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FINAL REPORT

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Screening of twenty five medicinal plant extracts for their polyphenol content and *in- vitro* antioxidant activity

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Contents

FINAL REPORT

Section 1

Information regarding Project/Project Personnel:

i) Contract Number - RG/2007/Hs/10

ii) Title of the Project - Screening of twenty five medicinal plant extracts for their polyphenol content and *in- vitro* antioxidant activity

iii) Principal Investigator - Dr. K.A.P.W. Jayatilaka

iv) Co-Investigators - not applicable

v) Institute(s) where research was being carried out – Department of Biochemistry, Faculty of Medicine, Galle

vi) Date of award - 03-12-2007

vii) Date of completion of Project - 31-01-2011

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ix) Total spent (Rs) - 179,267.20

x) Number of Research Students employed - None

xi) Post graduate degree completed with dates – Not applicable

xii) Number of Technical Assistants and/or labourers employed and period of service – Not applicable

xiii) Publications/Communications arising from the project during the reporting period – none so far

Section 2

Executive Summary of the Project:

Recent years have witnessed renewed interest in plants as pharmaceuticals across the world. This interest has channeled into the study of bioactivities of medicinal plant extracts. Oxidative stress is implicated in many chronic diseases. Plant antioxidants comprise an important role in defence against such oxidants.

A considerable body of literature supports the role for oxidative stress in pathogenesis of disease and contribution of dietary polyphenols to their prevention. The objective of this study was to determine the total polyphenol content of twenty five aqueous plant extracts and to determine the *in vitro* antioxidant activity of those extracts by DPPH assay, ferric reducing antioxidant power assay and by nitric oxide radical inhibition assay. These assays based on different chemical mechanisms were selected to take into account the wide variety and range of action of antioxidant compounds.

Twenty one hot water extracts out of the twenty five were analyzed. The most potent extracts out of the twenty one extract activities analyzed for the total polyphenol content were *Osbeckia aspera*, *Terminalia arjuna*, *Nauclea orientalis*, *Langus calcarata* and *Azadirachta indica*. Highest activities of the FRAP assays were followed by *Osbeckia aspera*, *Langus calcarata*, *Terminalia arjuna* and *Nauclea orientalis*. The low IC_{50} values for nitric oxide scavenging activity depicting high activity was found in *Osbeckia aspera, Terminalia arjuna*, *Nauclea orientalis* and *Adhatoda vasica*. The strongest activity

against DPPH radical was shown in *Osbeckia aspera, Nyctanthus arbor-tristis, Coscinium fenestratum, Nauclea orientalis* and *Terminalia arjuna*.

Regardless of the method of analysis the best antioxidant activity along with polyphenol contents were found in the extracts of *Osbeckia aspera, Terminalia arjuna* and *Nauclea orientalis*. Therefore they can be considered as rich sources of water soluble antioxidants and or phenolic compounds.

Section 3
Report in detail:

Introduction/background
Finding healing powers in plants is an ancient idea. People of all continents have long imbibed infusions of plants dating back to pre history. There is romance and mystique surrounding these traditional remedies, which is lacking from the white tablets and sophisticated techniques of modern medicine. New pharmaceuticals are often thought to arise from a 'black box' of synthetic chemistry or from recent drug design concepts such as biological receptors or the use of combinatorial chemistry techniques. But various active compounds from barks, leaves and roots are found in a new guise in existing treatments or may be used as a basis for design of novel medicinal molecules (1).

In spite of the scientific and commercial concerns, there is still considerable interest in ethno botany as a source of novel drugs and medicine for the world community. It should not be forgotten that a large proportion of the world's flora –probably 90% remains to be investigated scientifically. Most of these plants are used medicinally in some part of the world (1).

In addition to that the WHO has also stressed on the importance on medicinal plant research. In a monograph on medicinal plants by the WHO, it has emphasized the need of medicinal plant research as the information available on safety and efficacy data are very few (2).

Despite the wide distribution of plants, the health effects of polyphenols have come to the attention rather recently. Until mid 1990s the most widely studied antioxidants were antioxidant vitamins, carotinoids and minerals (3). A considerable body of literature supports the role for oxidative stress in pathogenesis of disease and contribution of dietary polyphenols to their prevention. Antioxidants are compounds that can delay or inhibit oxidation of lipids or other molecules by inhibition or propagation of oxidizing chain reactions. For many years polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging free radicals. However, this concept now appears to be an oversimplified view of their mode of action (4). It has been reported that cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modifications of redox status of the cell and may trigger a series of redox dependant reactions (5).

Polyphenols are a highly hydroxylated phenolic compounds present in higher plants. These molecules secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens. Polyphenols in plants are divided into several subclasses such as catechins, flavonols, flavanols, anthocyanins, proanthocyanidins, phenolic acids, stilbenes and lignans to name a few (6). It has also been pointed out that the evaluation of antioxidant activities of polyphenols from ethnomedicinal plants due to their neutraceutical effects (3).

Sri Lanka constitutes an apt example where medicinal plants are widely used in everyday life for culinary purposes, in preparing gruel with rice and as medicinal remedies. Sri Lankan flora is remarkable for its diversity and is a rich source of medicinal plants. However it is still needed to explore the usefulness of many of them for modern therapy. Little is known about the antioxidant potential of Sri Lankan medicinal plants. Limited research on medicinal plants and lack of facilities to do so has lead into another problem regarding the intellectual property rights of Sri Lankan flora. This is another reason as to why the scientists should embark on medicinal plant research.

A common denominator in pathogenesis of most chronic diseases is the involvement of oxidative stress related to the production of all aerobic organisms of reactive oxygen and nitrogen species, including free radicals (7, 8). In addition to having a role of intra and extracellular signaling these reactive molecular species may initiate damaging biochemical reactions (9) (10). In response to such damage, a complex antioxidant defence has developed, and plant antioxidants comprise an important role in defence (11, 12).

A simple perfect test that would be able to measure all possible mechanisms do not exist and all test methods are not immune to interferences present in complex plant extracts. Several methods have been developed recently for measuring the total antioxidant capacity of food and beverages and plant extracts. These assays differ in their chemistry (generation of different radicals/ and or target molecules) and in the way end points are measured (13, 14, 15, 16).

Therefore three methods were selected to measure *in-vitro* antioxidant activities. Those are DPPH radical scavenging activity; FRAP assay and nitric oxide radical inhibition assay. These assays differ in their chemistry and in the way end products are measured. It is important to note that radical scavenging methods and antioxidant capacity are listed under bioassay systems for the identification potential of chemopreventitive agents (17).

A key mediator released by activated macrophages tat has been implicated in toxicity is nitric oxide. A large number of laboratories have shown that increased of this highly reactive nitrogen intermediate are produced during tissue injury associated with inflammation (18, 19). It has been pointed out that modulating nitric oxide production can modify tissue injury (20). Nitric oxide radicals combine with superoxide radical and forms peroxynitrite which is cytotoxic. Due to lack of endogenous enzymes responsible for ONOO- inactivation, development of specific ONOO- scavengers or NO scavengers

is considered important (21). Recent studies by several authors have shown in vitro nitric oxide scavenging activity by fruits and isolated compounds from plant extracts (22, 23, and 24). Therefore it was decided to carry out an in vitro assessment of nitric oxide scavenging activity of the selected medicinal plants.

Scientific scope of the project (overall and specific objectives)

- To determine the total polyphenol content of aqueous extracts
- To determine the antioxidant activity of aqueous extracts by
    - 2,2'-diphenyl 1-picrylhydrozyl radical scavenging assay (DPPH assay)
    - ferric reducing antioxidant power assay (FRAP assay)
    - nitric oxide radical inhibition assay

i) Materials and methods

Selection and collection of plant material

Twenty-five medicinal plants used in traditional medicine were selected (Table 1) based on the literature given in (Jayaweera D.M.A.) (25) and on account of their availability and popularity among the traditional medical practitioners in the south. Priority was given to plants used internally. Some of these plants also have been appreciated and recognized for their aesthetic and ornamental value and/or usage as herbal food supplements. All the plants selected are used in treatment of different disorders. Plants were identified and a specimen is kept in the department. From each plant material three samples will be tested. *Benincasa hispidia* fruit, *Ruta graveolens* leaves, *Withania somnifera* leaves and roots, *Corriandrum sativum* seeds and *Nigellea sativa* seeds were purchased from the market.

Table 1

Scientific name	Family	Sinhala name	Part of plant tested
Adhatoda vasica	Acanthaceae	*agal aadhara, adathoda*	leaves
Alternathera sessilis	Amarantheceae	*mukunuwenna*	aerial parts
Aerva lanata	Amarantheceae	*polpala*	whole plant
Gymnema sylvestre	Asclepidaceae	*Bin-nuga, masbedda*	leaves
Hemidesmus indicus	Asclepidaceae	*iramusu*	aerial parts
Dregea volubilis	Asclepidaceae	*kirianguna*	aerial parts
Terminalia arjuna	Combretaceae	*kumbuk*	bark
Sphaeranthus indicus	Compositae	*mudamahana*	whole plant
Ipomea mauritiana	Convolvulaceae	*kiribadu*	aerial parts
Benincasa hispidia	Cucurbitaceae	*alupuhul*	fruit juice
Swertia chirata	Gentianaceae	*binkohomba*	aerial parts
Vetiveria zizanioids	Graminae	*sevenna*	root

Sevendra

Plectranthus zeylanicus	Libiatae	*iraweriya*	aerial parts
Osbeckia aspera	Melastomaceae	*heenbovitiya*	leaves
Azadirachta indica	Meliaceae	*kohomba*	leaves
Coscinium fenestratum	Menispermaceae	*baanwelgeta*	wood
Nyctanthus arbor-tristis	Oleaceae	*sepalika*	flowers
Nauclea orientalis	Rubiaceae	*bakmee*	bark
Oldenalandia biflora	Rubiaceae	*pepiliya*	whole plant
Pavetta indica	Rubiaceae	*pavetta*	leaves
Nigellea sativa	Rununculaceae	*kaluduru*	seeds
Ruta graveolens	Rutaceae	*aruuda*	leaves
Withania somnifera	Solanaceae	*Amukkara, ashwagandha*	leaves, roots
Corriandrum sativum	Umbelliferae	*kottamalli*	seeds
Langus calcarata	Zingiberaceae	*heenaratta*	rhizome

Preparation of plant extracts:

Different plant parts collected were washed and oven dried at 40^{0}C. Powdered plant extracts were stored under refrigeration. 2.5g of the relevant plant part was refluxed with 50mL of distilled water for two hours and the final volume adjusted to 50mL. The hot water extract used for the experiments.

Estimation of total polyphenol content

The total polyphenol content of extracts was determined using method described by Singleton et al (26). Appropriate dilutions of the plant extracts were used to estimate the total polyphenol content. The extracts were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue colour was measured at 760nm after 60min. The polyphenol content (TOC) was expressed as percentage by the dry weight using the gallic acid standard curve.

DPPH assay

The method described by Brand – Williams et al (27) was used with some modifications (28, 29). In cases where the structure of the electron donor is not known (e.g. plant extract), this method can afford data on reduction potential of the sample and hence can be helpful in comparing the reduction potential of unknown compounds (30). The radical scavenging activity of plant extracts against stable DPPH* (2, 2- diphenyl– 2–picrylhydrazyl hydrate) was determined spectrophotometrically. When DPPH* reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in colour (from deep violet to light yellow) was measured at 515nm using a spectrophotometer. L-ascorbic acid was used as the reference compound.
The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%).

The inhibition ratio percentage was calculated as
% inhibition = [(absorbance of control - absorbance of test sample)/absorbance of control] x 100%.

Ferric reducing power assay (FRAP assay)

The FRAP assay was performed as previously described by Benzie and Strain (15). In this assay, the antioxidants in the sample reduced Fe3+/ tripyridyltriazine complex present in stoichiometric excess to the blue coloured ferrous form with an increase in absorbance at 593nm. The antioxidant power of freshly prepared aqueous solution of ascorbic acid was measured as a reference.

$$\text{FRAP value } (\mu M) = \frac{\Delta A_{\text{sample}} \text{ (0 to 4 min)}}{\Delta A_{\text{standard}} \text{ (0 to 4 min)}} \times \text{FRAP value of standard (100 } \mu M)$$

FRAP value of ascorbic acid was taken as 2.

Nitric oxide radical inhibition assay

The method described by Marcocci et al was used (31). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which was reacted with Griess solution and the absorbance of the chromophore formed was measured immediately at 546nm. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside in phosphate buffered saline was mixed with different concentrations of the hot water extracts and incubated at 25^{0}C. The samples of the above were reacted with Greiss reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide was read at 5465nm and referred to the absorbance standard solutions of potassium nitrite treated with the same way with Griess reagent. Ascorbic acid was used as the reference compound.

Statistical analysis

The results were evaluated by using the statistical package Minitab. Results were expressed as ± SD.

(iv) **Results/outputs** – The water phase antioxidant activity and polyphenol content of twenty one extracts were studied. Figure 1 shows the gallic acid standard curve and fig 2 the total polyphenol content per gram of dry weight calculated corresponding to the gallic acid standard curve. The leaf extract of *Osbeckia aspera* showed the highest total polyphenol content followed by *Terminalia arjuna*, *Nauclea orientalis*, *Langus calcarata* and *Azadirachta indica*. The lowest total polyphenol content was in the seed extract of *Nigellea sativa*. A wide variation was observed in the range of total polyphenol contents of the twenty one extracts.

Pareth a
6
Induca

The IC_{50} values of DPPH assay are shown in fig 3. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH radicals. The low IC_{50} values for DPPH assay was found in *Osbeckia aspera*, *Nyctanthus arbor-tristis, Coscinium fenestratum, Nauclea orientalis* and *Terminalia arjuna*. Those IC_{50} values suggest that *Osbeckia aspera, Nyctanthus arbor-tristis, Coscinium fenestratum, Nauclea orientalis* and *Terminalia arjuna* extracts possess better radical scavenging activity than the other extracts analyzed. The highest IC_{50} value (lowest *in vitro* radical scavenging activity) was shown in *Withania somnifera.*

Highest values of the FRAP assays were found in *Osbeckia aspera*, *Langus calcarata*, *Terminalia arjuna* and *Nauclea orientalis* (fig 4). The lowest FRAP values were found in the extracts of *Alternathera sessilis* , *Plectranthus zeylanicus* , *Nigellea sativa* and *Withania somnifera.* The FRAP values of the extracts also showed a wide variation like in the total polyphenol contents of the extracts. The IC_{50} values for nitric oxide scavenging activity of the plant extracts are shown in fig 5. The hot water extracts of *Osbeckia aspera, Terminalia arjuna*, *Nauclea orientalis* and *Adhatoda vasica* have more potent nirtric oxide radical scavenging activities compared to the other extracts. The results are shown in fig 5. All the hot water extracts exhibited IC_{50} value less than 500 µg/mL. In the DPPH assay and nirtric oxide radical scavenging activity assay the reference compounds showed more pronounced activities than the crude water extracts.

Regardless of the method of analysis the best antioxidant activity along with polyphenol contents were found in the extracts of *Osbeckia aspera, Terminalia arjuna* and *Nauclea orientalis.*

ii) Discussion

In this preliminary investigation, hot water extracts were used for the analysis of selected plants. Plant extracts were prepared in the common way in which they are prepared for human consumption. For the use in traditional medicine or in food, plant extracts made with water are medicinally and nutritionally more relevant. Currently the availability of information on antioxidant properties of many tropical plants is sporadic and lacking.

In the present study twenty one hot water extracts of medicinal plants were studied for their total polyphenol contents. The hot water extracts of *Osbeckia aspera, Terminalia arjuna, Nauclea orientalis, Langus calcarata* and *Azadirachta indica* showed high total polyphenol contents. It has been reported that the antioxidant capacity is mainly derived from the water soluble antioxidants and there is a high correlation with polyphenols (32).

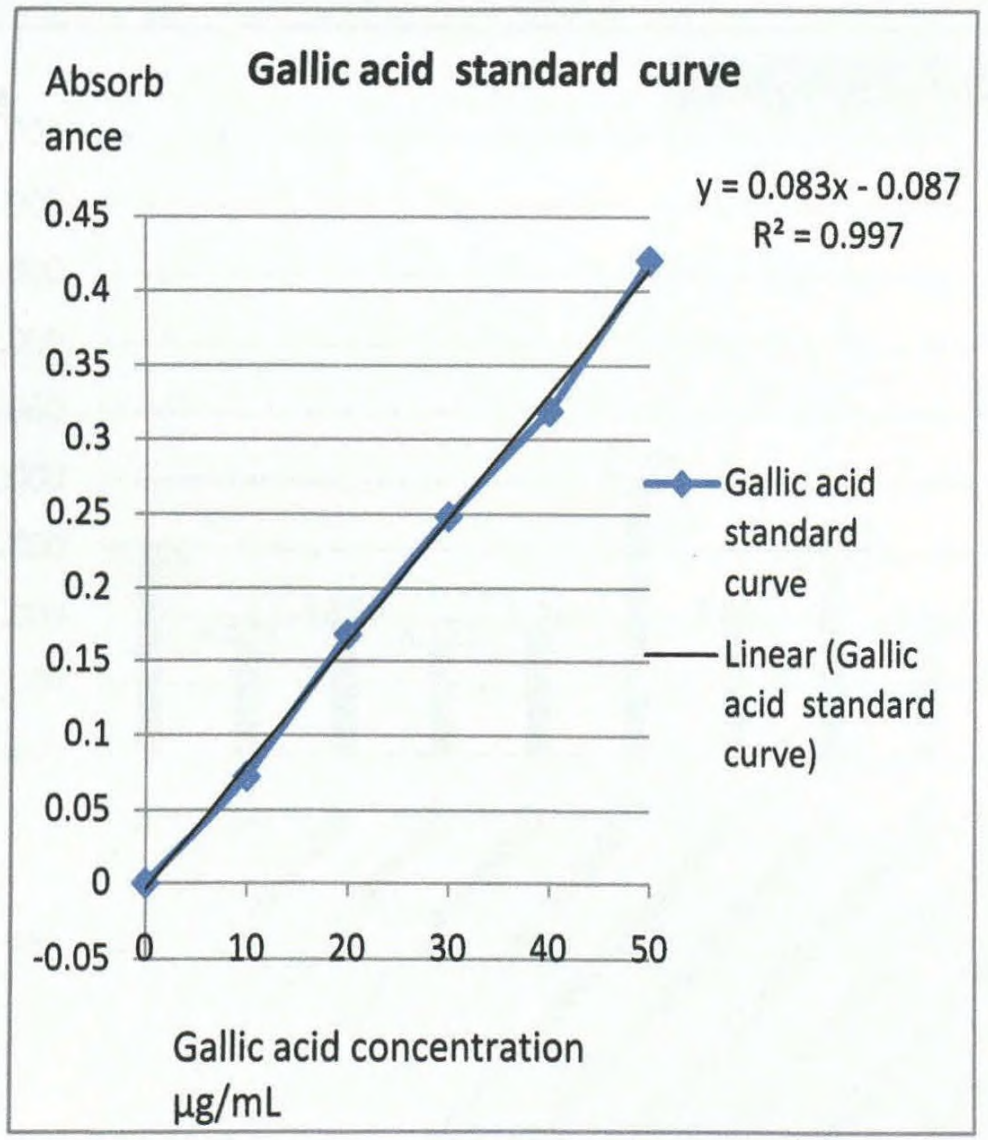
Over the past few years investigations for polyphenolic compounds in medicinal herbs have gained importance due to their high antioxidative activity (33). A large number of reports have demonstrated that these compounds are of great value in preventing the onset or progression of many diseases. Polyphenols have many favourable effects on

human health like inhibiting the oxidation of LDL, anti-inflammatory and anti-carcinogenic properties. Flavonoids and many other phenolic compounds of plant origin have reported as scavengers of reactive oxygen species and are viewed as promising therapeutic drugs for free radical pathologies. Thus measurement of polyphenols and antioxidants in medicinal plants have become important tools to understand the relative values of plant species, especially from a health point of view.

In our study the IC_{50} values of the extracts in the DPPH radical scavenging activity varied between 113-245 µg/mL and there are fourteen plant extracts which have the IC_{50} values less than 150 µg/mL. Where the DPPH radical scavenging activity is concerned all the plant extracts studied have exhibited high radical scavenging activities. DPPH assay is based on the ability of DPPH, a stable free radical to decolorize in the presence of antioxidants. This is reported to be a direct and a reliable method for determining radical scavenging activity. In cases where the structure of the electron donor is not known (e.g. plant extract), DPPH assay method can afford data on reduction potential of the sample and hence can be helpful in comparing the reduction potential of unknown compounds (30). The IC_{50} value of L - ascorbic acid which was used as the reference compound was found to be 6.97 µg/mL. This is in agreement with the findings of Raqulbul Hasa S.M. et al (34). The low IC_{50} value of L - ascorbic acid is understandable since L-ascorbic acid is in pure form while the crude extracts need to be processed in order to isolate the compounds responsible for the antioxidant activity. The *Osbeckia aspera* leaf extract possesses comparable IC_{50} value to *Ginko biloba* extract which has been reported as 106.14 µg/mL (35). *Ginko biloba* extract has been studied widely for its clinical efficacy. As other, we have found that the content of polyphenolics was consistent with the finding of DPPH radical scavenging activity. (36, 37). Further analysis will have to be carried out to find the correlation coefficient.

The FRAP assay is on the basis of the capacity of antioxidant to reduce Fe(III) to Fe(II) ions (15). Generally the FRAP assay method is simple and inexpensive. It can be applied to aqueous and alcoholic extracts to assess antioxidant capacity and offers fast reproducible results. In the present study the FRAP value varied from 15.84 µmol to 1.14 µmol Fe(II)/g dry weight of the plant material. It is interesting to note that the hot water extract of *Osbeckia aspera* leaves showed the highest polyphenol content and FRAP values and the lowest IC_{50} value for the DPPH radical scavenging activity.

In addition to reactive oxygen species, nitric acid is also implicated in inflammation, cancer and other pathological conditions (38). The plant or plant product may have the property to counteract the formation of nitric oxide and in turn may be of considerable interest in preventing the ill effects of excessive nitric oxide generation in the human body. The best IC_{50} values for nitric oxide scavenging activity were shown by the hot water extracts of *Osbeckia aspera, Terminalia arjuna*, *Nauclea orientalis* and *Adhatoda vasica* . The present results suggest that these extracts might be potent and novel therapeutic agents for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite. However the IC_{50} value of the positive control vitamin C (19.13 µg/mL) was more pronounced than the plant extracts.



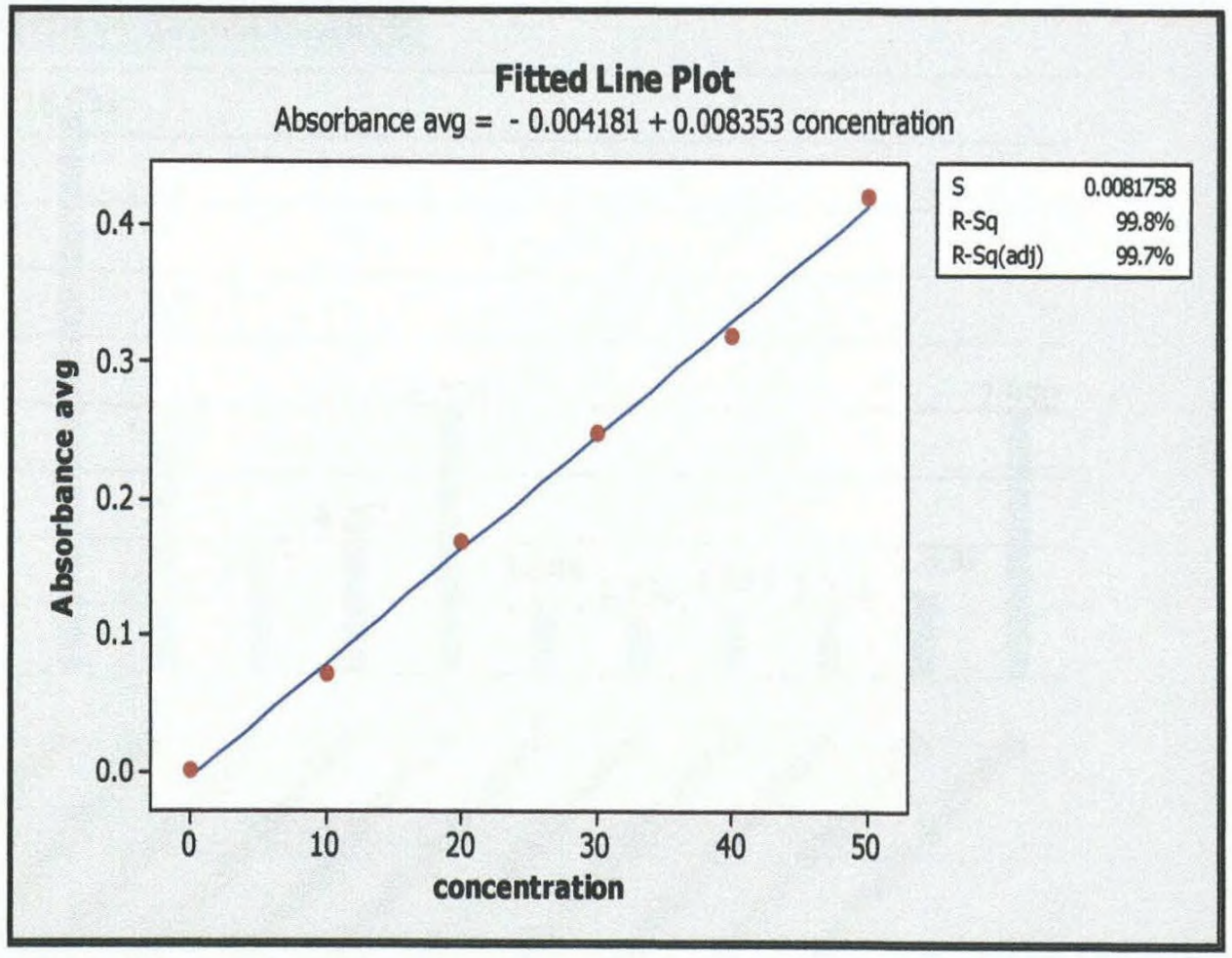


Fig: 1 Gallic acid standard curve and fitted line plot

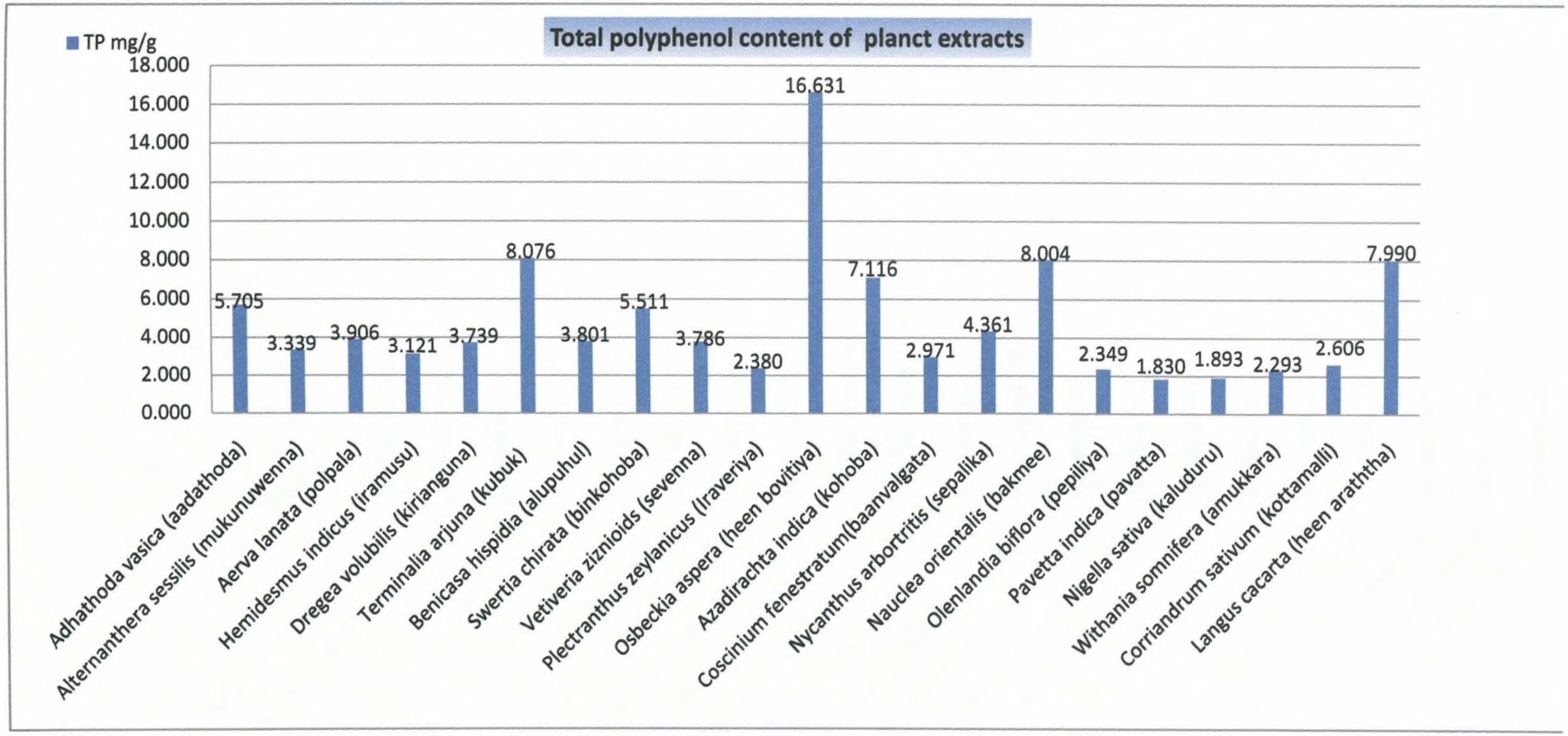


Fig 2 : Total polyphenol content of the hot water extracts of twenty one plant extracts. Results are mean of ± SD of three independent measurements. The SD varied between 0.61 – 1.7

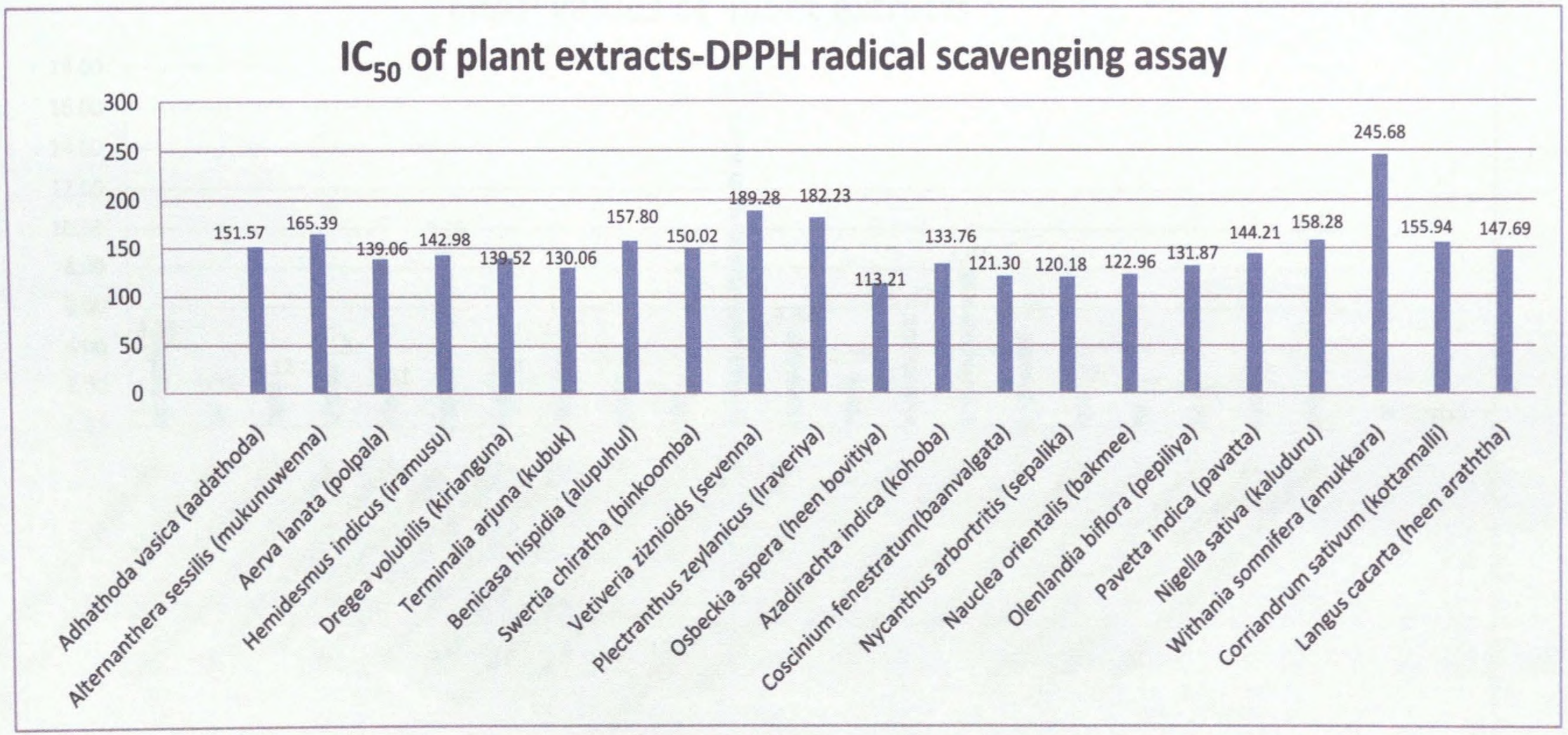


Fig 3 : Effects of hot water extracts of plants on the nitric oxide inhibition assay . IC_{50} (µg/mL) value was calculated and results are mean ± SD of three independent measurements.

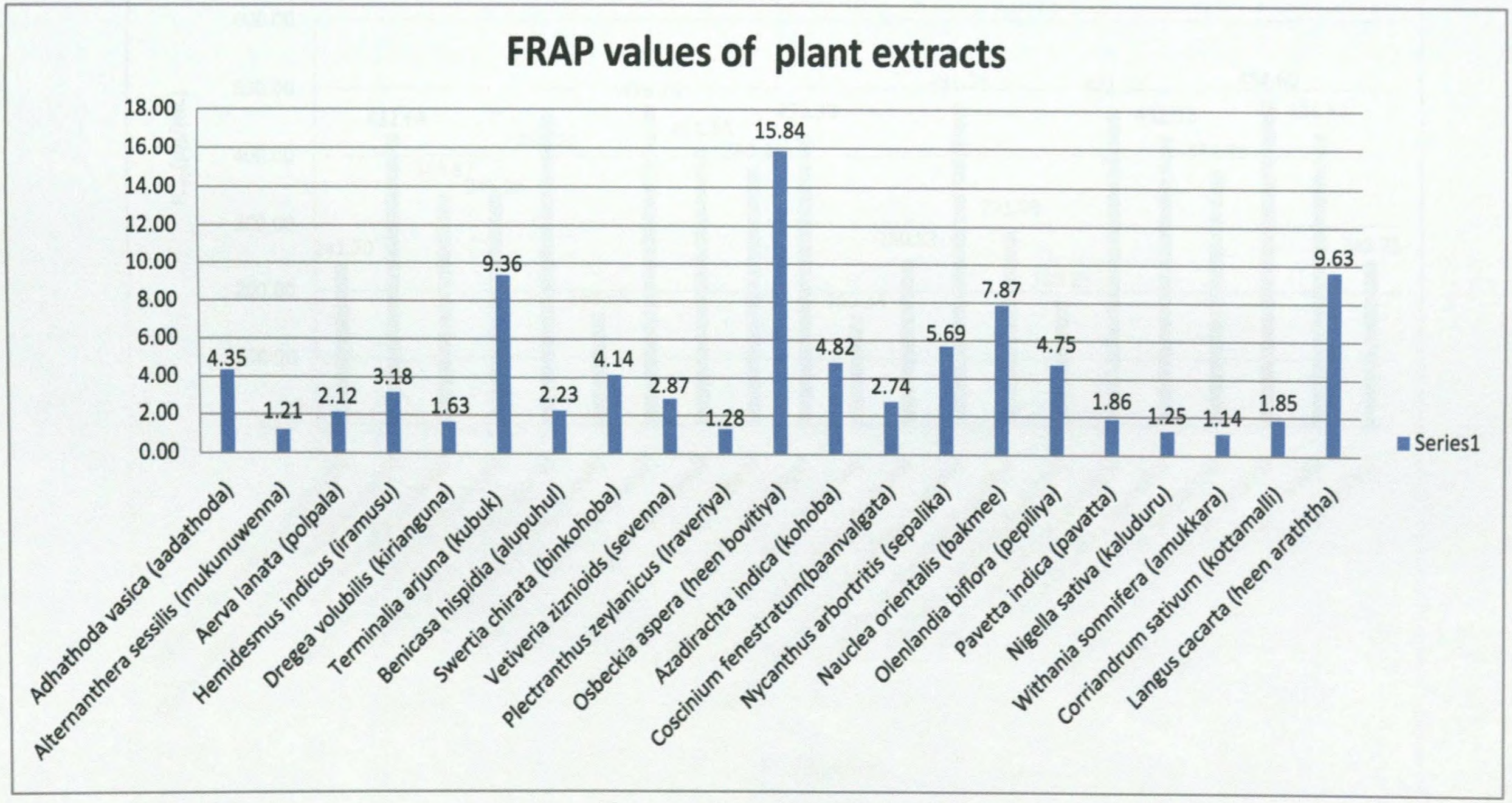


Fig 4 : FRAP values of hot water extracts of plants. The results are mean ± SD of three independent measurements. The SD varied between 0.08 – 1.7

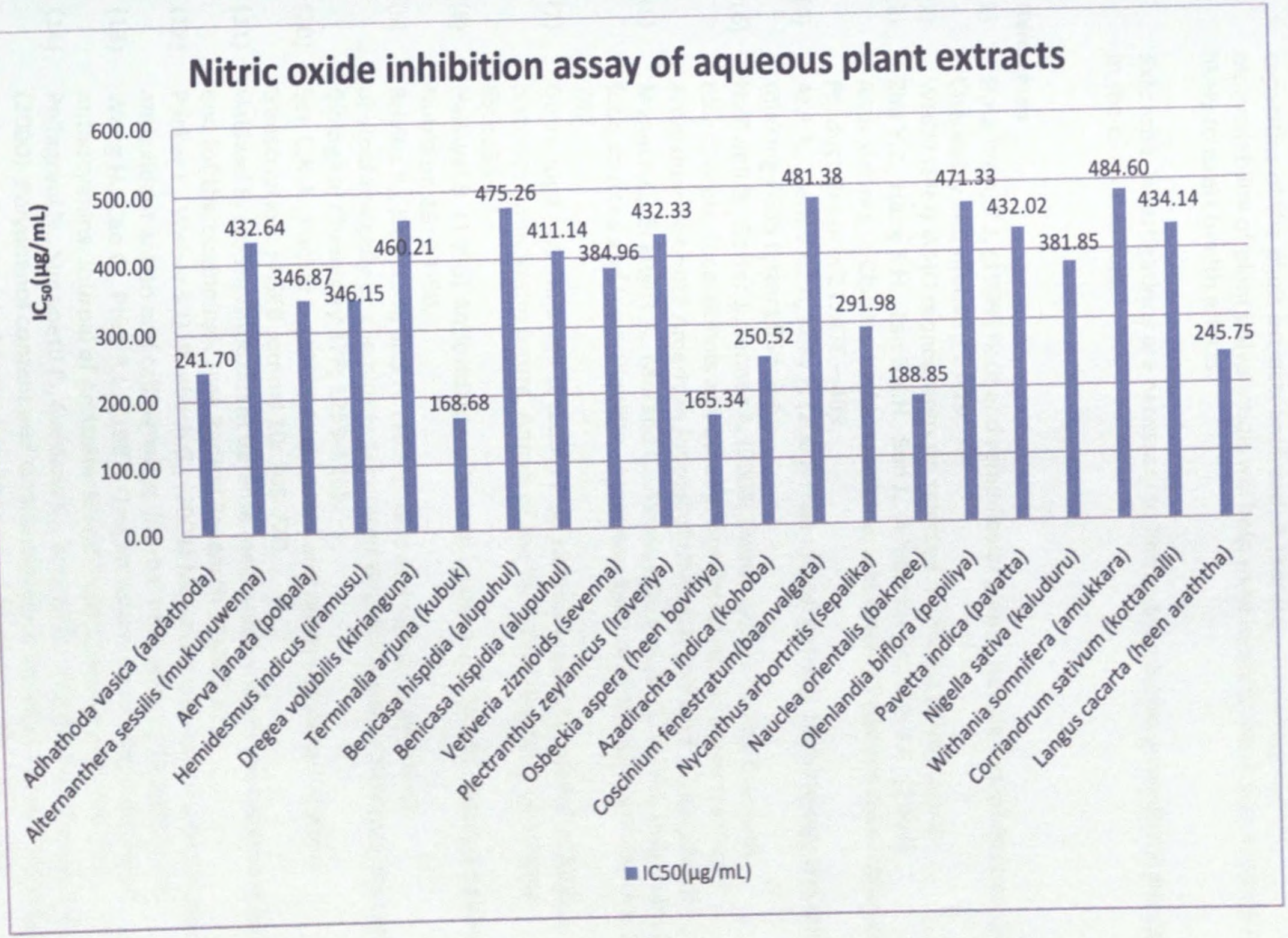


Fig 5 : Effects of hot water extracts of plants on the nitric oxide inhibition assay . IC_{50} (µg/mL) value was calculated and results are mean ± SD of three independent measurements. The SD varied between 6.17- 11.54

(vi) Conclusions

The present results suggest that the tested plant extracts have moderate to potent antioxidant activity *in vitro*. Further analysis will have to be carried out to evaluate the correlations between total polyphenol content and antioxidant capacities of the plant extracts analyzed. A thorough knowledge of the bioavailability of plant polyphenols will help us to identify those that are more likely to exert health effects.

Extensive investigations are necessary to find out the active antioxidant principles in the plant extracts.

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(viii) Problems if any, encountered during the implementation of the project

Staff shortage in the department and increased intake of students delayed the work. In addition to that I had to contribute as a coordinator of the MLS degree program commenced in 2009.

The orders placed for chemicals were not received on time.

Section 4

Impact of Research results:

i) Relevance of results achieved to scientific advancement

It provides information about the total polyphenol content and *in vitro* antioxidant activities of medicinal plants.

ii) Relevance of results achieved to national/socio-economic development

In Sri Lanka medicinal plants are natural living treasures. There are thousands that are indigenous and very few are scientifically validated for efficacy and safety.

The plant extracts studied in this investigation are used in combination or singularly in ayurvedic medicine which is becoming popular. The findings of this research will add information about the activities of the plant extracts.

Dissemination/application of research output

Medical plant use has been mostly on empirical grounds. There is need for validation of such empirical knowledge. The naturally occurring antioxidants can be formulated to give nutraceuticals which can help to prevent oxidative damage from occurring in the body.

Section 5
Miscellaneous

i) List of major equipment acquired during the project period and their functionality - none

ii) List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)
None so far

Section 6
Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and Subsistence and Miscellaneous)

Consumables

Gallic acid 100g
Sulfanilic acid 100g
Folin Ciocalteus phenol reagent
DPPH 0.5g x 2
N- -(naphthyl ethylenediamine 5g
TPTZ 0.5g
Pure Vitamin C 0.5g

Financial statement has been sent to the NSF.

Section 7

i) Grantees' signatures

Jayathlaka

Dr. K.A.P.W. Jayathlaka

ii) Comments of the Head of the Department/signature

Forwarded.

15.03.2011

Dean (As the grantee is the Head of the department)

Dean
Faculty of Medicine
University of Ruhuna
Galle.

iii) Head of the Institution's signature

18/03/2011

Acting Vice Chancellor
University of Ruhuna
Matara

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