In-vitro antioxidant activity and the total phenolics content of herbal plants used in cancer treatment of Sri Lankan ayurvedic medicine

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Abstract *- There are large number of both reported clinical studies and unreported ethnobotanical approaches related to herbal medicine with beneficial effects. However, the effectiveness of the decoctions depends on the method of its preparation basically from lyophilized and fresh plant materials which the traditional medical practitioners got to know from their experience. Thus, this study was carried out with the main objective of determining the effectiveness of antioxidant activity of freshly prepared (FHD) and lyophilized decoctions (LHD) of herbal plants, using to treat cancers in Sri Lankan ayurvedic medicine. Leaves of Annona muricata, Coleus amboinicus, flowers of Malvaviscus penduliflorus, leaves of Munronia pinnata, whole plant of Rauvolfia serpentia, Vernonia cinerea were used to prepare aqueous extracts of FHD and LHD. They were subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. LHDs were subjected to ferric ion reducing antioxidant power (FRAP) assay and the total phenolics content (TPC) was determined using Folin Deni's method. The DPPH radical scavenging activities of LHDs were significantly higher ($p<0.05$) than that of the FHDs. The decreasing order of EC_{50} for DPPH radical scavenging activity of plant extracts was C.amboinicus > A.muricata L. > M.penduliflorus > R.serpentia > M.pinnata > V.cinerea roots > V.cinerea aerial parts. The same order was obtained for the FRAP assay. There was no significant difference between the TPC of A.muricata, M.penduliflorus, M.pinnata, and R.serpentia L. and also between the aerial parts and the roots of V. cinerea. According to the results obtained, it could be concluded that using LHDs are more effective than using the FHDs in the generation of free radicals which the ultimate objective in cancer treatments.*

Keywords: lyophilized, phenolics, DPPH

I. INTRODUCTION

To improve the quality of cancer therapy and reduce side effects and complications arises due to conventional therapeutics, numerous herbal medicines are being used in combination with radiotherapy or chemotherapy (Yin *et al*., 2013). Extraction is the initial step to utilize a biological compound from a plant resource. Thus, proper actions must be taken to make sure that the potential active compounds not lost, destroyed or distorted during the preparation of the decoction**.** The major compounds which have been identified and extracted from terrestrial plants for their anticancer properties include polyphenols, brassinosteroids and taxols (Greenwell and Rahman, 2015). Polyphenols have the property of cytotoxicity on a range of cancer cells and their antioxidant properties have determined (Azmi *et al*., 2005 and Siriwatanametanon *et al*., 2010). Thus it is thought that by being natural antioxidants, polyphenols can improve person's health and reduce risk of cancers (Apostolou *et al*., 2013 and Azmi *et al*., 2006). Traditional medical practitioners extract these active compounds in their own way in order to treat their patients. However, the method of extraction differ from one another which there are two common ways of preparing decoctions (fresh and lyophilyzed). Even the general public tend to prepare decoctions using these two common methods without knowing the most effective method. Therefore the present study was carried out with the main objective of determining the most effective method of preparing decoctions using some herbal plants used in Sri Lankan ayurwedic medicine to treat cancers, depending on their antioxidant activity and the total phenolics content (TPC).

II. METHODOLOGY AND EPERIMENTAL DESIGN

A. Collection and preparation of plant materials

The leaves of *Annona muricata* L. (Anoda) were collected from Aththidiya (Colombo district). The plants of *Munronia pinnata* (Wall.) Theob. (Bin Kohomba) were collected from Kirindiwela (Gampaha District) and the leaves were collected from those plants. The plants of *Vernonia cinerea* (Monarakudumbiya) were collected from Boralesgamuwa and Piliyandala (Colombo District). The flowers of *Malvaviscus penduliflorus* (Sleeping hibiscus) were collected from Boralesgamuwa (Colombo District). The leaves of *Coleus amboinicus* Lour. Fl. Cochinch (Kapparawalliya) and *Rauvolfia serpentia* L. (Ekaweriya) were collected from Kirindiwela (Gampaha District). All sampling processes were performed between the months from May to July of the year 2017

and matured plants were chosen. All the plant materials were bought to the laboratory in safe containers (Zip-lock bags) where they were identified and authenticated. The collected plant parts were cleaned by washing with tap water, subsequently with distilled water followed by the deionized water. Then they were air dried on clean tissue papers in plastic trays until there were no trace of water. After that they were cut in to small pieces and freeze dried until a constant weight was obtained. Finally, dried parts were grinded to a fine powder using a clean kitchen blender.

B. Preparation of the extracts

1) Fresh Herbal Decoction (FHD): A stock solution was prepared to obtain a concentration of 20 mg/ml by dissolving in deionized water. All the stock solutions were then subjected to microwave assisted extraction and irradiated for 2 minutes and 30 seconds. At the end of the heating, the beakers were left for temperature stabilization. One step efficient technique was applied to remove floating and depositing debris of the extract using a disposable plastic syringe (5 cc). The plunger and the needle were removed and a cotton wool plug was placed at the bottom. The raw extract was loaded on the top of the cotton wool plug and centrifuged at 2000 g for 10 minutes at 4^0C. The supernatant (FHD) was collected to sterile tubes.

2) Lyophilized Herbal Decoction (LHD): The supernatants obtained in the above section, were freeze dried. All the freeze dried samples were weighed and stored in -20^0C in air tight vials which were later dissolved in deionized water to prepare LHD. The yields were calculated as a percentage of the total raw material weight.

C. Determination of antioxidant activity

1) 1,1-Diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity: DPPH radical scavenging assay was carried out as has been described by Blois (1958) with slight modifications (Perera *et al.,* 2008). Both FHD and the LHD were separately subjected to the DPPH radical scavenging assay. The 20 mg/ml stock solution of each FHD was diluted with deionized water to get the final concentrations of *A.muricata, M.pinnata, V.cinerea* roots*, V.cinerea* aerial parts*, M.penduliflorus, C.amboinicus and R.serpentia* samples which were analyzed in triplicates. From the LHD, fresh stock solutions were prepared separately diluting with deionized water. The dose response curve was plotted between percentage inhibitions (%) against concentration. The results were expressed as the percentage inhibition (%) using the following equation.

$$\text{Percentage Inhibition of DPPH free radical} = \frac{Ab_{Control} - Ab_{Sample}}{Ab_{Control}} * 100\%$$

The effective concentration of the sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis. From the two types of decoction preparation methods, the method that most effective in DPPH radical scavenging activity was selected for further use.

2) Ferric ion reducing power assay: The reducing power of the LHDs were performed according to the method described by Oyaizu, 1986 with slight modifications (Perera *et al*., 2008). Stock solutions of LHDs were diluted with deionized water in order to prepare different concentrations of the test samples. Ferric ion reducing power was compared using the dose response curve constructed between the absorbance and the concentration of sample and standard by linear regression analysis. L- Ascorbic Acid was used as the reference standard antioxidant. EC_{50} value was calculated according to the concentration of the LHD.

D. Determination of Total Phenolic Content

The total phenolic content of the LHDs were determined by the Folin-Denis method according to the Folin–Ciocalteu method as has been used by Makkar *et al.,* (1993) with slight modifications (Perera *et al.*, 2008). The phenolic content was calculated as w/w% Gallic acid equivalents on the basis of the standard curve which was constructed using Gallic acid standards (3-9 µg/ml).

E. Statistical Analysis

The results are presented as mean ± standard deviation (Mean ± SD). At least three independent experiments were carried out in all experiments. Linear regression analysis was carried out using Microsoft Excel (2013). In order to compare the means one way ANOVA and a Post Hoc test was carried out using the IBM SPSS software (version 20). Significance was determined at 5% level. Calibration curves of the standards were considered as linear if $R^2 > 0.90$. EC50 values were calculated from either linear or logarithmic dose response curves where $R^2 > 0.90$.

III. RESULTS AND DISCUSSION

Plants continue to be a major source for new chemical set-up to develop novel therapeutic agents and the most common forms of therapeutic use of plants worldwide are herbal infusions (Jayaweera, 1982). Recent preclinical and clinical studies have shown that different herbal infusions consumed in Asian countries and other countries have antiproliferative effects against human cancer cells (Monteiro *et al.,* 2014). According to the information that had been gathered from traditional medicinal practitioners in Sri Lanka and ethnobotanical information, *A.muricata, M.penduliflorus* and *V.cinerea*

are used for the treatment of cancer with or without effective results. But has been not reported that *C.amboinicus*, *M.pinnata*, and *R.serpentia* are using to treat cancers. According to previous research studies carried out on phytochemical properties, various standard laboratory procedures have been utilized for extraction such as gradient polarity solvent extraction technique with vacuum distillation using petroleum ether, chloroform, ethanol and distilled water as solvents (Patel *et al.*, 2008). The extraction procedure used in the present study was based on the ethnobotanical information and the common extraction procedures used by ayurvedic practitioners or the general public. Among all the samples, the highest extraction was obtained for *C. amboinicus* which was 20.52 % (w/w) whereas the lowest was obtained for *V. cinerea* roots which was 7.35 % (w/w) (Table 1).

Table 1. The extraction yield obtained for plant materials

Plant species	Extraction Yield (g) (w/w% of dry weight)
A. muricata	16.64
C. amboinicus	20.52
M. penduliflorus	13.21
M. pinnata	10.02
R. serpentia	7.92
V. cinerea aerial parts	9.67
V. cinerea roots	7.35

A. Determination of antioxidant activity

1) 1,1-Diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity: The antioxidant property of each type of decoction was analyzed using DPPH radical-scavenging assay where the most effective decoction was selected depending on the EC_{50} value obtained by each. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule; 2,2-diphenylpicrylhydrazine. Hence the positive DPPH test suggests that the hydrogen donating ability of the plant extracts used (Thanigaivel *et al.*, 2014). A higher DPPH radical-scavenging activity is associated with lower EC_{50} value.

DPPH free radical scavenging activity was increased in a dose dependent manner during both times of using FHD and LHD. When considering the results obtained for FHD; the EC_{50} values obtained for all the samples were significantly different ($p<0.05$) from the EC_{50} value of L-Ascorbic Acid. A significant difference between the mean EC_{50} values of two groups could be observed by which the EC_{50} values of LHDs were significantly less than the EC_{50} values of FHDs. Remarkably, the EC_{50} value of LHD of *C. amboinicus* (35.41 ± 4.20 µg/ml) did not show a significant difference from EC_{50} value of L-Ascorbic Acid (4.74 ± 0.54 µg/ml). Thus it can be concluded that LHD of *C. amboinicus* has a hydrogen donating capacity similar to a standard antioxidant such as L-Ascorbic Acid. Other than that the EC_{50} value of *C. amboinicus* was significantly higher, than the decoctions of *A. muricata* (57.10 ± 0.98 µg/ml), *M. penduliflorus* (100.42 ± 20.52 µg/ml), *M. pinnata* (188.22 ± 24.30 µg/ml), *R. serpentia* (120.32 ± 4.25 µg/ml), *V. cinerea* aerial parts (391.64 ± 27.05 µg/ml) and *V. cinerea* roots (211.94 ± 16.73 µg/ml). However the results further indicate that EC_{50} values of *V. cinerea* aerial parts and *M. pinnata* were not significantly different from each other. The present study

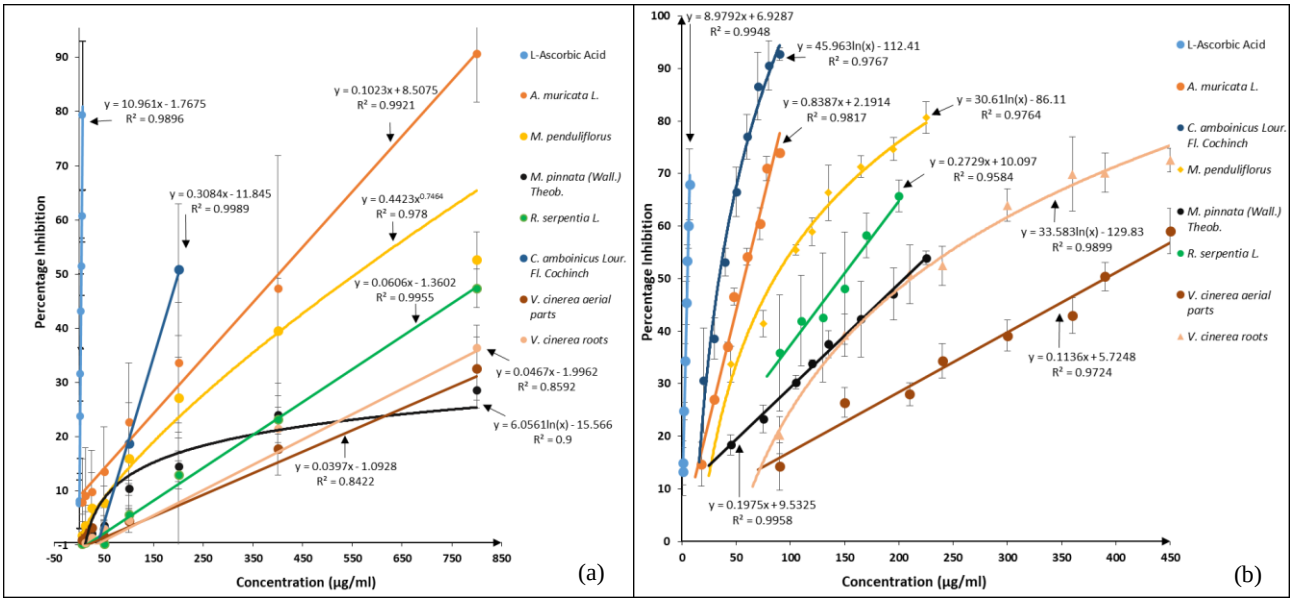


Figure 1. The dose response curves for percentage scavenging of DPPH by (a) FHD and (b) LHD in comparison with L-Ascorbic acid. The results are presented as mean ± SD for (n=6).

Table 2. Mean EC_{50} values for antioxidant tests for aqueous extracts of LHD and FHD and the TPC calculated as Gallic Acid Equivalent (GAE)

Plant species	DPPH assay		Fe^{3+} reducing power	TPC
	FHD (n=6)	LHD (n=6)	LHD (n=6)	
L-Ascorbic acid	4.74 ± 0.54	4.74 ± 0.54	5.18 ± 0.39	
A. muricata	411.14± 120.90	57.10 ± 0.98	70.97 ± 1.02	18.68 ± 1.71
C. amboinicus	200.51 ± 45.72	35.41 ± 4.20	49.82 ± 3.07	10.59 ± 2.18
M. penduliflorus	644.78 ± 37.74	100.42 ± 20.52	99.08 ± 12.52	17.96 ± 2.50
M. pinnata	1229.13 ± 79.14	188.22 ± 24.30	182.69 ± 19.52	17.16 ± 1.60
R. serpentia	845.15 ± 40.21	120.32 ± 4.25	175.82 ± 28.70	13.96 ± 2.97
V. cinerea aerial parts	1223.03 ± 71.18	391.64 ± 27.05	427.23 ± 31.63	3.27 ± 0.83
V. cinerea roots	1006.69 ± 49.13	211.94 ± 16.73	317.35 ± 23.55	9.68 ± 2.75

further indicate that hydrogen donating ability or the antioxidant activity of LHDs were significantly higher than that of the FHD.

This could be the reason behind using LHD of the plant extracts in almost all of the previous research studies. In the review of the Effect of Freeze-drying and its Implications for Botanical Medicine by Abascal *et al*. (2005), suggest that freeze drying has some potentially significant and unforeseen effects on the chemical composition and the medicinal activity of the plants. Furthermore it has been mentioned that there is an unwarranted and unexamined assumption in botanical research that freeze-drying properly and optimally preserves the plant's constituents which some of the studies had indicated that these kind of assumptions are erroneous. Abascal *et al.* (2005) suggest the need of further research on the effects of freeze-drying compared with the fresh plant material, with the intention of more accurate testing of the medicinal effects of botanicals. Hence, with accurate results obtained for the DPPH free radical scavenging activity of the above mentioned decoctions; the present study suggests that, freeze-drying properly preserves the chemical components of decoctions prepared from plant extracts and is superior to the fresh plant decoction. With that background the LHDs were used for further assays.

2) Ferric ion reducing power assay: There are number of assays used to determine the antioxidant activity other than the DPPH radical scavenging assay such as Ferric Reduction Activity Potential (FRAP) which is often being used in parallel with the DPPH assay, and also the Total Phenolics Content (TPC) as a representative marker for antioxidant capacity (Clarke *et al.,* 2013). FRAP assay measures the electron-donating capacity of an antioxidant. The presence of reducing agents (i.e. antioxidants) causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. The absorbance measured at 700 nm of the resultant blue-green colored solution is proportional to the amount of Fe^{2+} in the system. Therefore an increased absorbance is indicative of higher reducing power. The reducing power of the LHDs of *A. muricata*, *C. amboinicus*, *M. penduliflorus*, *M. pinnata*, *R. serpentia*, *V. cinerea* aerial parts and *V. cinerea* roots was observed to be increased progressively in dose dependent manner ($R^2 > 0.96$) over the concentration investigated (Figure 2). However the L-Ascorbic acid which was used as the standard, showed the highest reducing power than the decoctions. The strength of the ferric ion reducing power was observed to be *C. amboinicus* > *A. muricata* > *M. penduliflorus* > *R. serpentia* > *M. pinnata* > *V. cinerea* roots > *V. cinerea* aerial parts. Hence the results obtained for FRAP assay further prove the results obtained for the DPPH radical scavenging assay which the antioxidant activity would be high when decoctions are used as LHDs.

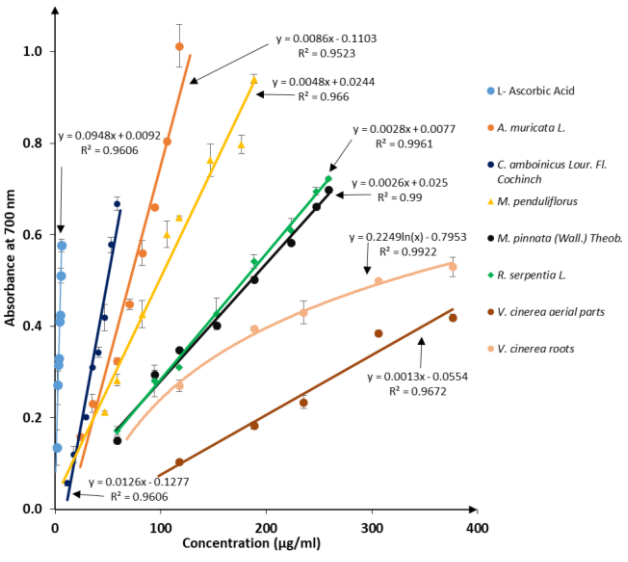
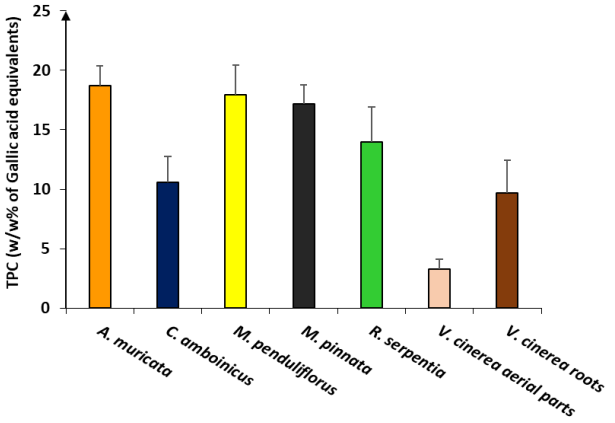


Figure 2. The reduction capability of different concentrations of LHDs in comparison with L-Ascorbic acid. The results are presented as mean ± SD for (n=3)

D. Determination of Total Phenolic Content

The most common compound in plant materials which gives a potentially high free radical scavenging activity to a decoction is the phenolic content. Thus the total phenolic content of all the samples were determined to test the relationship between the TPC and the antioxidant activity. The percentage of total phenolic content was expressed in Gallic Acid Equivalents (GAE) per dry weight of the decoctions. The results obtained for TPC of LHDs are tabulated in Table 2. When considering the TPC of two parts (roots and aerial part) of the same plant (*V. cinerea*) there was no significant difference observed. This result was contradicted with the values obtained for antioxidant activity which there was a significant difference between the DPPH radical scavenging activities between the LHD of roots and leaves of *V. cinerea*. However both the antioxidant activity and the TPC of root decoctions were higher than aerial parts. According to the study of Rajamurugan *et al.* (2011) the TPC of methanolic extract of *V. cinerea* has been 167.48 ± 0.57 Gallic acid equivalents mg/g or 16.75 w/w% of Gallic acid equivalents whereas the DPPH radical scavenging activity has been 56 ± 6.92 µg/ml. In the present study instead of leaves the entire aerial parts were used. Therefore both the antioxidant activity and TPC was expected to be higher than that of a leaf extract even with a different type of solvent. Because it has been reported that *V. cinerea* leaf extract and flower extract have moderate amount of polyphenols (Sonibare *et al.*, 2016). But the obtained values for antioxidant activity and TPC was apparently lower. One of the reason for this could be the conditions of the extraction procedure. For the analytical purpose the extraction method was kept uniform for all the samples. However the efficiency of releasing intracellular chemicals to a solvent by a plant material might be different from one species to another. Another reason could be the reactions take place within the decoction where a mixture of chemicals present from



all the aerial parts of the plant including flowers and seeds. *T*here was no significant difference ($p>0.05$) between the total phenolic contents of *A. muricata, M. penduliflorus, M. pinnata and R. serpentia.*

Figure 3. TPC of LHD presented as w/w% of Gallic acid equivalents. The results are presented as mean +SD for (n=6)

Considering the results obtained for DPPH radical scavenging activity and the TPC, a relationship between those two could be obtained (Figure 4). When considering all the samples it was not a very strong positive relationship (R^2=0.6623). However it was noticed that even though *C. amboinicus* had a high antioxidant activity which was similar to L-ascorbic acid the TPC obtained was considerably low (Table 2). There could be several reasons for this observation. One of them is the antioxidant activity might not be due to the presence of polyphenols. This could have been tested by removing the polyphenols. Another reason could be another molecule other than a polyphenol might have brought the high antioxidant activity of *C. amboinicus* leaf extract such as tannins. As has been shown in the study by

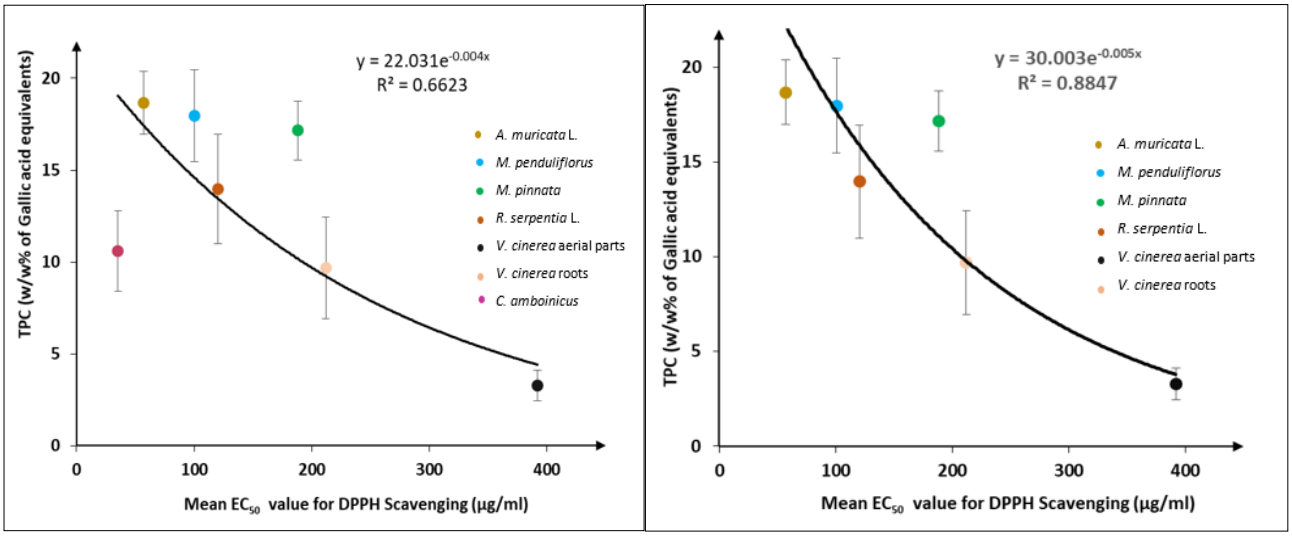


Figure 4. Relationship between TPC and DPPH radical scavenging activity (n=6) (a) with *C. amboinicus* (b) without *C. amboinicus*

Asiimwe *et al.* (2014), the aqueous leaf extracts of *C. amboinicus* contains tannins, saponins, flavonoids, steroid glycosides and polyuronides. In the same study a gas chromatography analysis has revealed the presence of 11 chemical compounds constituting 97.6% of the total extract composition. The principle constituents has been include linalool (50.3%), nerol acetate (11.6%), geranyl acetate (11.7 %) and carvacrol (14.3%). Linalool is a naturaly occurring monoterpenoid alcohol (Asiimwe *et al.,* 2014). However when excluding the results obtained for *C. amboinicus* a strong positive relationship (R^2=0.8847) could be observed between the TPC and the DPPH radical scavenging activity of lyophilized decoctions of A. muricata, *M. penduliflorus, M. pinnata*, *R. serpentia*, *V. cinerea* aerial parts and *V. cinerea* roots.

IV. CONCLUSION

With the results obtained for the antioxidant activity and the TPC of the LHD and the FHD of herbal plants; the present study suggests that, LHDs properly preserves the chemical components of plant extracts (*C. amboinicus, A. muricata, M. penduliflorus, R. serpentia, M. pinnata, V. cinerea* roots and *V. cinerea* aerial parts) and is superior to the FHD. Thus, the treatments will be more effective if the herbal decoctions are prepared as lyophilized decoctions.

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