); lipid profiles (cholesterol, triglycerides, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol and very low density lipoprotein (VLDL)-cholesterol) enzymatic and non-enzymatic antioxidants (catalase, superoxide dismutase, peroxidase, glutathione (GSH), lipid peroxidation) and metabolites (total protein, albumin and bilirubin) were evaluated in the plasma and liver homogenates of albino rats. Phytochemical screening of FF tested positive for the presence of flavonoids, saponins, anthraquinones and cardiac glycosides. The total phenolics and flavonoids in FF were (33.58 ± 0.11 mg TAE/g, 50.98 ± 0.89 mg RE/g), respectively. Analyses of plasma and liver homogenates of rats (control and experimental) revealed that acetaminophen caused metabolic dysfunction in acetaminophen-induced rats. Administration of FF reversed the altered biochemical parameters. The study concluded that FF of *P. angolense* increases plasma lipid profile level, exhibits moderate anti-oxidant activity and elicits hepatoprotective potential.

Key words: Hepatotoxicity, *Platycerium angolense*, acetaminophen, flavonoids, flavonoid fraction.

INTRODUCTION

Flavonoids belong to a group of polyphenolic compounds with diverse chemical structures and properties. They are found in fruits, nuts, seeds, vegetables, grains, bark, roots, stems, flowers of plants as well as in tea and wine (Middleton, 1998) and constitutes integral part of human diets (Agati and Tattini, 2010; Pollastri and Tattini, 2011). They are powerful antioxidants against free radicals and are described as radical scavengers (Pal et al., 2009), the

activity that is attributed to their hydrogen donating ability. The phenolic groups of flavonoids serve as a source of a readily available "H" atoms such that the subsequent radicals produced may be delocalized over the flavonoid structure (Tripoli et al., 2007; Heim et al., 2002). Flavonoids have been reported to exert wide range of biological activities which include anti-inflammatory, antibacterial, antiviral, antiallergic (Cushnie and Lamb, 2005) cytotoxicity, antitumour, treatment of neuro-protective, vasodilatory action (Tsuchiya, 2010; Chebil et al., 2006). Flavonoids and other phenolics have exhibited their effects as antioxidants, free radical scavengers and chelators of divalent cations (Cook and Shamman, 1996; Agati et al., 2012). In addition, flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities (Chebil et al., 2006).

Hepatotoxicity may be defined as injury to the liver that is associated with impaired liver functions caused by exposure to drugs or other infectious agents (Navarro and Senior, 2006). Acetaminophen (AAP) is a safe and effective analgesic/antipyretic drug when used at therapeutic levels (Rumack, 2004). However, an acute or cumulative overdose causes severe liver injury with the potential to progress to liver failure (Lee, 2004). Acetaminophen is extensively metabolized by conjugation with sulphate and glucuronic acid when a normal dose is used. N- acetyl - p- benzo- quinoneimine (NAPQI) a highly electrophilic metabolite that triggers liver damage is generated when a small fraction of the drug is subjected to oxidation reactions catalyzed by Cytochrome P_{450} enzymes in the liver (Chen et al., 2009). Normally, toxic oxidation metabolites generated in the liver are converted into non-toxic metabolites excreted in urine via conjugation with glutathione (GSH) containing sulphydryl groups. However, high doses of paracetamol limit the ability of GSH to detoxify NAPQI, and results in the consumption of liver GSH stores (Mitchell et al., 1973; Savides and Oehme, 1983).

Platycerium (*Platycerium angolense* Welw; Polypodiaceae family) is an epiphytic genus that grows naturally on branches and trunks of trees. It is native to tropical and temperate areas of South America, Africa, Southeast Asia, Australia and New Guinea. Bode and Oyedapo (2011) earlier reported that studies on the biological activity of the extract of *P. angolense* revealed that the plant contains bioactive molecules which exhibited potent and appreciable antioxidant and anti-inflammatory activities. This study was designed to evaluate the effect of flavonoid fraction of *P*. angolense against acetaminophen-induced liver damage in rats with a view of investigating further the activity of *P. angolense* and the possibility of using this extract for the management and treatment of liver-related disorders.

MATERIALS AND METHODS

Collection, identification and preparation of plant materials

Fresh leaves of *P. angolense*, Welw. Ex Hook, were collected from *Azadirachta indica*, (Neem) tree, Opposite Staff Club, OAU, Staff Quarters at the main campus of Obafemi Awolowo University, Ile-Ife, Nigeria. The plant was identified and authenticated by Dr. F. A. Oloyede at the IFE Herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria. The specimen sample was deposited at IFE Herbarium where specimen identification number (IFE 17340) was collected. Fresh leaves of *P. angolense* were cut into bits, air-dried in the laboratory over a period of 4 weeks and pulverized into powder using hand operated machine.

Reagents and chemicals

All the reagents and chemicals used in this study were of analytical grade, and were procured from British Drug House (BDH) Chemicals Limited, Poole, England. Diagnostic kits, for lipid profile, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transferase, alkaline phosphatase, total protein, albumin and bilirubin assays, were procured from Randox Laboratories Ltd, United Kingdom. Silymarin was obtained from Sigma Chemical Company and Acetaminophen was obtained from Drug Research and Production Unit (DRPU), Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife.

Experimental animals

Thirty (30) healthy Albino rats were purchased from Empire Farms, Osogbo, Osun State, Nigeria. The rats were acclimatized for two weeks and fed with Standard Rat Chow (Ladokun Feeds Limited, Ibadan) and watered *ad libitum*.

Preparation of methanolic extract (ME) and flavonoid fraction (FF) of *P. angolense*

The methanolic extract of *P. angolense* was prepared according to a modified method of Oyedapo and Amos (1997). Powdered plant (500 g) was macerated with 6 L of 70% (v/v) methanol for 24 h. The suspension was filtered through two layers of white cotton-cloth and the residue was re-extracted with same solvent for another 24 h. The filtrates were combined and centrifuged at 3,000 rpm for 10 min at 25°C, on a Table Centrifuge (Model 90-2 Microfield Instrument, Essex, England).

The supernatant was evaporated to dryness at 40°C under reduced pressure on Edward High Vacuum Pump, Model ED-100 (Edward Vacuum Components, Crawley, England). The brown residue termed methanolic extract (ME) was collected and kept in

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desiccator until required for analysis. Methanolic extract (50 g) was taken up in warm water (250 ml) until totally dissolved and filtered. The filtrate was partitioned successively with *n*-hexane (10 × 250 ml), chloroform (10 × 150 ml) and ethyl acetate (10 × 200 ml). The solvent and filtrate were shaken together and poured into the separating funnel, allowed to separate properly and each fraction was collected appropriately. This process was followed with other solvents. The fractions from the same solvent were pooled together and evaporated at reduced temperature (40°C), as earlier described**.** The residue from each fraction after evaporation was kept in a desiccator until required for analysis.

Phytochemical screening of fractions

The fractions (n-hexane, nHF, chloroform, CF, ethyl acetate, EAF and aqueous, AqF) were phytochemically screened using standard procedures earlier described by Sofowora (2002) and Oyedapo et al. (1999).

Estimation of total phenolic and flavonoid contents of fractions

The total phenolic contents of the fractions were carried out according to the method of Singleton et al. (1999). The reaction mixtures contained 0.2 ml (1 mg/ml stock) of the fraction, and Folin Ciocalteu's (phenol) reagent (1.5 ml, 1:10). 10% (w/v) $NaHCO_3$ (1.5 ml) was added. The mixture was further incubated for 1 h 30 min in the dark. The absorbance was read at 725 nm against the reagent blank. The standard calibration curve was prepared with tannic acid (0.02 mg/ml). The assays were carried out in triplicate and the mean of three readings was estimated. The concentrations of the phenolics were obtained from standard calibration curve by extrapolation and expressed as milligram tannic acid equivalent per g of extract (mg TAE/g extract) (Figure 1). The total flavonoid contents of the fractions were carried out according to the method of Singh et al. (2010). The reaction mixtures consisted of 0.5 ml of the fraction (1 mg/ml), distilled water (1.5 ml), 5% (w/v) $NaNO_2$ (0.3 ml), 10% (w/v) $AlCl_3$ (0.3 ml), and 4% (w/v) NaOH (2.0 ml). The reaction mixture was incubated at 25°C for 10 min after which the absorbance was read at 500 nm against the reagent blank. The assay was carried out in triplicates and the mean was obtained. Standard calibration curve was prepared by using rutin (0.1 mg/ml). The concentrations of the flavonoids were obtained from standard calibration curve and expressed as milligram rutin equivalent per g of extract (mg RE/g extract) (Figure 2).

Grouping and treatment of experimental animals

Albino rats (30) were divided into 6 groups of 5 animals per group and treated as follows:

Group 1: Rats served as control and received distilled water.
Group 2: Rats were administered acetaminophen150 mg/kg body weight (bwt).
Group 3: Rats were treated with standard drug (silymarin 50 mg/kg bwt) only.
Group 4: Rats were administered acetaminophen (150 mg/kg bwt) and silymarin 50 mg/kg bwt.
Group 5: Rats were pretreated with flavonoid fraction (125 mg/kg bwt) and administered with acetaminophen 150 mg/kg bwt an hour later.
Group 6: Rats were administered acetaminophen (150 mg/kg bwt) and treated with flavonoid fraction 125 mg/kg bwt an hour later.
The rats were orally treated once daily consistently for 15 days.

Sacrificing of experimental animals

The rats were sacrificed under slight ether anaesthesia on the 16th day. The rats were dissected; blood was collected by cardiac puncture, into an anticoagulant bottle containing (3.8% (w/v) trisodium citrate). Also, livers were aseptically collected, perfused in normal saline (0.85% (w/v) NaCl) and blotted on tissue paper. The livers were stored frozen until required for analysis.

Preparation of blood plasma

The blood samples were centrifuged at 3,000 rpm for 10 min on a Bench centrifuge (Model 90-2, Microfield Instrument, Essex, England) at room temperature. The plasma was carefully collected, stored frozen and used for biochemical analyses.

Preparation of liver homogenates (post mitochondrial fraction)

Liver (1.0 g) was weighed, cut into bits and thoroughly homogenized in 10 ml of freshly prepared 100 mM phosphate buffer, pH 6.8 to prepare 10% (w/v) homogenates. The homogenates were centrifuged at 3,000 rpm for 10 min using table centrifuge (as earlier described). The supernatants were carefully decanted into clean tubes, labelled, stored frozen and used for protein determination and other biochemical analyses.

Biochemical analyses

i. Plasma and liver alanine and aspartate aminotransferases activities were estimated using Randox Diagnostic kit, according to the procedure of Reitman and Frankel (1957). The activities of the enzyme was extrapolated from the ALT and AST standard calibration curve, respectively and expressed as U/L or U/g of liver.
ii. Gamma Glutamyl Transferase (GGT) activity (Teitz, 1987). The enzyme activity was calculated using the expression:

GGT activity (U/L) = 1158 × ∆A 405 nm/min; where 1158 (extinction coefficient) and ∆A 405 nm/min (change in absorbance per minute).

iii. The activities of AlkPase and AciPase were assayed according to the procedures of Saini and Van-Etten (1979) and Oyedapo (1996). The activities were estimated as:

AlkPase activity (U/L) = 2760 × ∆A 405 nm/min; where 1260 (extinction coefficient) and ∆A 405 nm/min (change in absorbance per minute).

The phosphatase activity was calculated as:

$$\text{Activity } (\mu\text{mol p} - \text{nitrophenol/ml}) = \frac{\text{Abs (410 nm)}}{\varepsilon \text{ X } \ell} \text{ X } \frac{1}{t} \text{ X } 10^6 \text{ X } \frac{TV}{SV}$$

Where Abs (absorbance at 410 nm), t (period of assay), Ɛ (extinction coefficient, 18.8×10^3 $M^{-1}cm^{-1}$), ℓ (1 cm) path length of cuvette, SV (sample volume) and TV (total assay volume), respectively.

iv. The total cholesterol concentration in the plasma was estimated according to the procedure of (Richmond, 1973), HDL-c (Tietz, 1987). Cholesterol concentration of the plasma was calculated using the expression:

$$\text{Conc. of Cholesterol (mg/dL)} = \frac{\Delta Abs_{sample}}{\Delta Abs_{standard}} \text{ X conc. of standard (mg/dL)}$$

Where ΔAbs_{sample} (change in absorbance of sample) and $\Delta Abs_{standard}$ (change in absorbance of standard).

v. Plasma triacylglycerol (Tietz, 1990). Triacylglycerol concentration was calculated using the expression:

$$\text{Triacylglycerol conc. (mg/dL)} = \frac{\Delta Abs_{sample}}{\Delta Abs_{standard}} \text{ X Standard conc. (mg/dL)}$$

Where ΔAbs_{sample} (change in absorbance of sample) and $\Delta Abs_{standard}$ (change in absorbance of standard).

vi. Plasma HDL- cholesterol concentration was calculated using the expression:

$$\text{Conc. of HDL} - \text{c (mg/dL)} = \frac{\Delta Abs_{sample}}{\Delta Abs_{standard}} \text{ X Conc. of standard (mg/dL)}$$

Where ΔAbs_{sample} (change in absorbance of sample) and $\Delta Abs_{standard}$ (change in absorbance of standard).

vii. VLDL-cholesterol concentration in the plasma was calculated as described by Friedewald et al. (1972).

$$\text{VLDL} - \text{c .(mg/dL)} = \frac{\text{Triglyceride}}{5}$$

viii. LDL- cholesterol concentration in the plasma was calculated as described by Friedewald et al. (1972).

$$\text{LDL} - \text{c (mg/dL)} = \text{TC} - (\text{VLDL} - \text{c} + \text{HDL} - \text{c})$$

Where, LDL-c = low density lipoprotein cholesterol concentration, TC = total cholesterol, HDL-c = high density lipoprotein cholesterol concentration, VLDL-c = very low density lipoprotein cholesterol concentration.

Assays of antioxidant enzymes

ix. Catalase (Sinha, 1972) (Figure 3).
x. Peroxidase activity (Reddy et al., 1995). The activity of peroxidase was calculated as:

$$\text{Peroxidase Activity} = \frac{\text{OD/ min X Total assay vol. X dilution factor}}{\text{extinction coefficient X vol. of enzyme}}$$

Where extinction coefficient was 11.3

xi. Superoxide dismutase activity (Misra and Fridovich, 1972).

One unit of SOD activity was defined as the amount of protein necessary to cause 50 % inhibition of the oxidation of epinephrine.
xii. Total proteins were estimated using Biuret reaction method according to the procedure of Tietz (1995).

$$\text{Total Protein Concentration (g/dL)} = \frac{Abs_{sample}}{Abs_{standard}} \text{ X Standard concentration (g/dL)}$$

Where (+) represented present and (-) represented absent. EA (ethyl acetate fraction) and AqF (aqueous fraction).

Where Abs_{sample} (absorbance of sample) and $Abs_{standard}$ (absorbance of standard).

xiii. Albumin concentration (Doumas et al., 1971). The concentration of albumin was calculated as:

$$\text{Albumin Conc. (g/dL)} = \frac{Abs_{sample}}{Abs_{standard}} \text{ X conc. of Standard (g/dL)}$$

Where Abs_{sample} (absorbance of sample) and $Abs_{standard}$ (absorbance of standard).

xiv. Total bilirubin (Jendrassik and Groff, 1938). Total bilirubin concentration was calculated as:

Total Bilirubin (mg/dL) = 10.8 × Abs (578 nm); where Abs is the absorbance of sample at 578 nm. and 10.8 (extinction coefficient)

xv. Lipid peroxidation assay was carried out according to the method of Buege and Aust (1978) with MDA as biomarker. The amount of MDA released was calculated using the expression:

$$\text{MDA (µmol/L)} = \frac{\text{Abs X Total assay vol.X dilution factor}}{\text{extinction coefficent of MDA X sample vol.}}$$

Where extinction coefficient of MDA (1.56 X 10^5 M^{-1} CM^{-1}) and Abs (absorbance at 532 nm).

xvi. Glutathione concentration was estimated according to the method of Moron et al., (1979). The concentrations of glutathione were expressed as µg GSH/ml sample (Figure 4).

Statistical analysis

The data was expressed as mean ± SEM of n = 5 readings. Differences between mean values were determined by One-way analysis of variance (ANOVA), using Graph Pad prism. Differences were considered to be significant if $p < 0.05$.

RESULTS AND DISCUSSION

The phytochemical screening of the fractions (chloroform [CF], ethyl acetate [EAF], and aqueous [AqF]) and methanolic extract revealed the presence of flavonoids, saponins, tannins, anthraquinone and cardiac glycosides but tannins were not detected in the ethyl acetate fraction (Table 1). Moreover, the levels of total phenolic and flavonoid in the ethyl acetate fraction were 33.58 ± 0.11 mg TAE/g and 50.98 ± 0.89 mg RE/g. This agreed with earlier reports of Wojdylo et al. (2007), who investigated 32 plants for antioxidant activity and phenolic contents. It was noted that 9 of the 32 plants had a higher flavonoid contents than total phenolics. Earlier studies have revealed that flavonoids and phenolics exhibit potent and appreciable biological activities (Olaleye and Rocha, 2008; Lebda et al., 2013).

Due to their simple, accurate reproducibility, paracetamol

Table 1. Phytochemical Constituents of the Fractions of Methanolic Extraction of *P. angolense*

Phytochemicals	CF	EAF	AqF
Flavonoids	+	+	+
Alkaloids	-	-	-
Saponins	+	+	+
Tannins	+	_	+
Anthraquinone	+	+	+
Cardiac glycosides	+	+	+

Where (+) represented present and (-) represented absent. EA (ethyl acetate fraction) and AqF (aqueous fraction).

and carbon tetrachloride are commonly used to induce hepatotoxicity in animal models (Olaleye and Rocha, 2008; Colle et al., 2012). Evaluation and assays of hepatic bio-marker enzymes such as alanine amino-transferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase are largely used in the assessment of liver damage by the agents bilirubin and protein (plasma) (Ujah et al., 2014). Membrane damage or necrosis releases the enzyme into the blood and other fluids which are then measured. The elevated levels of these enzymes and metabolites are indication of cellular leakage and loss of functional integrity of the hepatocytes (Rajkapoor et al., 2008; Parmar et al., 2010). In this study, the activity of plasma ALT decreased (4.35%) as a result of the administration of aceta-minophen but there was an increase of 10.11% in the liver ALT activity (Table 2). There was a slight increase in the activity of plasma ALT of the treated groups when compared to the acetaminophen-induced group with concomitant decrease in the liver ALT activity. Also, plasma and liver AST activities decreased as a result of the administration of acetaminophen by 9.0 and 26.0%, respectively. However, administration of FF reduced the activities of plasma AST in both pre- and post-treated groups when compared to the acetaminophen induced group which could be due to the protective effect of FF. The administration of FF slightly reduced liver AST activity of the pre-treated group and slightly increased that of the post-treated group when compared to the acetaminophen-induced group. It could be suggested that FF exhibits both anti-oxidant and pro-oxidant depending on the organ and the response of animals and mode treatment.

Gamma glutamyl transferase (GGT) and alkaline phosphatse (AlkPase) are membrane bound enzymes, which are released unequally depending on the pathological phenomenon. The elevation of plasma GGT concentrations is regarded as one of the most sensitive indices of hepatic damage (Muthulingam, 2012). The administration of acetaminophen caused increase in the activity of GGT by 82.7% (Table 3) due to leakage of the enzyme from the membrane to the plasma as a result of damage to the membrane which was reduced by 12.74% by the administration of FF. Also elevation of the activity of AlkPase was attributed to the damage of the structural integrity of liver because it is cytoplasmic enzyme and it is released into circulation indicating development of hepatotoxicity (Parmar et al., 2010). The activity of the enzyme was lowered by FF both in pre- and post-treated groups when compared to the acetaminophen induced group. Similar trends were observed in the activities of plasma and liver acid phosphatases in both pre and post treatment groups.

Liver injury of different aetiology is often accompanied by disorders of lipid metabolism in the liver and is often reflected in altered plasma lipids and lipoproteins (Steinberg, 1989; Skottova and Krecman, 1998). In the present study, administration of acetaminophen led to decrease in plasma total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-c), very low-density lipoprotein cholesterol (VLDL-c) but increase in low-density lipoprotein cholesterol (LDL-c) levels (Table 4). This was in agreement with earlier observations of Lebda et al. (2013) and Kobashigania and Kasiska (1997) that oral administration of acetaminophen brought about a decrease in serum triglyceride, HDL-c and VLDL-c levels but an increase in serum LDL-c level with a non-significance difference in the total cholesterol level.

The increase in the TC and LDL-c levels in the pre- and post-treated groups could be due to the synergetic effects of acetaminophen and FF. The increase in TC of plasma may also be due to the increase in the concentration of acetyl CoA arising from probably enhanced β-oxidation of fatty acid. High blood cholesterol is one of the major risk factors of cardiovascular disease (CVD) (Afolayan et al., 2009). HDL-c is considered to have anti-atherogenic properties; therefore increase in HDL-c could be clinically beneficial to the animals. The increase in plasma LDL- c is understandable since an increase in plasma total cholesterol should normally result in increase in plasma LDL-c (Yakubu et al., 2008). Atherogenic index is referred to as the ratio of LDL-c to HDL-c which has been used as indicator of cardiovascular disease and the cut-offs for high risk of atherosclerosis was put at atherogenic index of greater than 5 (Yakubu et al., 2008). In this study the atherogenic index value was less than 5, it could be therefore inferred that the animals were not likely prone to atherosclerosis. Hence, the use of the extract of *P. angolense* may be a useful therapeutic agent with treatment and management of CVD.

Cells which are under constant threat of toxic effect of reactive oxygen species (ROS) have a way of eliminating these toxic oxygen species. One major mechanism of this elimination is the antioxidant enzymes cascade (Karan et al., 1999; Radhika et al., 2012). Super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)

Table 2. Plasma and liver ALT and AST activities.

Group	ALT		AST	
	Plasma (U/L)	Liver (U/g liver)	Plasma (U/L)	Liver (U/g liver)
I. Distilled water (control)	79.72±1.52	0.89±0.01	43.68±4.47	1.12±0.05
II. Acetaminophen (150 mg/kg bwt)	76.25±3.01*(4.35% ↓)[a]	0.98±0.01*(10.11% ↑)[a]	39.74±4.84* (9.02% ↓)[a]	0.83±0.06* (25.89% ↓)[a]
III. Silymarin (50 mg/kg bwt)	78.21±2.27	1.02±0.03	44.11±5.56	0.99±0.16
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	77.30±3.29(1.38% ↑)[b]	1.03±0.01(5.10% ↑)[b]	34.21±2.37(13.92% ↓)[b]	0.73±0.04 (12.05% ↓)[b]
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	91.49±6.59**(20.09% ↑)[b]	0.97±0.02**(1.02% ↓)[b]	15.58±6.99#(60.80% ↓)[b]	0.76±0.04** (8.43% ↓)[b]
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	84.70±2.93**(11.08% ↑)[b]	0.98±0.02**(0.0%)[b]	18.95±1.67**(52.32 % ↓)[b]	0.88±0.11** (6.02% ↑)[b]

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**" not significantly different (group II vs. group V to VI).

Table 3. Plasma GGT, AlkPase and AciPase plasma and liver activities.

Group	GGT	AlkPase	AciPase	
	Plasma (U/L)	Plasma (U/L)	Plasma (µmol/min/mg protein)	Liver (µmol/min/mg protein)
I. Distilled water (control)	1.16±0.33	9.66±0.80	2.69±0.97	2.51±1.30
II. Acetaminophen (150 mg/kg bwt)	2.12±0.39*(82.76% ↑)[a]	11.04±0.0* (14.29% ↑)[a]	3.64±0.53* (35.32% ↑)[a]	5.00±0.53* (99.20% ↑)[a]
III. Silymarin (50 mg/kg bwt)	1.16±0.41	7.82±0.46	2.80±0.30	3.93±0.23
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	1.74±0.0(17.92% ↓)[b]	10.58±1.22 (4.17% ↓)[b]	2.55±0.43 (29.95% ↓)[b]	2.26±0.19 (54.80% ↓)[b]
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	2.17±0.36**(2.36% ↑)[b]	7.94±0.35** (28.08% ↓)[b]	3.39±0.90** (6.87% ↓)[b]	2.82±0.56** (43.60% ↓)[a]
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	1.85±0.22**(12.74% ↓)[b]	10.70±1.04** (3.08% ↓)[b]	2.75±0.48** (24.45% ↓)[b]	3.37±0.70** (32.60% ↓)[a]

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**" not significantly different (group II vs. group V to VI).

serve to eliminate primary products of partially reduced oxygen. SODs are found mainly in the intracellular compartment and exist in different forms in the mitochondria matrix, cytosol, and cytoplasm, to lesser extent in the lysosomes and at low concentrations in the extracellular fluid (Geller and Winge, 1984; Halliwell and Gutteridge, 1990).

SODs are involved in the dismutation of the superoxide anion which is an immediate oxygen metabolite with a high biological activity to yield hydrogen peroxide and oxygen (Kharpate et al., 2007). Catalase is present in subcellular peroxisomes and catalyzes the decomposition of hydrogen peroxide to yield oxygen and water (Olalelye and Rocha, 2008; Rapkan et al., 2008). Glutathione peroxidase (GPx) on the other hand catalyses the decomposition of hydrogen peroxide or organic peroxides and reduces glutathione which forms oxidized glutathione (GSSG). GSSG is again reduced to GSH by GPx thus forming redox cycle. These enzymes are found in both cytosol and mitochondria (Afolabi et al., 2012).

In Table 5 is the summary of the effects of the

Table 4. Plasma lipid profile concentration.

Groups	Total cholesterol (mg/dl)	Triacylglycerol (mg/dl)	HDL-c (mg/dl)	VLDL-c (mg/dl)	LDL-c (mg/dl)	LDL-c/HDL-c ratio
I. Distilled water (control)	58.97±2.74	34.40±6.09	44.26±4.77	6.88±1.22	7.83±0.81	0.18
II. Acetaminophen (150 mg/kg bwt)	33.45±2.33## (43.28% ↓)[a]	23.46±3.90* (31.80% ↓)[a]	18.71±2.53* (57.73% ↓)[a]	4.69±0.78* (31.83% ↓)[a]	10.05±2.26* (28.35% ↑)[a]	0.54
III. Silymarin (50 mg/kg bwt)	41.28±4.96	21.73±3.92	21.43±1.00	4.35±0.78	15.51±5.30	0.72
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	69.73±2.94 (108.46% ↑)[b]	45.24±8.62 (92.84% ↑)[b]	30.12±9.41 (60.98% ↑)[b]	9.05±1.73 (92.96% ↑)[b]	30.56±6.70 (204.08% ↑)[b]	1.01
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	67.47±6.70# (101.70% ↑)[b]	76.55±1.84# (226.30% ↑)[b]	32.00±10.86** (71.03% ↑)[b]	15.31±0.37# (226.44% ↑)[b]	20.15±4.67** (100.50% ↑)[b]	0.63
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	70.09±4.11# (109.54% ↑)[b]	73.38±3.18# (212.79% ↑)[b]	30.64±4.72** (63.76% ↑)[b]	14.68±0.64# (213.01% ↑)[b]	24.78±4.83** (146.57% ↑)[b]	0.81

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**" not significantly different (group II vs. group V to VI).

Table 5. Liver catalase, peroxidase and sod activities.

Groups	Catalase	Peroxidase	SOD
	Liver (µmol/min./mg protein)	Liver (U/min./mg protein)	Liver (U/min./mg protein)
I. Distilled water 5 mg/ml (control)	3.67±0.90	45.40±4.99	59.27±3.20
II. Acetaminophen (150 mg/kg bwt)	1.58 ± 0.06* (56.95% ↓)[a]	11.73±1.55## (74.16%↓)[a]	9.84±0.52## (83.40% ↓)[a]
III. Silymarin (50 mg/kg bwt)	1.53±0.07	11.84±1.95	10.74±0.91
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	1.39±0.09 (12.03% ↓)[b]	13.51±3.42(15.17% ↑)[b]	9.52±0.65 (3.25% ↓)[b]
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	2.09±0.12** (32.28% ↑)[b]	23.98±2.97** (104.43% ↑)[b]	15.71±5.22** (59.65% ↑)[b]
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	1.61 ± 0.20** (1.90% ↑)[b]	15.65±1.17** (33.42% ↑)[b]	11.28±0.52** (14.63% ↑)[b]

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**" not significantly different (group II vs. group V-VI).

acetaminophen, silymarin and flavonoid fraction on the antioxidant enzymes. It was observed that administration of acetaminophen caused drastic reduction in the activities of the enzymes which implied overwhelming of the antioxidant systems by generated ROS. However, treatments (pre and post) led to increase in the activities of the enzymes. The hepatocytes synthesize most proteins found in the plasma and secretes them into circulation. The reduction in plasma and hepatic tissues total protein level could be a result of possible damage to the hepatocytes induced by ingested toxins damage (Radhika et al., 2012). The induction of liver damage with acetaminophen decreased the plasma total protein concentration

Table 6. Total protein (plasma and liver), albumin and bilirubin plasma concentrations.

Group	Total Protein		Albumin	Bilirubin
	Plasma (g/dl)	Liver (mg/g)	Plasma (g/dl)	Plasma (mg/dl)
I. Distilled water 5 mg/ml (control)	3.71±0.50	17.48±1.22	1.78±0.48	0.10±0.03
II. Acetaminophen (150 mg/kg bwt)	2.15±0.16* (42.05% ↓)[a]	33.94± 5.01* (94.16% ↑)[a]	1.60±0.10* (10.11% ↓)[a]	0.20±0.01* (100.00% ↑)[a]
III. Silymarin (50 mg/kg bwt)	4.65±0.17	44.71±1.69	2.52±0.24	0.19±0.03
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	3.2±0.45 (54.42% ↑)[b]	47.96±3.25 (41.31% ↑)[b]	1.30±0.13 (18.75% ↓)[b]	0.17±0.05 (15.00% ↓)[b]
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	3.74±0.58** (73.95% ↑)[b]	31.97±1.78** (5.80% ↓)[b]	1.78±0.46** (11.25% ↑)[b]	0.22±0.08** (10.00% ↑)[b]
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	4.79±0.13# (122.79% ↑)[b]	42.88±4.64** (26.34% ↑)[b]	1.90±0.21** (18.75% ↑)[b]	0.18±0.09** (10.00% ↓)[b]

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**"not significantly different (group II vs. group V to VI).

Table 7. Liver lipid peroxidation and glutathione concentrations.

Group	MDA	GSH
	Liver (µmol)	Liver (µg/ml)
I. Distilled water 5 (control)	1.42 ± 0.02	7.53±0.58
II. Acetaminophen (150 mg/kg bwt)	1.75±0.12* (23.24% ↑)[a]	5.14±0.58* (31.74% ↓)[a]
III. Silymarin (50 mg/kg bwt)	1.0±0.15	6.63±0.79
IV. Acetaminophen (150 mg/kg bwt) + Silymarin (50 mg/kg bwt)	0.89±0.30 (49.14% ↓)[b]	7.95±0.59 (54.67% ↑)[b]
V. FF (125 mg/kg bwt) + Acetaminophen (150 mg/kg bwt) pre-treatment	0.39±0.14# (77.71% ↓)[b]	6.54±0.69** (27.24% ↑)[b]
VI. Acetaminophen (150 mg/kg bwt) + FF (125 mg/kg bwt) post-treatment	0.88±0.17** (49.71% ↓)[b]	6.37±0.82** (23.93% ↑)[b]

Each value represented Mean ± SEM of n = 5. ↑ = increase, ↓ = decrease; a vs. control and b vs. acetaminophen. ##p < 0.05 compared to control animals; #p < 0.05 compared to group II; "*" not significantly different (group I vs. II); "**" not significantly different (group II vs. group V-VI).

but increased the liver total protein concentration (Table 6). Administration of FF increased the concentration of total protein in the plasma and the liver in the pre- and post-treated groups.

Albumin is the most abundant plasma protein synthesised in the liver, maintains water balance in the plasma and serum, transports and stores a wide variety of ligands (Quinlan et al., 2005). The administration of acetaminophen slightly lowered the concentration of albumin (Table 6) and the concentration was slightly increased in the pre- and post-treated groups.

Total bilirubin a by-product of the breakdown of red blood cells in the liver, is a good indicator of liver function. High levels will cause icterus (jaundice) and are indicative of damage to the liver and bile ducts (Saleem et al., 2008). There was 100% increase in the concentration of bilirubin as a result of acetaminophen administration (Table 6). It was noted that the drug, silymarin and flavonoid fraction altered the concentrations of bilirubin which implied that the compounds might be eliciting adverse effects on the haemoglobin content of the blood and metabolic functions of the kidney. Malondialdehyde (MDA) is one of the end-products of polyunsaturated fatty acid peroxidation and is a good indicator of the degree of lipid peroxidation, which is related to APAP-induced tissue damage (Fakurazi et al., 2012; Lodi et al., 2012). The amount of MDA formed increased by 23.24% with the administration of acetaminophen as a result of lipid peroxidation (Table 7). Administration of FF

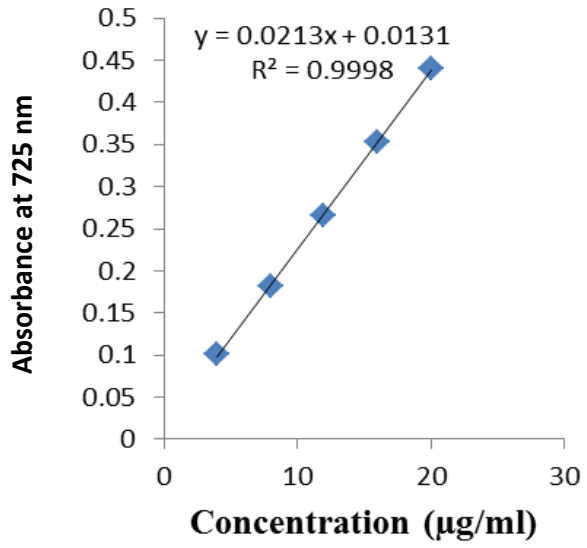

Figure 1. Total phenol standard calibration curve

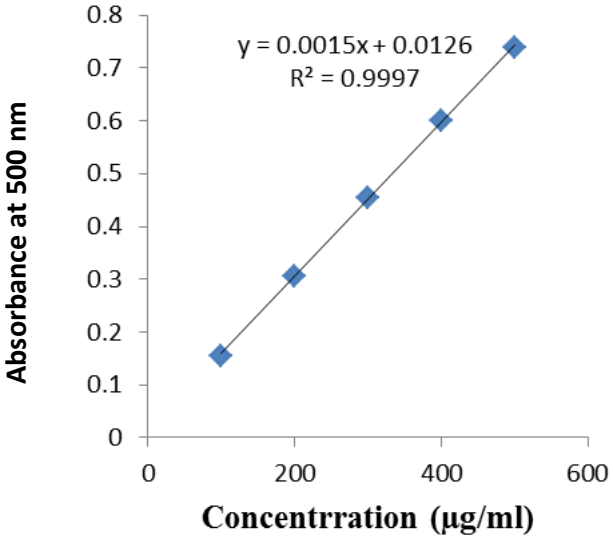

Figure 2. Total flavonoid standard calibration curve.

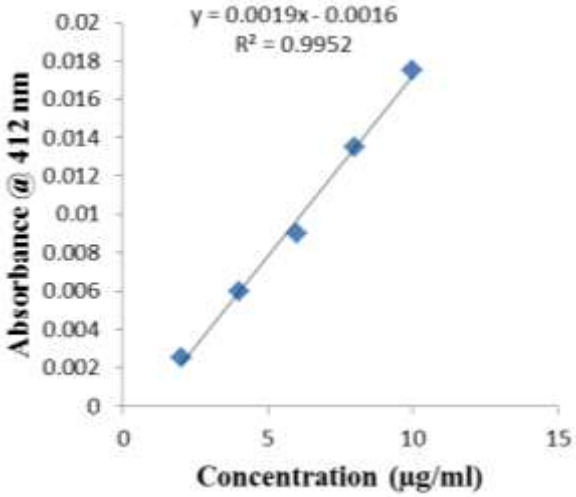

Figure 3. Catalase standard calibration curve.

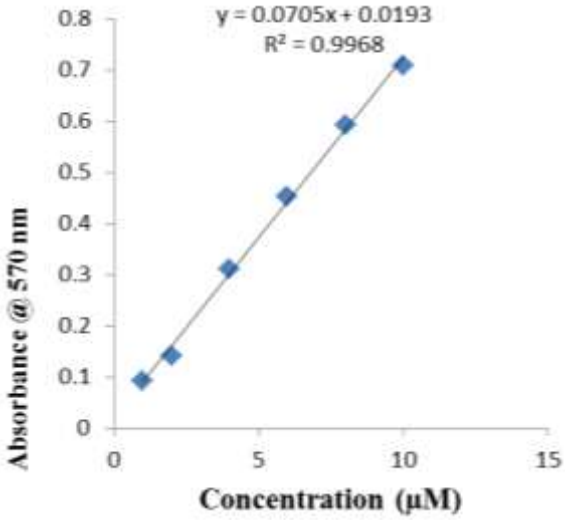

Figure 4. GSH standard calibration curve

was able to reduce the amount of MDA formed in both the pre- and the post-treated groups which could be due to ability of the FF to mop up free radicals that exert lipid peroxidation. The results agreed with the earlier observations of Ratty and Das (1988) that flavonoids and phenolic compounds inhibit and reduced the formation of MDA and lipid peroxidation.

Glutathione (GSH) is a tripeptide which plays prominent role in metabolism of both endo and xenobiotics and functions to remove potentially toxic electrophilic compounds and protects the organ against dysfunction (Radhika et al., 2012). The depletion of GSH concentration (Table 7) could not be unconnected with generation of endogenous ROS that are involved in the initiation of lipid peroxidation, membrane breakdown and cell death (Lebda *et al*., 2013). Administration of FF led to increase in the concentration of GSH in both pre- and post-treated groups which could be as a result of synergetic action of both GSH and FF to mop up excess ROS generated by the acetaminophen. The FF might also be acting by stimulating the activity of glutathione peroxidase to regenerate GSH from GSSG a potent non-enzymatic antioxidant. Flavonoids and phenolics compound impact their biological effects as antioxidants

and free radical scavengers (Afolabi et al., 2012).

Conclusion

It is surmised that flavonoid fraction (FF) of *P. angolense* exerts its mode of action by eliciting moderate, potent anti-oxidant, free-radical activity and hepatoprotective potential.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Distribution of phenolic content and screening for antioxidant capacity of different parts of tea plant (*Camellia sinensis* L.)

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Tea is a medicinal beverage manufactured from the plant, *Camellia sinensis* L. The bud and two adjacent leaves (tea flush) are generally harvested in this process. Currently, it is also used as an ingredient in cosmetic products. The present study was carried out to screen the antioxidant activity and the distribution of phenolic content in different parts of the tea plant for their utilization in other industries. The parts of *C. sinensis* L. used were tea flush, leaves, branches, seed, pericarp and root. Total phenolic content and antioxidant properties of the aqueous extracts were determined by Folin-Denis method and DPPH assay, respectively. The DPPH scavenging capacity in descending order was bud and first two leaves (tea flush)>tender leaves>mature leaves>seed=pericarp>tender branches>mature branches>root. There was no significant difference between tender leaves and the tea bud with two leaves regarding antioxidant activity. The phenolic content was highest in tea flush and least in root. The antioxidant capacity of the different parts were significantly correlated (r^2=0.91; p<0.0001) with their polyphenolic content. Independent of the part of the plant, polyphenols are the principal constituents which are responsible for antioxidant capacity. Tender leaves just below the tea flush cannot be disregarded in cosmetic or food related industries.

Key words: *Camellia sinensis* L., phenolic content, antioxidant potential, tea.

INTRODUCTION

Tea is the most widely consumed medicinal beverage in the world. The bud and two adjacent leaves of the tea plant (*Camellia sinensis* L.) referred to as the `tea flush' are generally harvested during tea manufacture.

C. sinensis L. plant is unique for having a number of pharmacologically active compounds (Modder and Amarakoon, 2002; Chaturvedula and Prakash, 2011). The main constituents of tea leaves belong to the polyphenol groups. Among them, (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin- 3-gallate (ECG) and (-)-epicatechin (EC) are the major tea polyphenols (Yang and Hong, 2013). Tea catechins are a great source of natural antioxidants which can react with reactive oxygen species (ROS) in

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terminating free radical formation and reducing oxidative stress (Yang and Hong, 2013; Kanwar et al., 2012). The oxidative stress is associated with several diseases including skin aging and dermal problems. Tea is not only used as a beverage, it is also used in external applications for skin care (Hara, 2011; Gianeti et al., 2013).

Not only the tea leaves, it has been found that tea seed oil also has strong antioxidant activity which is similar to sesame oil (Rajaei et al., 2008; George et al., 2013). Saponin isolated from roots of *C. sinensis* has also shown antioxidant activity (Sur et al., 2001).

In tea industry, tea bud and the extending two tea leaves are used for quality tea manufacture. Remaining tender leaves as well as the fruits are left in the bush or discarded. Even though many studies have been confined to tea flush, the ethnobotanical importance of other parts of the plant have to be considered due to the raising challenge of finding commercial applications as well.

The objective of the present study was to screen the whole plant of *C. sinensis* for total phenolic content and antioxidant activity. The findings of this analysis are useful in utilizing the parts of tea plant in development of other industries such as cosmetics and food additives.

MATERIALS AND METHODS

Parts of the tea plant were collected (April-July) from the 8 years old well grown mature plants of the clone TRI2026 of *C. sinensis* L. from Akuranakande Estate, Ehaliyagoda, Sri Lanka. The clone was identified and authenticated by the plant breeding division of Tea Research Institute, Ratnapura, Sri Lanka.

Preparation of the extract

Each plant (n=4) was separated into different parts (root, apical bud and two adjoining leaves, next tender leaves, mature leaves, tender branches (branch up to 8 inches from the tea flush), mature branches (branch below 8 inches from the tea flush). Collected mature fruits from ten individual plants were pooled, pericarp was removed from the fruits and considered as two different parts as pericarp and seed. Fresh samples were washed with deionized water and air-dried at room temperature. Each part of the plant was cut into small pieces and freeze dried separately. Percentage of dry weight of each part was determined. Freeze dried samples were blended into powder using a kitchen blender and stored at -20°C until further analysis.

Each part of the plant (1 g) were mixed with deionized water (8 mL) separately, and sonicated for 2 h to disrupt the cells and release its content. The mixture was then centrifuged at 9000 rpm for 30 min and the extracts were filtered through a Whatmann No 1 filter paper. Aqueous extracts of apical bud and two leaves (tea flush), tender leaves, mature leaves, tender branch, mature branch, seed, pericarp and root were prepared freshly from four separate plants for all experiments.

Determination of total phenolic content

Total phenolic content of different samples were determined by the Folin-Denis met hod described by Singleton and Rossi (1965), with slight modifications. The calibration curve was plotted using gallic acid as the standard to determine the phenolic content of test samples. The results were expressed as w/w % gallic acid equivalent.

Determination of antioxidant activity

Antioxidant activity was determined by DPPH free radical scavenging assay, described by Blois (1958), with slight modifications. Different concentrations of tea flush were prepared by diluting the tea flush (125 mg/mL). L-Ascorbic acid was used as the standard antioxidant. The results were expressed as percentage of inhibition (I %) using the following equation:

$$I\% = (A_{control}-A_{sample})/A_{control} \times 100$$

The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was determined by linear regression analysis of dose response curve obtained between percentage inhibitions against concentrations (µg/L). The values obtained were transformed into antioxidant potency defined as -log EC_{50} values (Anesini et al., 2008).

Data analysis

A minimum of four independent experiments were carried out for each assay and results are expressed as mean ± standard deviation (Mean ± SD). Linear regression analysis of the dose response curves were applied to calculate EC_{50} values for DPPH. The level of significance was calculated by T-test. Linear and nonlinear regression analysis and all the statistical evaluations were carried out using Microsoft Excel. The p value <0.05 was considered as significant.

RESULTS

All the parts were subjected to freeze drying to minimize the enzymatic oxidation which can occur during air drying. The highest dry weight was observed in the seed as compared to the other parts of tea plant (Table 1). Water soluble phenolic content was determined and the highest value was observed with tea flush (Figure 1). The dose dependent increase in DPPH scavenging activity was shown with all the parts of tea plant with different capacities and the highest scavenging capacity was also observed with the tea flush. However, the values were lower than the L-Ascorbic acid. The EC_{50} value calculated for the percentage dry weight of the plant material are illustrated in Table 2 and the value is > 625 µg/mL for the root. Polyphenolic content of the individual part of the plant were significantly correlated (r^2=0.9059; p<0.0001) with the DPPH radical scavenging potency. The mean polyphenol concentration against antioxidant potency calculated from the EC_{50} values are illustrated in the Figure 2.

DISCUSSION

Tea is a traditional food consumed by many countries.

Table 1. Percentage dry weight of different parts of *C. sinensis* L. plant. Results are presented as the mean ± SD (n=4).

Part of the plant	Dry weight (%)
Tea flush	22.45 ± 1.59
Tender leaves	35.58 ± 3.27*
Mature leaves	37.25 ± 4.57*
Tender branches	22.81 ± 3.34
Mature branches	24.47 ± 4.54
Root	33.76 ± 1.71*
Pericarp	24.40 ± 2.12
Seed	44.22 ± 2.30*

Results are expressed as mean ± SD (n=4). *Significant difference at p<0.05 between tea flush and other parts.

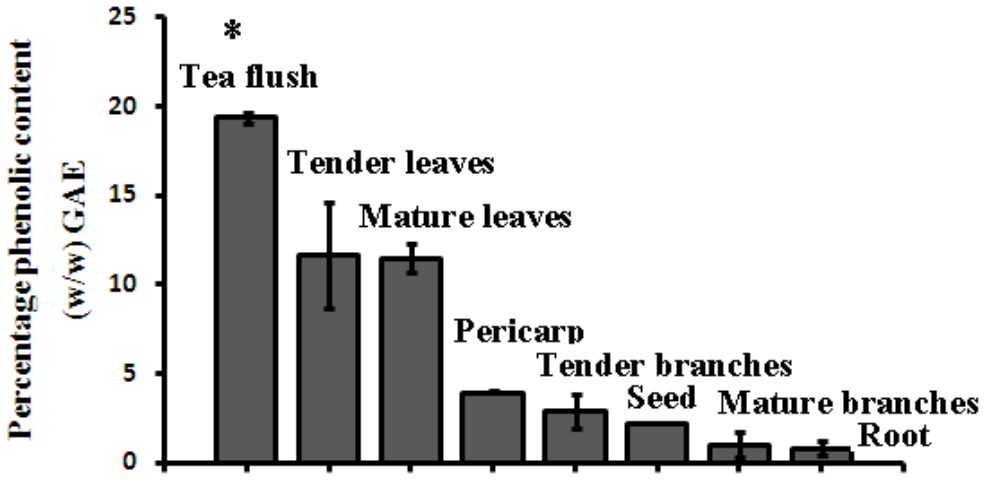

Figure 1. Total phenolic content (mean ± SD) of different extracts: tea flush, tender leaves, mature leaves, tender branches, mature branches, seed, and pericarp extracts calculated with regards to their dry weight. The bars represent the mean values of four independent experiments. *Significant difference at p<0.05 between tea flush with all the other parts.

Medicinal effects of tea are mainly provided by polyphenols (Yang and Hong, 2013). It was observed that, extraction of polyphenols and the antioxidant activity during tea brewing are time dependent (Fernando and Soysa, 2015). Polyphenols and their ability to scavenge reactive species are associated with prevention of diseases including cancer and atherosclerosis (Chen et al., 2008; Brieger et. al., 2012). The present study was focused on the phenolic distribution and antioxidant activity of different parts of the tea plant grown in some countries. Ultrasound assisted extraction (Altemimi et al., 2016) was conducted for all the experiments to minimize oxidation of plant materials.

The highest dry matter content was found in seed, which could be associated with the accumulation of starch in the embryos as reported by Bhattacharya et al. (2002). Tea flush and branches showed the lowest dry weight. The distribution of total phenolic content and antioxidant potential depend on the tissue part of the plant. It was observed that the phenolic content and antioxidant capacity decrease from the apical parts to lower parts of the tea plant. The total phenolic content as well as the antioxidant activity depends on the age of the tea leaf. No significant difference in DPPH scavenging capacity was shown between the tea flush and the tender leaves which could be attributed to their levels of maturity. However, the antioxidant capacity is significantly higher (p<0.05) in tea flush as compared to the dark green mature leaves as well as in the other parts of the plant except for tender leaves. Tea flush and the leaves

Table 2. DPPH scavenging activity (EC_{50} values) of different parts of *C. sinensis* L. plant. Results are presented as the mean ± SD (n=4).

Part of the plant	DPPH scavenging activity EC_{50} (µg/mL of the dry weight)
Tea flush	16.42 ± 4.04*
Tender leaves	30.86 ±10.06
Mature leaves	37.84 ± 4.89
Tender branches	248.25 ± 38.00
Mature branches	390.38 ± 28.43
Root	>625.00
Pericarp	209.50 ± 7.21
Seed	200.08 ± 10.02
L-Ascorbic acid	4.08 ± 0.66

Results are expressed as mean ± SD (n=4). *Significant different at p<0.05 between tea flush and other parts except for tender leaves.

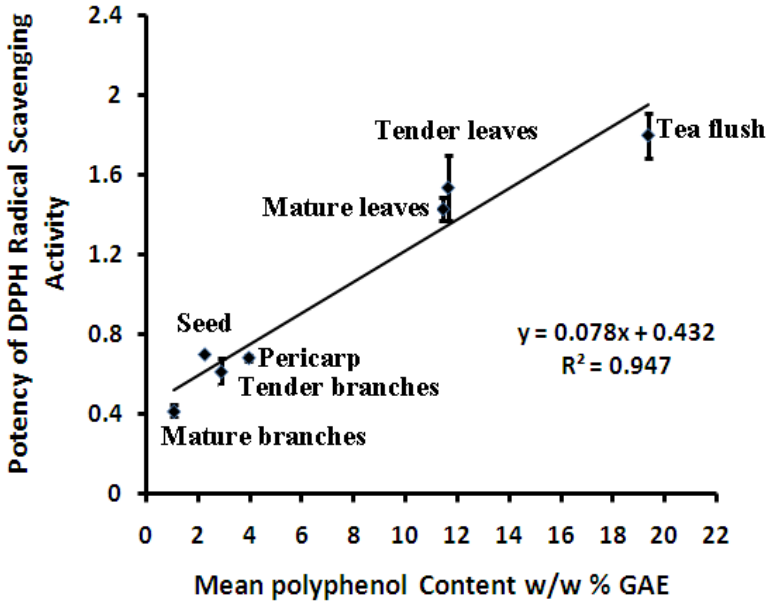

Figure 2. Potency of DPPH radical scavenging activity (-log EC_{50}) with total phenolic content and extracts of tea flush, tender leaves, mature leaves, tender branches, mature branches, seed and pericarp. The curve represents the mean values (± SD) of four independent experiments.

collected from the branch up to 8 inches below from the tea flush did not show any significant difference in DPPH scavenging capacity. The reason for this is the variation found in the levels in maturity of the leaves. Selecting the tender leaves from the appearance (light green) may be more useful as compared to the length of the branch in selecting the parts to minimise the variation (SD) found in antioxidant activity. Tea leaves collected from Malysian tea plantation, have shown that the highest antioxidant activity and phenolic content were found in the ethanolic extracts of tea flush followed by tender and mature leaves (Izzreen and Fadzelly, 2013). However, the percentage of phenolic content they reported was much lower than that of the present study. It has been reported that secondary metabolites of tea leaves vary depending on the genotype, climate, soil type, growing condition, agricultural practices, harvesting period and physical structure of the leaves (Kerio et al., 2013; Jayasekera et al., 2011; Okal et al., 2012). DPPH scavenging ability of the tea root was very low (Table 2) which could be due to the low phenolic content.

The total phenolic content and antioxidant activity

showed similar values in pericarp, seed and tender branches (Figure 1). Water and methanol extract of pericarp and seed have been shown as potential resources for antioxidant ingredients (Jo et al., 2012). It has been also shown that tea seed oil has antioxidant activity *in vivo* (Sahari and Amooi, 2013). However, the present study did not show high antoxidant capacity or phenolic content in the water extract of pericarp or seed as compared to the tea flush or tender leaves. It was reported that the extraction efficiency of phenolics depends on the time of extraction and the solvents used (Rusak et al., 2008). Forrest and Bendall (1969) have also reported that the polyphenol concentration mainly catecheins, flavanols and flavones which contribute to antioxidant activity are high in the aerial apical regions (leaves) of the tea plant and deficient in roots. The content of saponin present in the root could be very low to show antioxidant activity as reported by Sur et al. (2001). However, Pearson correlation analysis in the present study showed that polyphenolic content was significantly correlated (r^2=0.9059; p<0.0001) with the potency of DPPH scavenging capacity (-log EC_{50}) proving that major contribution for antioxidant capacity is provided by polyphenols of each part of the plant (Figure 2).

In conclusion, this study shows that the fresh tea flush which is used in tea industry to produce black tea and green tea in Sri Lanka shows the highest antioxidant activity and phenolic content as compared to the other parts of the tea plant and decreases with the age of the leaf. There was no significant difference between tender leaves and the tea bud. It is strongly reflects that polyphenols present in tea significantly contribute to the antioxidant capacity of the different parts of the tea plant. Tender leaves just below the tea flush could be used in cosmetic or food industries.

Conflict of Interests

The authors have not declared any conflict of interests.

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