ANTIOXIDANT ACTIVITY OF SELECTED WILD ORCHIDS OF NEPAL

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RECOMMENDATION

This is to certify that the dissertation work entitled "**Antioxidant Activity of Selected Wild Orchids of Nepal**" is submitted by Mr. Mukesh Babu Chand for the partial fulfillment of Master Degree in Botany with special paper 'Plant Biotechnonology and Biochemistry' from Tribhuvan University. The result of the present investigation was carried out by him under my supervision in the Plant Biotechnology and Biochemistry Laboratory of Central Department of Botany, Tribhuvan University, Kirtipur. Also, it has not been submitted for any other degree to the best of my knowledge. I recommend this dissertation for the final evaluation and acceptance.

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Abbreviations and Acronyms

BHT – tert-butyl 4-hydroxy toluene

CDB – Central Department of Botany

DE – Dry Extract

DEYF – Dry Extract Yield from Fresh material

DEYP – Dry Extract Yield from Powder

DNA – Deoxyribonucleic Acid

DPPH – 2, 2-Diphenyl-1-picryl hydrazyl

DP – Dry Powder

DPY – Dry Powder Yield

DW – Dry weight

EC_{50} – Half Effective Concentration

$FeCl_3$ – Ferric Chloride

FW – Fresh Weight

g – gramme

HCl – Hydrochloric Acid

H_2SO_4 – Sulfuric Acid

$HgCl_2$ – Mercuric Chloride

IBM SPSS Version 20 - International Business Machines Corporation Statistical Package for the Social Sciences Version 20

KI – Potassium Iodide

IC_{50} – Half Inhibition Concentration

m – meter

mg – milligramme

mg GAE/g – milligramme Gallic Acid Equivalent per gramme

mg QE/g – milligramme Quercetin Equivalent per gramme

mg RE/g – milligramme Rutin Equivalent per gramme

µg - microgramme

ml – milliliter

mm - millimeter

mM - millimolar

Mol. Wt. – Molecular Weight

$NaHCO_3$ – Sodium Bicarbonate

NAST – Nepal Academy of Science and Technology

nm – Nanometer

TFC – Total Flavonoids Content

TPC – Total Polyphenolics Content

TU – Tribhuvan University

UV – Ultraviolet

VDC – Village Development Committee

w/v – Weight by volume

Abstract

The inhibitory or delaying action of both the synthetic chemicals and naturally occurring phytochemicals against oxidative damage to tissues by free radicals produced in biological system of living organisms is known as antioxidant activity. Since some phytochemicals are responsible for biological as well as medicinal activities, nine wild orchids of Nepal were assessed for total polyphenolics and flavonoids content along with the antioxidant activity. The ethanolic extract of *Eria graminifolia* pseudobulbs, *Gastrochilus acutifolius* leaf and root, *G. distichus* whole plant, *Luisia trichorhiza* leaf and root, *Otochilus albus* pseudobulbs, *Papillionanthe uniflora* whole plant, *Pholidota articulata* leaf and pseudobulbs, *Rhynchostylis retusa* leaf, and *Trudelia cristata* leaf and stem were prepared by Soxhlet extraction. Phytochemicals were detected by previously established protocols with minor modifications. The total flavonoids were estimated with aluminium chloride method and total polyphenolics content with Folin-Ciocalteu phenol reagent method. Antioxidant activity was assessed by DPPH (2, 2-diphenyl-1-picryl hydrazyl) free radical scavenging assay. There was significant variation of total flavonoids, total polyphenolics content and antioxidant activity among the orchid extracts at $P = 0.05$. The total flavonoids varied with highest in *Rhynchostylis retusa* leaf (110.68 ± 4.52 mg QE/g) and lowest content in *Gastrochilus acutifolius* root (22.32 ± 1.10 mg QE/g); total polyphenolics with highest in *Trudelia cristata* stem (69.68±2.78 mg GAE/g) and lowest content in *Gastrochilus acutifolius* leaves (11.89±0.64 mg GAE/g). Also, the antioxidant activity varied with highest in *Trudelia cristata* stem (IC_{50} 79.69 μg/ml) and lowest DPPH radical scavenging activity in *Gastrochilus acutifolius* leaf (IC_{50} 341.79 μg/ml). However, none of the orchid extracts were as effective as quercetin – the reference compound – in radical scavenging activity (IC_{50} 32.90 μg/ml). Total polyphenolics and flavonoids content and antioxidant activity of selected orchid extracts in this study were higher or lower than medicinal plant and orchid extracts of previous studies with considerable margin. Again, their antioxidant activity was positively associated with total flavonoids and total polyphenolics content. Hence, this study claims that the ethanol extract of selected wild orchids perform significantly varying antioxidant activity with further possibilities of pharmacognistical and pharmacological studies.

CHAPTER ONE: INTRODUCTION

1.1.Background

1.1.1. Antioxidants and antioxidant activity

Some chemicals inhibit or delay the oxidizing activity of free radicals generated in biological system of living organism as by-products viz. superoxide anion, hydroxyl radicals and hydrogen peroxide on biomolecules. Such chemicals are known as antioxidants and their inhibitory or delaying action against oxidative damage to tissues is known as antioxidant activity. Basically, antioxidants are of two categories i.e., synthetic and natural. Generally, synthetic antioxidants are compounds with phenolic structures of alkyl substitution while natural antioxidants are phenolic compounds such as tocopherols, flavonoids, phenolic acids etc., (Velioglu *et al*. 1998; Zhao *et al*. 2006).

Plants produce a diverse array of phytochemicals which are grouped as terpenoids, alkaloids, steroids, glycosides and phenolics on the basis of biosynthetic origin. Among these phytochemicals, plant phenolics often said as polyphenols that possess one or more acidic phenolic hydroxyl groups protect biological system from oxidative stress and function as antioxidants. The major classes of plant phenolics are the hydroxycinnamic acids, flavonoids, anthocyanins and tannins. These are found in all higher plants, often at high levels (Grace 2005).

Polyphenols are commonly found in both the edible and nonedible plants. Mainly, they are found in flowering tissues, leaves, stems and barks, berries and fruits (Kiselova *et al*. 2006). Natural antioxidants especially flavonoids show multiple biological effects including antioxidant activity, antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory actions (Velioglu *et al.* 1998).

Various medicinal plants that have been used to treat human diseases in the different parts of the world for centuries are found with singnificant amount of phenolics and level of antioxidant activity (Cai *et al.* 2003; Li *et al.* 2008; Roy *et al.* 2011; Awah *et al.* 2013). They are also shown with significant activities in anti-inflammatory, anti-tumor, anti-

allergic, anti-viral and antibacterial assays. Such biological effects could be partly attributed to their antioxidant and free radical scavenging activities (Li *et al.* 2008).

1.1.2. Preliminary phytochemical screening

Since the plants accumulate enormous variety of organic compounds the screening of the biologically active phytochemicals is primary step for phytochemical and pharmacological studies. Phytochemicals are detected in a homogenised plant material adopting rapid and accurate method of screening. Such rapid and accurate methods of detection perform the test for the presence of alkaloids, glycosides, flavonoids, polyphenols, proteins, and saponins etc. (Habourne 1973). So, the phytochemical screening gives prior hint for the presence of biologically active phytochemical constituents.

1.1.3. Determining plant phenolics content and antioxidant activity

As antioxidant activity of plant extracts is attributed to biological and medicinal properties its determination along with plant phenolics content is of prime importance. So antioxidant activity and amount of plant phenolics are determined by numbers of methods. However, total flavonoids content determination by aluminium chloride spectrophotometric method, total polyphenol content by Folin-Ciocalteu reagent method, and antioxidant activity by DPPH free radical scavenging assay are rapid and effective methods (Chang *et al.* 2002; Koleva *et al*. 2002; Roy *et al*. 2011).

The aluminium chloride forms acid stable complexes with keto group and hydroxyl group of flavones and flavols. Again, it forms acid labile complexes with the ortho-dihydroxyl groups in the rings of flavonoids. So, these aluminium chloride complexes of flavones, flavonols, and flavonoids show maximum absorbance at 415 nm (Chang *et al*. 2002). This colour intensity of aluminium chloride complex is utilized to determine the total flavonoids content in a particular extract.

Folin-Ciocalteu reagent is a yellow acidic solution with complex polymeric ions formed by phosphomolybdic and phosphotungstic heteropoly acids. This reagent is reduced by phenolate anion from phenolics in a basic medium to form blue-coloured molybdenum

oxide. The intensity of blue colouration is directly proportional to the total phenolic contents (Awah *et al*. 2012). Again, this direct proportionality of blue colour with total polyphenolics content is emplyoed to determine total polyphenolics content in a given extract.

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) is a purple coloured stable nitrogen radical; its solution in methanol or ethanol exhibits highest absorbance at 515 to 520 nm. Specific compounds or extracts containing antioxidant (AH) when allowed to react alcoholic $DPPH^{\bullet}$ (i.e., DPPH radical in ethanol or methanol solution) then yellow coloured 2,2 – diphenyl-1-picryl hydrazine or non-radical form DPPH-H is formed as in the equation below and decrease in absorbance occurs. This phenomenon of decrease in absorbance of alcoholic $DPPH^{\bullet}$ is applied to determine radical scavenging or antioxidant activity of specific compound or extract (Koleva *et al*. 2002).

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

1.2. Wild Orchids and their medicinal properties

Orchids are monocotyledonous plants belonging to family Orchidaceae. They are of diverse importance including medicinal value with vast majority in number. In Nepal, orchids are recorded with 107 genera and 450 species. Among these 450 species of orchids over 100 species are used to cure varoius diseases and ailments in Nepal. Different organs viz., leaf, stem, pseudobulbs, bulbs, rhizome, tuber, root and whole plant of Nepalese orchids are reported to heal wound, rheumatism, tuberculosis, arthritis, nervous disorders, nervous debility, fever, diarrhea, dysentery, fracture, dislocated bones, headache, backache, bodyache, stomachache, and muscular pain; also they are aphrodisiac, blood purifier, emetic, congenity enhancer, tonic, and vermifuge (Rajbhandari 2014). In this study, some of the orchid species have been selected for preliminary phytochemical screening, total polyphenols and flavonoids content estimation and antioxidant activity assay.

1.3. Hypothesis

Different plant species have various phytochemicals in varied amount, so the orchids show variation in total flavonoids content, total polyphenolics content and antioxidant activity.

1.4. Objectives

A. General objective

- To evaluate the antioxidant activity of selected wild orchids of Nepal

B. Specific objectives

- To know the presence or abscence of selected phytochemical classes;
- To estimate the total polyphenol and total flavonoids content;
- To determine the antioxidant activity of selected orchids;

1.5. Rationale of the study

Orchids are known to cure some diseases and ailments in human beings and animals since long ago; also, they are valuable to Nepalese due to their aesthetic, food, and medicinal properties. Some of the selected orchids are medicinal in folklore medicine; others may show biological activity. Several studies have been done regarding their ethnomedicinal uses, conservation status, few phytochemical studies, and fewer biological properties. However, the knowledge and information of pharmacological and biological properties of Nepalese orchids is still obscure. This study may provide information about phytochemical classes, pharmacognostic properties and biological activity of selected wild orchids of Nepal. Further, the antioxidant activity assessment on selected medicinal orchids will provide further validation of their medicinal importance. Moreover, study on randomly selected species will open a way further in research for their biological activity.

1.6. Limitations of the study

The plant materials are collected on the basis of their uses in folklore medicine and their availability in natural populations considering their minimum impact on natural population. The solvent used in extraction is only ethanol. So the tested extracts may not have wide ranges of phytochemicals that can be extracted using different solvents viz., acetone, chloroform, and hexane etc., on a same material. Since the preliminary phytochemical screening, total flavonoids content estimation, total polyphenols content estimation and antioxidant activity assay belong to plant biochemistry, pharmacognosy, and pharmacology the study may cover only a field of plant biochemistry, pharmacognosy, and pharmacology.

CHAPTER TWO: LITERATURE REVIEW

2. 1. Phytochemical constituents

Previous studies showed numerous plant species including orchids with varieties of phytochemical classes. Maridass *et al.* (2008) carried out phytochemical survey on 61 orchid species of south India. They did test for the presence of flavonoids, reducing sugars, cyanogenic glycosides, terpenoids, and tannins. The chemical test on methanolic extract of *Dendrobium aqueum*, *D. barbatulum*, *D. didon*, *D. herbaceum*, *D. heterocarpum*, *D. macrostachyum*, *D. microbulbon*, *D. nanum*, *D. nutans*, *D. panduratum*, *D. waghtii*, *Eria muscicola*, *E. nana*, *E. reticosa*, *Gastrochilus acaulis*, *Luisia zeylanica*, *Papilionanthe subulata*, *Vanda tessellata*, *Vanda testacea* showed the presence of flavonoids in all above mentioned species; presence of reducing sugars in *D. barbatulum*, *D. herbaceum*, *D. heterocarpum*, *D. heyneanum*, *D. microbulbon*, *D. nanum*, *D. panduratum*, *D. wightii*, *E. muscicola*, *G. acaulis*, *L. zeylanica*, *P. subulata*, *V. testacea*; absence of cyanogenic glycosides in *D. macrostachyum*, *E. reticosa*, *Vanda testacea*; presence of terpenoids in *V. tessellata*, *V. testacea*,; absence of tannins in *D. barbatulum*, *D. heterocarpum*, *D. macrostachyum*, *D. nutans*, and *D. wightii*.

An epiphytic orchid *Vanda tessellata* (Roxb.) Hook. Ex Don contains glycoside (melianin) and complex withanolide, alkaloids, glucosides, bitter principle, tannins, resins, saponins, sitosterols, colouring matters (Singh 2009).

Phytochemical screening on various orhchid species have been done for the preliminary assessment of phytochemicals. The qualitative phytochemical analysis by Shanmugavalli *et al.* (2009) on petroleum ether, benzene, chloroform, and ethanol extracts of leaf and stem of *Vanilla planifolia* showed the presence of alkaloids, anthraquinone glycosides, flavonoids, steroids, and terpenoids. However, catachins, sapnonis, and tannins are absence in all extracts; anthraquinone glycosides and terpenoids are absence in stem extracts.

The mixture of from petroleum ether extract, ethyl acetate extract, acetone extract, benzene extract, and methanol extract of *Papilionenthes teres* (Roxb.) Schltr. stem was

screened for the phytochemical constituents by Mazumder *et al.* (2010). They found the presence of alkaloids, reducing sugars, flavonoids, steroids, saponins, and tannins.

The preliminary phytochemical screening by Gurucharan *et al.* (2012) on petroleum ether, chloroform and ethanol extract of orchid *Acampe praemorsa* showed the presence of alkaloids, steroids and terpenoids in all solvent extracts. However, flavonoids are presence in chloroform and ethanol extract and tannins in petroleum ether and chloroform extracts.

The preliminary phytochemical analysis on methanol extract of *Satyrium nepalense* tubers by Mishra *et al.* (2012) showed the presence of alkaloids, carbohydrates and glycosides, flavonoids, unsaturated sterols and triterpenes but absence of resin, saponins, and tannins.

The tuber part of *Dactylorhiza hatagirea* was detected with glycosides, bitter substances, starch, mucilage, albumen, a trace of volatile oil and ash (Pant *et al.* 2012). *Dendrobium macrostachyum* was found with alkaloids, flavonoids, glycosides, sterols, tannins, and phenols in a study by Nimisha *et al.* (2012). The ethyl acetate extract of *Eria pseudoclavicaulis* which was again extracted by ethanol and water was found to contain maximum phytochemical constituents, viz. alkaloids, carbohydrates, phytosterols, phenolic compounds, saponins, terpenoids, and tannins in a preliminary phytochemical screening by Sahaya *et al.* (2012). Kurapa *et. al.* (2012) did qualitative phytochemical analysis of aquous, ethanolic, and methanolic extract of whole plant of *Eulophia nuda* Lind. Their result showed the presence of alkaloids, cardiac glycosides, steroids, terpenoids in both the ethanolic and methanolic extracts; flavonoids in methanolic and ethanolic extracts; saponins in aquous extract only. However, tannins and phlobatanins were absent in all the extracts. The hexane, chloroform, and methanol extracts of *Cymbidium aloifolium* leaf contain tannins, alkaloids, triterpenoids, caumarins, flavones, flavonines, carbohydrates, amino acids, and proteins (Radhika *et al.* 2013).

In a phytochemical evaluation of different extracts of pseudobulb and stem of *Flickingeria nodosa* (Dalz.) Seidenf by Nagananda *et al.* (2013) showed the presence of alkaloids, phytosterols, phenolics, and flavonoids. Flavonoids and Phenolics were found

in all the petroleum ether, chloroform, acetone, ethanol and water extracts prepared by cold and Soxhlet extraction. Alkaloids were found in all the extracts except ethanol and water extracts from both extraction process. Phytosterols were detected in all extracts except ethanol and water extracts. Glycosides and saponins were not found in all extracts.

So, the orchids contain alkaloids, amino acids, anthraquinones, bitter principle, carbohydrates, caumarins, colouring matters, flavanones, flavonoids, glucosides, proteins, resins, saponins, sitosterols, tannins, triterpenoids, steroids and terpenoids as phytochemical constituents.

2. 2. Total polyphenols and total flavonoids contents

Numbers of plants including orchids were estimated with varying amount total polyphenols and flavonoids content in previous studies. Here, the reports on the estimation of total polyphenols and flavonoids contents are mainly focused on few orchids from different countries due to lesser availability of literatures and Nepalese medicinal plants only.

Ghimire *et al.* (2011) determined total flavonoids contents with 13.53 ± 0.85 (in in *Withania somnifera* leaf) to 100.33 ± 1.53 mg QE/ g extract (in *Artemisia vulgaris* leaf) of total flavonoids and 23.80 ± 1.11 (in *Drymaria cordata* leaf) to 321.23 ± 1.06 mg GAE/g extract (in *Artemisia vulgaris* leaf) of total phenols in root, bark, leaf, fruit and seed of 24 different plants belonging different families.

Some Nepalese medicinal plants were estimated with total phenolic contents of 0.4 (in *Fritillaria delavayi*) to 10.9 mg GAE/100 g DW (in *Rhododendron anthopogon*) Maharjan *et al.* (2013) as lowest and lowest contents respectively among the selected plant extracts.

Methanolic extracts of *Terminalia bellirica* fruits*, T. chebula* fruits*, Phyllanthus emblica* fruits*, Bergnia ciliata* rhizome*, Adhatoda vasica* leaf*,* and *Vitex negundo* leaf which are medicinally used in Nepal were estimated with 109.512 ±9.589 to 304.00 ±18.180 mg/g

GAE of total phenolic content. Also, total phenolic content and IC_{50} of selected extracts were found with negative association with R^2 value 0.999 (Genwali *et al.* 2013).

Methanol extract of *Dendrobium speciosum* leaf and stem were found to contain good percentage of total polyphenols i. e., 1.15 ± 0.10 % and 1.06 ± 0.12 %, respectively and flavonoids content i.e., 0.21 ± 0.08% and 0.12 ± 0.07% respectively (Moretti *et al.* 2013).

Mythili (2014) found total phenol content with 12.3± 0.31 (in petrolium ether extract) and 29.43±0.31 (in ethyl acetate extract) mg of GAE/ g of extract as lowest and highest content in different extracts of *Calanthe triplicata* - an orchid. However, it was estimated with 23.53±0.25 mg GAE/ g of extract in methanolic extract. Again, the total flavonoids contents were estimated with 90.24±0.04 mg QE/ g of extract as highest in ethyl acetate extract and 38.93±0.05 mg QE/ g of extract in chloroform extract.

Methanolic extracts of *Ageratum conizoides* whole plant, *Alium cepa* outer scales, *Elaeocarpus shpaericus* leaves, *Ficus benghalensis* leaves and *Ipomea carnea* leaves which are medicinally important in Nepal were determined with total phenolic and total flavonoids content. Among them, *Ageratum conizoides* whole plant was estimated with lowest (12.7±2.24 mg QE/g) while *Ficus benghalensis* leaves was determined with highest (78.2±2.71 mg QE/g) total flavonoids contents. Still, the *Elaeocarpus shpaericus* leaves was obtained with highest (247.6±3.91 mg GAE/g) but *Ipomea carnea* leaves with lowest (47.0±3.93 mg GAE/g) total phenolic contents. Also, the IC50 value for the DPPH radical scavenging activity was negatively associated with total phenolic contents with R^2 value 0.931 (Pandey *et al.* 2014).

Methanol extract of different plant parts viz., petals, sepals, androecium, gynoecium, flowers, twigs, leaves, bark and stem of *Rhododendron arboreum* - the national flower in Nepal and medicinally valuable plant - were estimated with total phenolic and flavonoids contents. Leaves were estimated with 495.0±8.66 mg GAE/g as highest and stem with 187.0±8.29 mg GAE/g as lowest total phenolic contents while sepals were determined with 17.2±4.18 mg QE/g and leaves were with 150.0±0.00 mg QE/g of total flavonoids

content. Also, the the IC_{50} value for the DPPH radical scavenging activity were negatively associated with total phenolic and flavonoids contents with R^2 value 0.923 and 0.9653 respectively (Bhandari *et al.* 2014).

Previous studies show that the total polyphenolic contents can be present from 0.4 mg GAE/100 g DW to 495.0±8.66 mg GAE/g and total flavonoids content from 12.7±2.24 mg QE/g to 150.0±0.00 mg QE/g in different extracts of Nepalese medicinal plants. Also, the total polyphenolic in same extracts are present in more amount than the total flavonoids content.

2. 3. Antioxidant activity by DPPH assay

Different plant and orchid extracts in previous studies were estimated with antioxidant activity of varied percentage scavenging activity and half inhibition concentration (IC_{50}). Štajner *et al.* (2010) assessed the absolute ethanol extract of flowers and above ground parts of *Anacamptis pyramidalis* L. for the antioxidant and radical scavenging capacity by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. The percentage scavenging activity by flowers and above ground parts was (32.82 ± 1.11) % and (54.16 ± 2.51) % respectively.

Mukherjee *et al.* (2012) on the aquous extracts of *Dendrobium aqueum* for the antioxidant activity using DPPH free radical scavenging assay showed the increase in the percentage free radical scavenging potential in a dose dependent manner with highest activity of 49% at a dose of 100µg/ml.

Sahaya *et al.* (2013) examined antioxidant activity by DPPH free radical scavenging assay and FRAP on petroleum ether, chloroform, ethyl acetate, ethanol, and ether extracts of *Coelogyne nervosa* leaf. They observed the best DPPH free radical scavenging activity in the aqueous extract (IC_{50} value 126 µg/ml), followed by the ethanol and ethyl acetate extract (IC50 value 206µg/mL and 312µg/ml) respectively.

Radhika *et al.* (2013) assessed antioxidant activity assay on methanolic extract of *Rhynchostylis retusa* and *Cymbidium aloifolium* using different assays including DPPH

free radical scavenging assay. They observed higher percentage inhibition of DPPH free radical in *Rhynchostylis retusa* (84.657 %) than that of *Cymbidium aloifolium* (82.514 %).

Moretti *et al.* (2013) carried out the DPPH free radical scavenging assay on methanolic extract of leaves and stem of *Dendrobium speciosum*. They obtained IC_{50} value of leaf and stem extracts 0.026±0.004 mg/mL and 1.054±0.047 mg/ml as respectively.

Nagananda *et al.* (2013) performed the DPPH radical scavenging assay on ten different extracts (prepared by cold and hot successive extraction using petrolium ether, chloroform, acetone, ethanol and water as solvent) of pseudobulb stems extract of *Flickingeria nodosa.* They observed IC_{50} value of 1083.88 µg/ml and 46.49 µg/ml for hot successive extraction by water and cold successive extraction by acetone as highest and lowest respectively.

The aqueous, methanol, aqueous-methanol and acetone extracts of *Eulophia nuda* tubers showed DPPH radical scavenging activity. The aqueous-methanol extract showed maximum scavenging (87%) at 1 mg/ml concentration (Kumar *et al.* 2013).

Different extracts of medicinal plants of Nepal - *Terminalia chebula* fruits, *T. bellirica* fruits, and *Berginia ciliata* rhizome were estimated with IC_{50} 109.78, 213.11, 21.11µg/ml respectively for antioxidant activity (Genwali *et al*. 2013).

The methanol extracts of bark, stem, twigs, leaves, flowers, and petals of *Rhododendron arboreum* were estimated with IC_{50} value 65.45, 67.83, 46.02, 8.34, 25.15, and 16.83µg/ml respectively (Bhandari *et al*. 2014).

Methanolic extracts of different medicinal plant parts *Ageratum conizoides* whole plant, *Elaeocarpus sphaericus* leaves, *Ficus benghalensis* leaves, and *Ipoemea carnea* leaves were estimated with IC_{50} value 312, 34, 135, 238 µg/ml respectively (Pandey *et al*. 2014).

Based on previous studies it can be noticed that the IC_{50} value of DPPH free radical scavenging varies from 0.026 mg/ml to 1.083 mg/ml; scavenging rate differes from 32.82 % to 87 % in orchid extracts. In other plant extracts, the IC_{50} value varies from 8.34 to 312 µg/ml. Also, the IC_{50} is negatively associated with total flavonoids content and total phenolic content.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Materials

3.1.1. Selected wild orchids

3.1.1.1. *Eria graminifolia* Lindl.

Eria graminifolia Lindl. is an epiphytic orchid. It has cylindrical pseudobulbs and oblong-lanceolate leaves with acuminate apex. The inflorescence bearing white flowers with yellow spotted lip arises near the apex of pseudbulb. Its flowering time is June to July. It grows on tree trunks of cool places and prefers partially shaded areas. It occurs in the subtropical forest at altitudes of 1500 – 2000 m. In Nepal, it is found in eastern and central part (Raskoti 2009).

The *Eria graminifolia* has not been reported for medicinal properties yet.

3.1.1.2. *Gastrochilus acutifolius* (Lindl.) Kuntz

The *Gastrochilus acutifolius* (Lindl.) Kuntz, an epiphytic orchid has slender stem covered by leaf sheaths. Leaves are leathery, oblong-lanceolate with acute apex. Axillary, corymbose inflorescence has dense, numerous, dull green flowers with yellow centered and reddish purple spotted lip. It blooms in October-December. It is semipendulous on mossy trunks or main branches of tree growing in warm to cool places. It occurs in the subtropical forest at altitudes of 1200-2000 meters. It is distributed through the central and western Nepal, Bhutan, and India. It is distributed in central Nepal (Raskoti 2009).

The *Gastrochilus acutifolius* has also not been reported for medicinal value yet.

3.1.1.3. *Gastrochilus distichus* (Lindl.) Kuntze

The *Gastrochilus distichus* (Lindley) Kuntze is an epiphytic orchid with pendulous, slender, often branched stems. The leaves are many, distichous; have lanceolate or falcate-lanceolate blade with acute apex and 2 or 3 awns. Inflorescences are several, opposite to leaves, subumbellate, and with 2-4 pale green flowers having reddish brown spots. Its flowering time is January-May. It grows on tree trunks in forests of 1100-2800

m altitude. It is distributed in Bhutan, China, Northern East India, and Nepal (Chen *et al.* 2009). It is distributed in eastern and central Nepal (Raskoti 2009).

The *Gastrochilus distichus* has not been reported reported for medicinal use yet.

3.1.1.4. *Luisia trichorhiza* (Hook.) Bl.

The *Luisia trichorhiza* (Hook.) Bl. is also an epiphytic orchid with 10-25 cm tall stem. It has terete, fleshy leaves with tapered apex. Spike has broad, acuminate stout floral bracts and 4 - 5 flowers possessing pale green sepals lined with faint purple lines and dark purple tip. It blooms in May. It grows trunks or major branches of tree in hot to warm regions but likes bright light. Its habitat is the subtropical open forest at altitudes of 1000-1400 meters. It is distributed in eastern and central Nepal, Bhutan, India, Myanmar, and Thailand (Raskoti 2009).

Paste made from *Luisia trichorhiza* leaves is used for the treatment of chronic wound and leaf paste is applied externally in case of muscular pain (Raskoti 2009). Some ethnic groups of India viz. *Boxas*, *Tharus*, *Jaunsaris*, and *Rhajis* use it to treat bone fracture of cattles (Pande *et al.* 2007); to cure jaundice, and as anti-diarrhoea for cattles (Dash *et al.* 2008).

3.1.1.5. *Otochilus albus* Lindley

The *Otochilus albus* Lindley, an epiphytic orchid possesses pseudobulbs which are enclosed in tubular sheaths when they are young, subcyindrical, grooved, and are usually with roots at joint. Leaves are petiolate, narrowly oblong or narrowly elliptic, and acuminate. Synanthus inflorescence has long sheathed peduncle. Rachis is slender, weakly zigzag; with pedicellate, uniformly white, laxly arranged 8 – 9 flowers and has caduceus, ovate – lanceolate floral bracts. The flowering occurs in June to July (Chen and Wood 2009). It is distributed in the range of 1300 – 1500 m in China, India, Myanmar, Nepal, Thailand, and Vietnam. In Nepal, it is found through out the country (Raskoti 2009).

The pseudobulbs of *Otochilus albus* are used in fracture (Raskoti 2009).

3.1.1.6. *Papilionanthe uniflora* (Lindl.) Lindl.

The *Papilionanthe uniflora* (Lindl.) Lindl is also an epiphytic orchid having slender, pendulous stem and terete leaves with acuminate apex. It has axillary inflorescence with 1-3 white flowers having pink flushed spur. It blooms in September.It grows on major branches of mossy tree, cool growing, likes air exposed areas and bright light. It thrives in cloudy wet temperate forest at altitudes of 2000-2400 meters. It is distributed in central Nepal, Bhutan, and India (Raskoti 2009).

The *Papilionanthe uniflora* has not been reported reported for medicinal use yet.

3.1.1.7. *Pholidota articulata* Lindl.

The *Pholidota articulata* Lindl., has stemlike, subcylindric, pseudobulbs connected to each other at both ends. Pseudobulbs are slightly narrowed and branched sometimes, with very short rhizomes between them and a few roots. Two leaves which are petiolate, obovate-elliptic, oblong, or narrowly elliptic with plicate veins and subacute or obtuse apex present at the apex of new pseudobulb. Nearly flexuous rachis with 10 or more flowers of greenish white or white and slightly tinged with red colour lies at the apex of new pseudobulb as an inflorescence. Its flowering time is June-August and fruiting time is October – December. It is epiphytic on trees in forests and on shaded rocks covered by adequate organic matters. It occurs in 800-2500 m altitude. It is distributed in Bhutan, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, central Nepal, Thailand, and Vietnam (Chen and Wood 2009a).

The whole plant of *Pholidota articulata* is used as tonic; juice berries is used to treat skin ulcers and skin eruptions; the paste from the pseudobulb is applied on dislocated bones (Pant and Raskoti 2013).

3.1.1.8. *Rhynchostylis retusa* (L.) Bl.

The *Rhynchostylis retusa* (L.) Bl. is also an epiphytic orchid with ascending stems enclosed in leaf sheaths. Leaves are broadly lorate, unequally bilobed. Densely many flowered 1-3 pendulous inflorescences bear thick rachis with reflexed and broadly ovate

floral bracts. Its flowering time is May-June and fruiting time June-July. It thrives on tree trunks in open forests or at forest margins of 300-1500 m altitude. It is distributed in Bhutan, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Philippines, Sri Lanka, Thailand, and Vietnam (Chen and Wood 2009a). In Nepal, it is distributed throught out the country.

The leaves of *Rhynchostylis retusa* are used to treat rheumatism and root juice is applied to cuts and wounds (Pant and Raskoti 2013). It is used as emollient; paste made of its root with leaf buds of *Pisum sativum* is taken orally on an empty stomach to cure bloody dysentery; the leaf paste is applied externally to cure wounds (Dash *et al.* 2008).

3.1.1.9. *Trudelia cristata* (Lindl.) Senghas

The *Trudelia cristata* (Lindl.) Senghas has oblong, leathery leaves with unequally bifid apex. Axillary inflorescence has rachis with 2 – 6 yellowish-green flowers with brown red striped golden yellow lip and ovate and obtuse floral bracts. It blooms in March-May. It is epiphytic on mossy trunks or large branches of tree. It grows from warm to cool places and like bright light. It occurs in the subtropical and temperate forest at altitudes of 1200 – 2300 meters. It is distributed in Nepal, Bhutan, China, and India. In Nepal, it is found through out the country (Raskoti 2010).

The root paste of *Trudelia cristata* is applied in cuts, wounds, boils and dislocated bones (Pant and Raskoti 2013) and leaf powder used as expectorant, paste applied to cuts and wounds (Subedi *et al.* 2013).

3.1.2. Chemicals used

The chloroform used for Salkovaski test and Ferric chloride (96 %) were from s. d. Fine-Chem Limited, India. The ethanol used in extraction was bought from Bengal Chemicals, India while it was from Hangsu Kangyuan Chemicals Co. Ltd., China for phytochemical screening, total polyphenols and flavonoids content estimation and antioxidant activity assay. Glacial acetic acid was from E. Merck (India) Limited, India; Hydrochloric acid and Aluminium chloride from Thermo Fisher Scientific India Pvt. Ltd.; Gallic acid from Moly Chem, Mumbai; Mercuric chloride, Sodium bicarbonate, and Sulfuric acid (97 %)

from Qualigens Fine Chemicals; Methanol from MERCK India ; Potassium Iodide from Ranbaxy India; Follin-Ciocalteu reagent was brought from NIKE CHEMICALS INDIA. Quercetin and DPPH (2, 2-diphenyl -1 - picrylhydrazyl radical) were obtained from HI MEDIA Pvt. Ltd. India.

3.1.3. Orchid samples

The orchid extracts were *Eria graminifolia* pseudobulbs (**Egp**), *Gastrochilus acutifolius* leaf (**Gal**) and root (**Gar**), *Gastrochilus distichus* whole plant (**Gdw**), *Luisia trichorhiza* Leaf (**Ltl**) and root (**Ltr**), *Otochilus albus* pseudobulbs (**Oap**), *Papillionanthe uniflora* whole plant (**Puw**), *Pholidota articulata* leaf (**Pal**) and pseudobulbs (**Pap**), *Rhynchostylis retusa* leaf (**Rrl**), and *Trudelia cristata* leaf (**Tcl**) and stem (**Tcs**).

3.1.4. Glassware and instruments

All the glassware including test-tubes, beakers, conical flasks, funnel, and Soxhlet apparatus were products of Borosil Glass Works Ltd. India and extraction thimble was Whatman thimble. The rotary evaporator was Rotavapor-BüChi (Made in Switzerland) and UV-Visible spectrophotometer was Thermo Fisher Scientific, Genesystem-10.5 (Made in India).

3.2. Methods

3.2.1. Collection and identification of plant materials

The plant samples of orchid species were collected from Kathmandu, Makawanpur and Salyan districts of Nepal during the October, April, June, August, and September (when they bear matured fruits) of 2012 and 2013. The whole plant of each orchid species was collected for investigation. The orchid species used for present study are *Eria graminifolia* Lindl., *Gastrochilus acutifolius* (Lindl.) Kuntz, *Gastrochilus distichus* (Lindl.) Kuntz, *Luisia trichorhiza* (Hook.) Bl., *Otochilus albus* Lindley, *Papillionanthe uniflora* (Lindl.) Lindl., *Pholidota articulata* Lindl., *Rhynchostylis retusa* (L.) Bl., and *Trudelia tristata* (Lindl.) Sehghas.

The *Rhynchostylis retusa* (L.) Bl. and *Pholidota articulata* Lindl., growing on *Prunus cerasoides* D. Don were collected from Central Department of Botany, Tribhuvan University Garden (1360 m). The support trees were getting direct sunlight from morning to noon during collection period.

The *Luisia trichorhiza* (Hook.) Bl. and *Trudelia tristata* (Lindl.) Sehghas were collected from mixed broadleaved forests of Darmakot VDC, Salyan (1400 m). They were epiphytic on *Castanopsis indica* tree which was growing on south west facing hill. Although there was not falling direct sunlight but they were exposing to plenty of light during noon to evening. There were some lichens and mosses growing on the surface of tree.

The *Gastrochilus acutifolius* (Lindl.) Kuntz which was growing on *Olea ferruginea* Royle and *Otochilus albus* Lindley found on *Lyonia ovalifolia* (Wall.) Drude tree and were collected from Jimali VDC, Salyan (1900 m). The *Olea ferruginea* Royle was growing on north east facing slope of while *Lyonia ovalifolia* (Wall.) Drude was thriving on north facing slope. There was a bright sunlight from morning to noon in that area.

Other orchid species *Eria graminifolia* Lindl. and *Gastrochilus distichus* (Lindl.) Kuntz - both the orchid species growing on *Quercus leucotrichophora* A. Camus - were collected from Daman-Simbhanjyang area of Makawanpur District. The former orchid was found on support trees thriving on north facing slope of 2000 m and later one on a east facing slope 2500 m altitude. There was foggy weather all the day during collection period. The trees were with thick barks, some lichens, and mosses. The collected orchid species were authenticated by Mr. Bhakta Bahadur Raskoti (Ph. D. Scholar, Institute of Botany, Chinese Academy of Sciences, Beijing, China) and Dr. Bijaya pant (Associate professor of CDB, TU).

3.2.2. Drying and extraction

The whole plant of each orchid species was cleanly washed in a tap water, and kept under shade till the complete drying of tap water. Then, the plant body was weighed in digital balance. The parts of each orchid plant was finely chopped and kept under shade for

drying. These finely chopped samples were frequently weighed till the constant mass was measured. A plant material that acquired a constant weight was made to fine powder by commercially available electrical blender.

Individual plant sample was extracted in Soxhlet extraction procedure using acetone (70%) and ethanol (70%) as solvents. The fresh weight of plant sample was taken from100 g to 500 g according to availability of species. They were reduced to 25 g to 50 g after complete drying. The dried powder of each species was introduced to extraction in the ratio of 1:10 w/v with solvents (i.e., 25 g dried powder dissolved with 250 ml of solvent).

The concentrated plant extract was operated to rotavapor (BuChi, made in Switzerland) for solvent drying. The dried plant extracts were obtained to two to five grams according to original amount of plant materials. Thus, obtained pure extract of plant materials were kept in tiny plastic bottles and stored in refrigerator at 4˚ C for further uses.

3.2.3. Estimation of dry powder yield

Mass of each fresh selected orchid part was measured and the fresh weight (FW) was noted. The fresh pieces of orchid were kept under shade for two weeks. The sample was weighed (DW). Again, they were powdered and weighed. Final mass of dried powdered (DP) of each sample was weighed. Then, the result was expressed as Dry Powder Yield in percentage of fresh mass using following formula:

$$\text{Dry Powder Yield (DPY)} = [(DP/FW) \times 100] \%$$

3.2.4. Estimation of extract yield

The mass of each dried powder (DP) of sample was kept in thimble for Soxhlet extraction. Then, the mass of extract of each orchid (E) was measured after removing its solvent using rotavapor. Finally, the dry extract yield was expressed in percentage of dried powder (DP) and Fresh weight (FW) of orchids using following formula:

a) $\text{Extract Yield from Fresh material (EYF)} = [(E/FW) \times 100] \%$
b) $\text{Extract Yield from Powder (EYP)} = [(E/ DP) \times 100] \%$

3.2.5. Reagent preparation

3.2.5.1. Preliminary phytochemical screening

Ferric chloride solution: Ferric chloride (Mol. Wt. 270.2957, 0.1 g) was dissolved in distilled water (90 ml). Then, the final volume was adjusted to 100 ml to prepare 0.1 % ferric chloride solution for tannins detection.

Mayer's Reagent: Mercuric chloride ($HgCl_2$; Mol. Wt. 271.50; 1.358 g) was dissolved in distilled water (60 ml) and Potassium iodide (KI; Mol. Wt. 166.00277; 5 g) dissolved in distilled water (10 ml) were mixed and final volume was maintained to 100 ml.

3.2.5.2. Estimation of total flavonoids content

Aluminium chloride solution: Aluminium chloride (10 g) was dissolved in distilled water (80 ml) and final volume was made to 100 ml.

Stock solution of Quercetin: The quercetin (15 mg) was dissolved in ethanol (15 ml; absolute) to make stock solution of quercetin (1 mg/ml).

Plant Extract Solution: Each plant extract (10 mg) was dissolved in ethanol (10 ml; absolute) to make a solution with strength of 1 mg/ml.

3.2.5.3. Estimation of total polyphenols content

Folin-Ciocalteu phenol reagent: The Folin-Ciocalteu phenol reagent (10 ml) was dissolved in distilled water (90 ml) to prepare 10 % strength.

Sodium bicarbonate solution: The sodium bicarbonate (7.5 g; $NaHCO_3$) was dissolved in distilled water (80 ml) and the final volume was made to 100 ml to make 7.5 % solution.

Stock solution of Gallic acid: The gallic acid (25 mg) was dissolved in ethanol (absolute; 25 ml).

Plant extract: Stock solution (1 mg/ml) was prepared by dissolving of plant extract (10 mg) in ethanol (10 ml). Then, it was diluted to 2.5 mg/ml adding by ethanol.

3.2.5.4. Antioxidant activity

DPPH-ethanol solution: The DPPH powder (mol. Wt. 394.32; 0.00973 g) was dissolved in ethanol (90 ml) and final volume was made to 97.28 ml to make 0.25 mM of DPPH-ethanol solution. The total volume was made as per required to test all the orchid extracts. DPPH-ethanol solution was freshly prepared for experiments.

Orchid extract solution: Each orchid extract (10 mg) was dissolved into absolute ethanol (10 ml) to make stock solution (1 mg/ml). Then, series of orchid extract solution (50 µg, 100 µg, 200 µg, 400 µg, and 800 µg/ ml) was prepared by diluting stock solution.

Reference Compound Solution: Quercetin (10 mg) was dissolved in absolute ethanol (10 ml) to prepare stock solution (1 mg/ml). Again, the series of solutions of reference compound was prepared by diluting stock solution into series of 50 µg, 100 µg, 200 µg, 400 µg, and 800 µg/ ml solutions.

3.2.6. Preliminary phytochemical screening

Preliminary phytochemical analysis was carried out using protocols Habourne (1998), Trease and Evans (1983) with slightly modifications.

A. Test for tannins

The plant extract (100 mg) was dissolved in distilled water (10 ml) in a clean test tube. The resulted aqueous plant extract solution (2 ml) was taken in a separate test tube. Then, the ferric chloride solution ($FeCl_3$; 0.1%; 2 ml) was added. The observation for the appearance of blue-black or greenish brown colouration was done.

Plant Extract (100 mg) + Distilled water (10 ml)

↓

Plant Extract Solution (2 ml) + Ferric Chloride Solution (0.1 %; 2 ml)

↓

Appearance of blue-black or greenish brown colouration
(**Presence of tannins**)

B. Test for saponins

The aqueous solution of plant extract (0.5 ml) prepared by plant extract (100 mg) and distilled water (10 ml) was added to with distilled water (5 ml) in a clean test tube. It was shaken vigorously for five minutes. The observation for the persistent froth for 5 minutes was done.

Plant Extract (100 mg) + Distilled Water (10 ml)

↓

Plant Extract Solution (0.5 ml) + Distilled Water (5 ml)

↓

Shaken vigorously to make frothing

↓

Persistent Froth
(**Presence of Saponins**)

C. Test for steroids and terpenoids

The plant extract (100 mg) was dissolved in methanol (4 ml) in a clean test tube. The resulted methanolic solution was completely dried by the help of boiling hot water bath. Then, chloroform (2 ml) was poured into methanolic solution dried test tube. Again, concentrated sulfuric acid (2 ml) was added to it to from side wall of a test tube in order to make two layers. Then, the observation for the yellowish ring between two layers and converting yellow colour to red or dark red was made.

Plant Extract (100 mg) + Methanol (4 ml)

↓

Completely dried by hot water bath

↓

Dried sample + chloroform (2 ml) + Conc. H_2SO_4 (2 ml)

↓

Yellowish ring between two layers and conversion of yellow colour to red or dark red (**Presence of steroids and terpenoids**)

D. Test for flavonoids

The plant extract (100 mg) was dissolved into methanol (10 ml). The resulted methanolic solution of plant extract (2 ml) taken in a separate test tube and concentrated hydrochloric acid (HCl; 2 – 3 drops) was added to it. Then, the magnesium ribbon pieces (3 mm×5 mm) were added to the resulted solution. The resulted mixture was allowed to stand for 10 minutes. The observation for the appearance of pinkish colouration on a mixture was done. A portion of methanolic solution (2 ml) in another test tube was taken as positive control to distinguish pinkish colouration appearance on a test solution.

Plant Extract (100 mg) + Methanol (10 ml)

↓

Resulted solution (2 ml) + Concentrated HCl (2 – 3 drops) + Magnesium ribbon pieces (3 mm × 5 mm)

↓

Pinkish Colouration
(**Presence of flavonoids**)

E. Test for alkaloids

The plant extract (100 mg) was dissolved in methanol (10 ml) in a test tube. The mixture was filtered through Whatman Number 1 filter paper. Thus, obtained filtrate was mixed with HCl (1 %, 2 ml) in a test tube. Then, the mixture was heated in a steam bath. After that, the mixture was treated with Mayer's reagent (6 drops in each replica). Finally the observation for the appearance of turbidity on a mixture was done.

Plant extract (100 mg) + methanol (10 ml)

↓ ← Filtration

Filtrate + HCl (1 %, 2 ml)

↓ ← Steam bath

Steam heated mixture + Mayer's reagent (6 drops in each replica)

↓

Turbidity

(Presence of alkaloids)

3.2.7. Estimation of total flavonoid content

The total flavonoid content of the plant extract was estimated using the standard protocol (Quettier *et al.* 2000 and Stankovic 2011) with slight modification. Shortly, each plant extract (2 ml; 0.5 mg/ml) was separately mixed with aluminium chloride solution (1 ml; 2 %). The reaction mixture was allowed to stand for an hour at room temperature. Then absorbance of the mixture was measured at 415 nm using the UV-spectrophotometer. The calibration curve was obtained using quercetin solution series (25, 50 75, and 100 µg/ml) as a standard (see Fig.4.). Here, the reagent mixture with same volume of ethanol instead of plant extract solution was used as a blank. Thus, obtained curve was calibrated to estimate the concentration of total flavonoid in each plant extract. The total flavonoids content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/ g). The test was done in triplicates.

Plant extract (2 ml; 0.5 mg/ml) + Aluminium chloride solution (1 ml; 2 %)

↓

Allowed to stand for 1 hour

↓

Absorbance at 415 nm

3.2.8. Estimation of total polyphenols content

The total polyphenol content was estimated by a standard protocol (Singleton *et al*. 1999 and Stankovic 2011) with slight modification. Briefly, each plant extract (0.5 ml; 1 mg/ml) was separately mixed with the Folin-Ciocalteu phenol reagent (2.5 ml; 10%) and aqueous sodium bicarbonate solution (2.5 ml; 7.5%). The reaction mixture was allowed to stand for about 45 minutes and the absorbance was measured at 765 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10.5).The calibration curve was plotted using gallic acid (see Fig.6.) as standard in ethanol (absolute) using the concentration series of 25, 50, 75, and 100 µg/ml. Here, the reagent mixture with same volume of ethanol instead of plant extract solution was used as a blank. On the basis of this calibration curve, the concentration of the individual samples was calculated. The

total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE/ g). The test was done in triplicates.

Plant extract (0.5 ml; 1 mg/ml) + Folin-Ciocalteu phenol reagent (2.5 ml; 10%) + aqueous sodium bicarbonate (2.5 m; 7.5 %) solution

↓

Incubated for 45 minutes at room temperature

↓

Absorbance at 765 nm

3.2.9. Antioxidant activity

The antioxidant activity of orchid extracts was performed following standard protocol according to Zhao *et al.* (2006) with slight modification. Briefly, each plant extracts was made to a series of solution with concentration 50 µg, 100 µg, 200 µg, 400 µg, 800 µg/ml. The sample solution was made by mixing plant extract solution (1.5 ml of each concentration of a series) and DPPH free radical solution (1.5 ml; 0.25 mM in ethanol); blank solution by plant extract solution (1.5 ml of each concentration) and absolute ethanol (1.5 ml); control solution by DPPH free radical solution (1.5 ml; 0.25 mM) and absolute ethanol (1.5 ml). The quercetin was taken as a reference compound. The solutions of reference compound were made as it was done in case of plant extract solution. These solutions were vigorously shaken and allowed to stand for 30 minutes under dark immediately after preparation. Then, absorbance of each solution was taken at 517 nm using UV- visible spectrophotometer. The experiment was done in triplicate.

To calculate scavenging activity of plant extracts and reference compound the following equation was applied:

$$\text{Scavenging rate} = [1-(A_1-A_2)/A_0] \times 100\ \%$$

Here, A_0 = Absorbance of control solution; A_1 = Absorbance in sample solution (i.e., in presence of plant extract); A_2 = Absorbance of blank solution (i.e., without DPPH).

Finally, the IC_{50} value of each plant extract was determined by non linear regression equation of plot of percentage antioxidant activity (AA %) against concentration.

Plant extract (1.5 ml; series of 50-800 µg/ml) + DPPH Solution (1.5 ml; 0.25 mM)

↓

Incubate in dark at room temperature for 30 minutes

↓

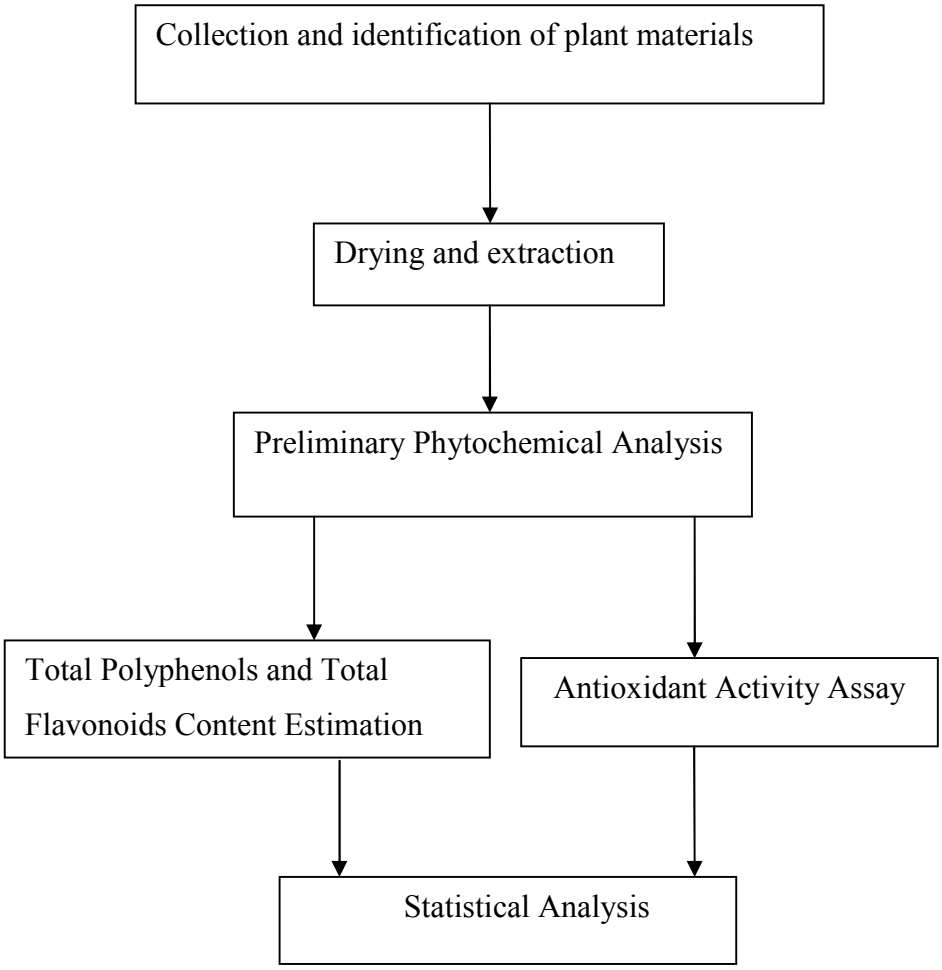
Absorbance at 517 nm

3.2.10. Statistical analysis

The tests were carried out in triplicate form for total flavonoids content, total polyphenolics content and DPPH free radical assay. The values of total flavonoids content and total polyphenolics content are presented as mean ± SD. For antioxidant activity, regression model second or third order polynomial equation was used in calculating IC_{50} of extracts. The variation in total flavonoids, total polyphenols content and antioxidant activity among ethanolic extract was analysed by one sample t-test at P = 0.05. The post hoc test by Tuckey HSD test at P = 0.05 was carried to distinguish actual difference between means ± SD values of total flavonoids content and total polyphenolics content.

The data was collected in MS Excel 2007 and one sample t-test was carried out to test the variation in total polyphenols and flavonoids content in IBM SPSS Version 20. A statistical software R version 3.1.2 was used to calculate IC_{50} value and to test variation of antioxidant activity among the extracts and with the quercetin (reference compound). The association of antioxidant activity and total flavonoids content and total polyphenolics content was done by obtaining regression quation ($y = mx + c$) in MS Excel 2007.

Flow chart 1: Overall scheme of methods

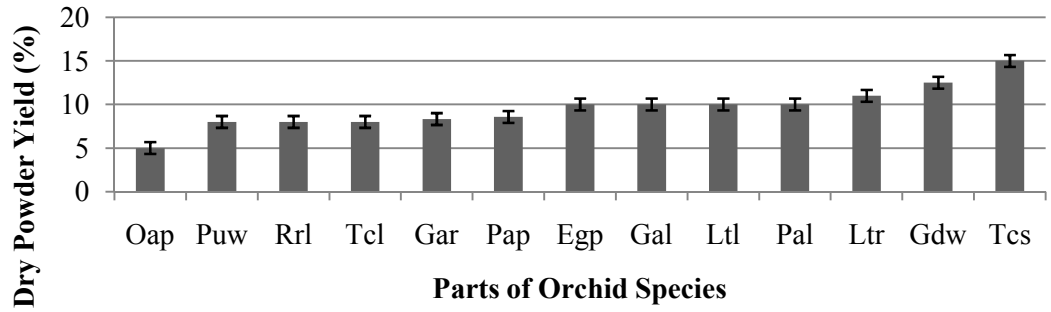


CHAPTER FOUR: RESULTS

4.1. Estimation of dry powder yield

The stem of *Trudelia cristata* and pseudobulbs of *Otochilus albus* provided highest (15%) and lowest (5%) dry powder yield from fresh materials. Rest of the selected orchids yielded from 8% to 12.5 %. The details of dry powder yield are presented in Figure 1.

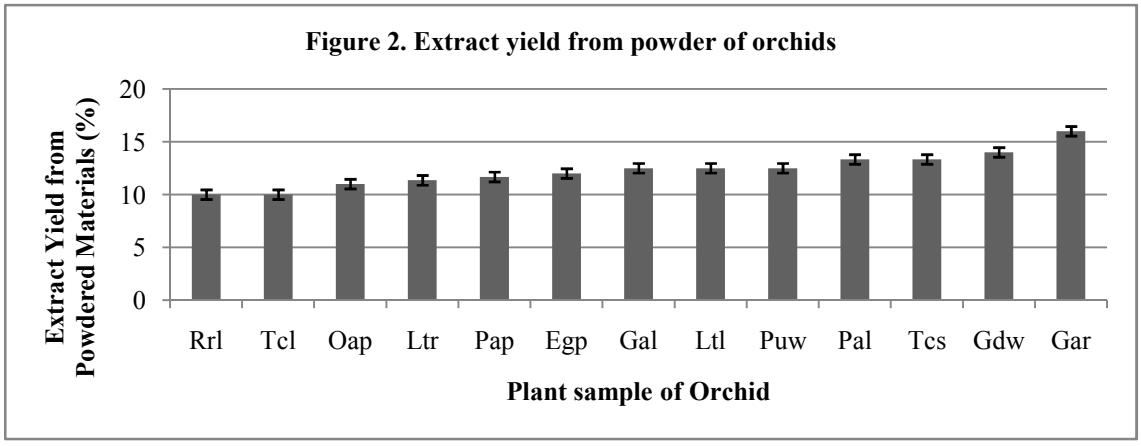
Figure 1. Dry powder yield in different orchid species



Here, **Oap** = *Otochilus albus* pseudobulbs; **Puw** = *Papilionanthe uniflora* whole plant; **Rrl** = *Rhynchostylis retusa* leaf; **Tcl** = *Trudelia cristata* leaf; **Pap** = *Pholidota articulata* pseudobulbs; **Egp** = *Eria graminifolia* pseudobulbs; **Gal** = *Gastrochilus acutifolius* leaf; **Ltl** = *Luisia trichorhiza* leaf; **Pal** = *Pholidota articulata* leaf; **Ltr** = *Luisia trichorhiza* leaf; **Gdw** = *Gastrochilus distichus* whole plant; **Tcs** = *Trudelia cristata* stem.

4.2. Extract yield from powdered materials

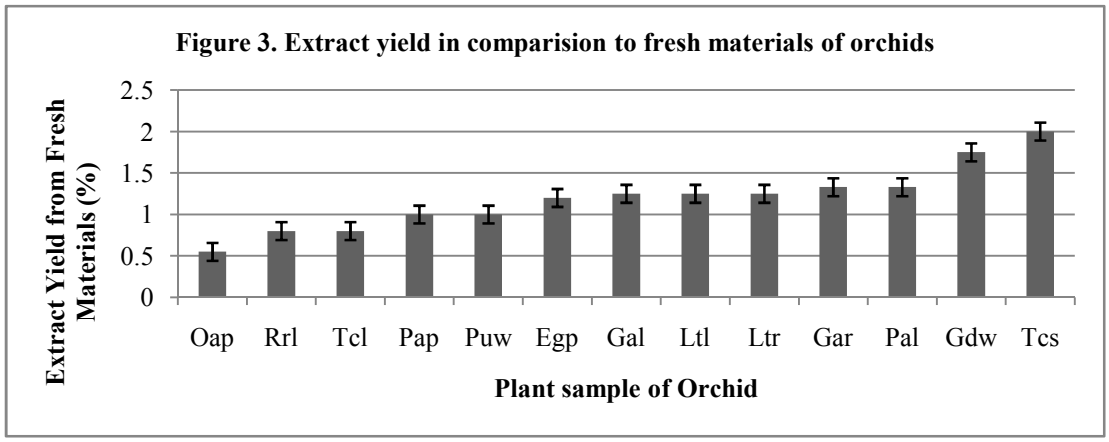
Rhynchostylis retusa leaf and *Trudelia cristata* leaf provided only 10% of dried extract from powdered material while *Gastrochilus acutifolius* root yielded 16% as lowest and highest yield respectively. Rest of the orchids gave dried extract ranging from 11 to 14%. The details are shown in the Figure 2.



Here, **Oap** = *Otochilus albus* pseudobulbs; **Puw** = *Papilionanthe uniflora* whole plant; **Rrl** = *Rhynchostylis retusa* leaf; **Tcl** = *Trudelia cristata* leaf; **Pap** = *Pholidota articulata* pseudobulbs; **Egp** = *Eria graminifolia* pseudobulbs; **Gal** = *Gastrochilus acutifolius* leaf; **Ltl** = *Luisia trichorhiza* leaf; **Pal** = *Pholidota articulata* leaf; **Ltr** = *Luisia trichorhiza* leaf; **Gdw** = *Gastrochilus distichus* whole plant; **Tcs** = *Trudelia cristata* stem.

4.3. Dry extract yield in comparison to fresh material

The pseudobulbs of *Otochilus albus* and Stem of *Trudelia cristata* gave lowest (0.55%) and highest (2%) amount of dried extract when they are compared with original fresh material. Rest of the orchids provided 0.8 to 1.75 % of dried extract (Figure 3).



Here, **Oap** = *Otochilus albus* pseudobulbs; **Puw** = *Papilionanthe uniflora* whole plant; **Rrl** = *Rhynchostylis retusa* leaf; **Tcl** = *Trudelia cristata* leaf; **Pap** = *Pholidota articulata* pseudobulbs; **Egp** = *Eria graminifolia* pseudobulbs; **Gal** = *Gastrochilus acutifolius* leaf; **Ltl** = *Luisia trichorhiza* leaf; **Pal** = *Pholidota articulata* leaf; **Ltr** = *Luisia trichorhiza* leaf; **Gdw** = *Gastrochilus distichus* whole plant; **Tcs** = *Trudelia cristata* stem.

4.4. Preliminary phytochemical screening

A. Alkaloids

The ethanolic extract of *Eria graminifolia* stem, *Gastrochilus acutifolius* root and stem; *Luisia trichorhiza* root and stem; *Otochilus ablus* pseudobulbs; *Pholidota articulata* leaf and pseudobulb; *Rhynchostylis retusa* leaf; *Trudelia cristata* leaf and stem; *Papillionanthe uniflora* whole plant showed the turbidity on Mayer's reagent as a presence of alkaloids. However, the ethanolic extract of *Gastrochilus distichus* did not show such turbidity indicating absence of alkaloids. The details are shown in a Table.1.

B. Flavonoids

All the tested extracts of orchid species provided pink – tomato colour except *Luisia trichorhiza* root. This indicates the presence of flavonoids in all the selected orchid species.

C. Saponins

The persistent frothing was observed only in the leaf and root extract of *Gastrochilus acutifolius* and whole plant extract of *Gastrochilus distichus* which indicates the presence saponins.

D. Steroids and terpenoids

The appearance of yellow ring between the two layers and converting of that to red to dark red colour was observed in leaf and root extract of *Gastrochilus acutifolius*, whole plant extract of *G. distichus*, leaf and root extract of *Luisia trichorhiza*, pseudobulb, and leaf extract of *Pholidota articulata*, leaf and root extract of *Rhynchostylis retusa*, stem

extract of *Trudelia cristata* and whole plant extract of *Papillionanthe uniflora* which indicates presence of the steroids and terpenoids.

E. Tannis

The appearance of blue-black precipitate was observed in leaf extract of *Trudelia cristata*, leaf and root extract of *Rhynchostylis retusa*; brown colouration was observed in root extract of *Luisia trichorhiza*, stem extract of *Trudelia cristata,* dark brown colouration in leaf and pseudobulbs extract of *Pholidota articulata* which indicates the presence of *tannins* in all the extracts of orchid species investigated.

The result of presence of phytochemical constituents is summarized in the Table.1.

Table.1. Photochemical constituents in selected orchid species

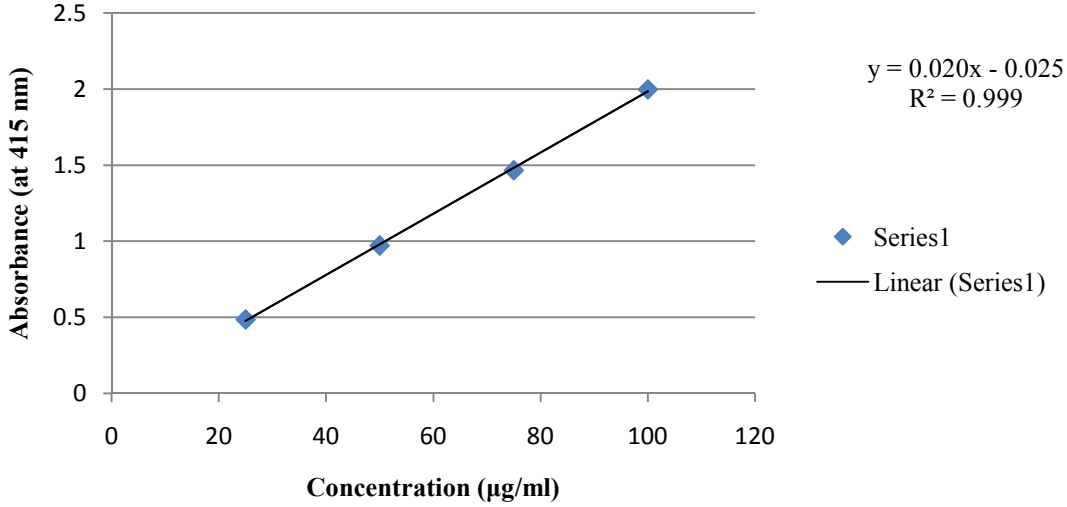
S. N.	Name of Orchid	Plant Part	Code	Alkaloids	Flavonoids	Saponins	Steroids & Terpenoids	Tannins
1	*Eria graminifolia*	Pseudobulbs	Egp	+	+	-	-	+
2	*Gastrochilus acutifolius*	Leaf	Gal	+	+	+	+	+
		Root	Gar	+	+	+	+	+
3	*Gastrochilus distichus*	Whole plant	Gdw	-	+	+	+	+
4	*Luisia trichorhiza*	Leaf	Ltl	+	+	-	+	+
		Root	Ltr	+	-	-	+	+
5	*Otochilus albus*	Pseudobulbs	Oap	+	+	-	-	+
6	*Papillionanthe uniflora*	Whole plant	Puw	+	+	-	+	+
7	*Pholidota articulata*	Pseudobulb	Pap	+	+	-	+	+
		Leaf	Pal	+	+	-	+	+
8	*Rhynchostylis retusa*	Leaf	Rrl	-	+	-	+	+
9	*Trudelia cristata*	Stem	Tcs	+	+	-	+	+
		Leaf	Tcl	+	+	-	-	+

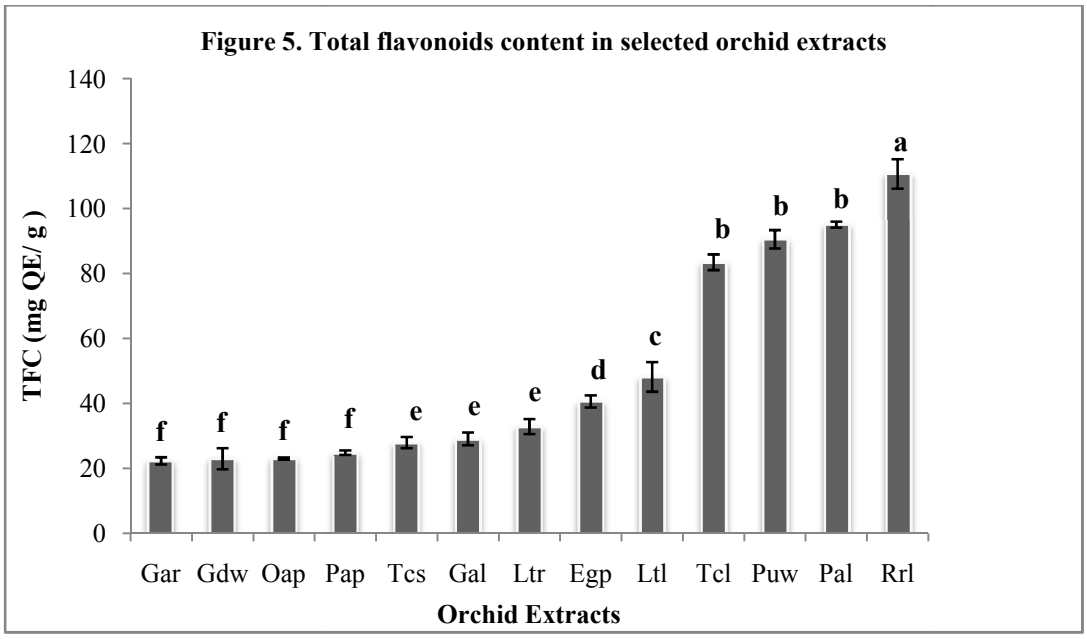
Here, "+" = Presence; "-" = Absence

4.5. Total flavonoids content

The ethanol extracts of selected orchids contained total flavonoids content (TFC) with significant variation among them (t-statistic = 9.88, df = 38, P < 0.01). The highest TFC was estimated in leaf extract of *Rhynchostylis retusa* (110.68 ± 4.52 mg QE/ g) but lowest in root extract of *Gastrochilus acutifolius* (22.32 ± 1.1 mg QE/ g). The extract of *Pholidota articulata* pseudobulbs, *Otochilus albus* pseudobulb, *Gastrochilus acutifoilius* leaf, *Trudelia cristata* stem, *Gastrochilus distichus* whole plant, *Eria gramminifolia* pseudobulbs, *Luisia trichorhiza* root and leaf were found below 50 mg QE/ g. Although there was no statistical difference at P = 0.05 among rest of the extracts they were estimated between 80 and 96 mg QE/ g with the order of *Trudelia cristata* leaf < *Papillionanthe uniflora* whole plant < *Pholidota articulata* leaf. The details are shown in the Figure 5.

Figure 4. Calibration curve of Quercetin at 415 nm

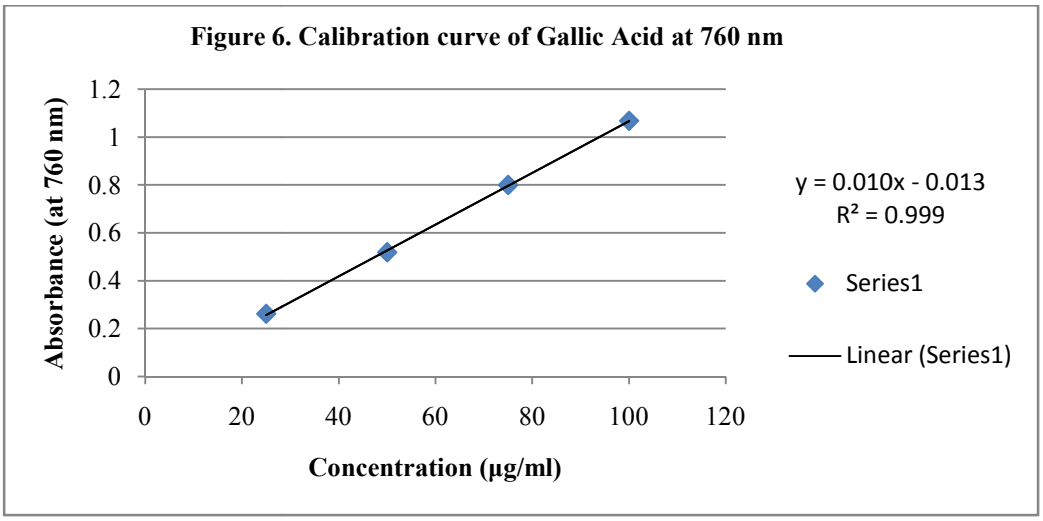


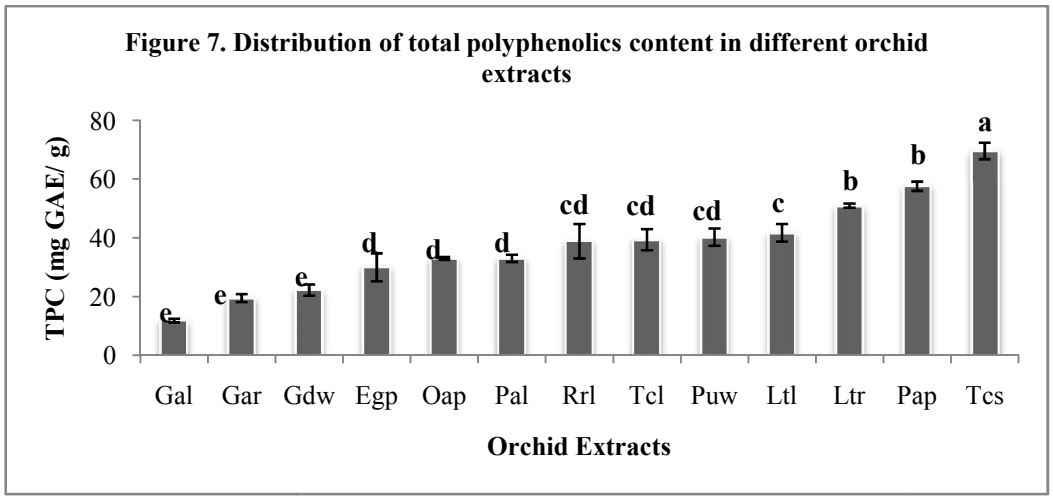


[a-f] Means with a same superscript letter are not significantly different at $P \leq 0.05$.

4.6. Total polyphenols content

The ethanol extracts of selected orchids showed total polyphenolics content (TPC) with significant variation (t-statistic = 15.74, df = 38, $P < 0.01$). The *Trudelia cristata* stem was found with highest total phenolic content (69.68 ± 2.78 mg GAE/ g) while *Gastrochilus acutifolius* leaf with lowest content (11.9 ± 0.65 mg GAE/ g). The other extracts – *Pholidota articulata* pseudobulbs, *Luisia trichorhiza* root, *Luisia trichorhiza* leaf, *Papillionanthe uniflora* whole plant, *Trudelia cristata* leaf, *Rhynchostylis retusa* leaf, *Pholidota articulata* leaf, *Otochilus albus* pseudobulbs, *Eria gramminifolia* pseudobulbs, *Gastrochilus distichus* whole plant, and *Gastrochilus acutifolius* root contained total polyphenolic content in decreasing order. The details with the significance of their difference are given in the Figure 7.





[a-e] Means with a same superscript letter are not significantly different at $P \leq 0.05$.

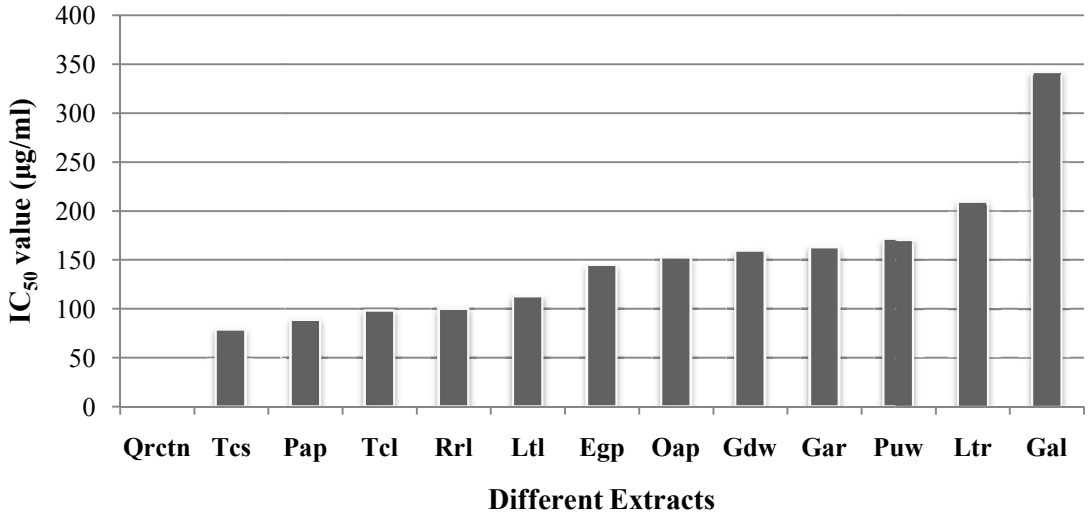
4.6. Antioxidant activity

The antioxidant activity was significantly varying among ethanol extracts of selected orchid (t - statistic = 7.3794, df = 13, p < 5.35e-06). The stem of *Trudeila cristata* was found with lowest (79.69 µg/ ml) and leaf of *Gastrochilus acutifolius* with highest IC_{50} value (341.79 µg/ ml) for the DPPH radical scavenging activity assay. It means ethanol extract of *T. cristata* stem has highest while *G. acutifolius* leaf shows lowest antioxidant activity. Rests of the extracts were found with IC_{50} values ranging from 89.18 µg/ ml (*Pholidota articulata* pseudobulbs) to 209.78 µg/ ml in (*Luisia trichorhiza* roots). The

other extracts arranged in following order of antioxidant activity: *Trudelia cristata* leaf > *Rhynchostylis retusa* leaf > *Luisia trichorhiza* leaves > *Eria graminifolia* pseudobulbs > *Otochilus albus* pseudobulbs > *G. distichus* whole plant > *G. acutifolius* roots > *Papillionanthe uniflora* whole plant > *Pholidota articulata* leaves.

The IC_{50} value for DPPH radical scavenging activity of each extract was noticeably lower than Quercetin (reference compound). Since IC_{50} value of Quercetin was 32.90 µg/ ml in this experiment the difference with antioxidant activity of all orchid extracts was statistically significant. The details is given is Figure 8.

Figure 8. Antioxidant activity of quercetin and selected orchids



4.7. Relationship of antioxidant activity with total polyphenolics and flavonoids content

The IC_{50} for DPPH radical scavenging activity of ethanol extract of selected orchids was statistically negatively associated with total polyphenols and flavonoids content in this study. When IC_{50} values were plotted against total flavonoids content the regression equation $y = -0.4247x + 174.71$ and $R^2 = 0.0405$ was obtained. The graph showing regression equation of IC_{50} and total flavonoids content is presented in Figure 9. Again, there was highly negative association of IC_{50} and total polyphenols content with

regression equation y = -2.74x + 256.43 and R^2 = 0.4022 in this study. Here, the plot showing negative association of IC_{50} and total polyphenols content is shown in Figure 10.

The negative association of IC_{50} of orchid extracts with flavonoids content and IC_{50} of orchid extract with polyphenol content was found in this study. So, increase in the polyphenols and flavonoids content decrease the IC_{50} that means increase in the antioxidant activity.

Figure 9. Regression of IC_{50} with total flavonoids content

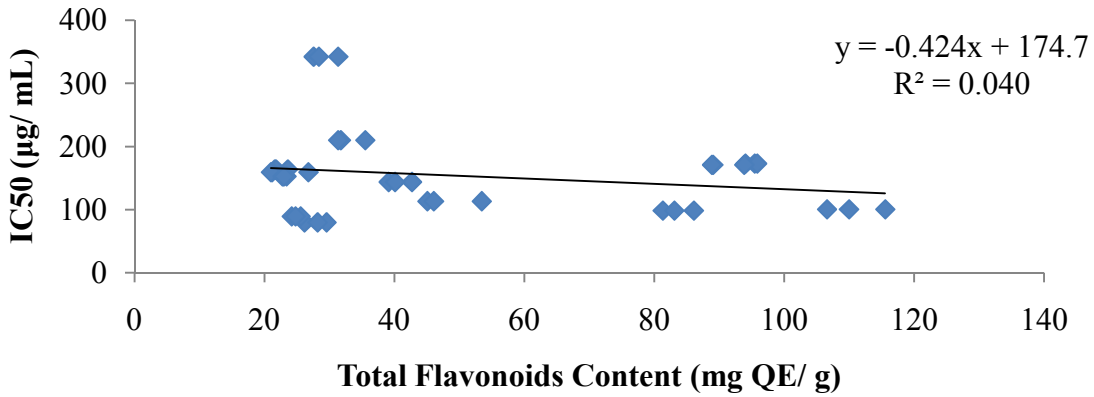
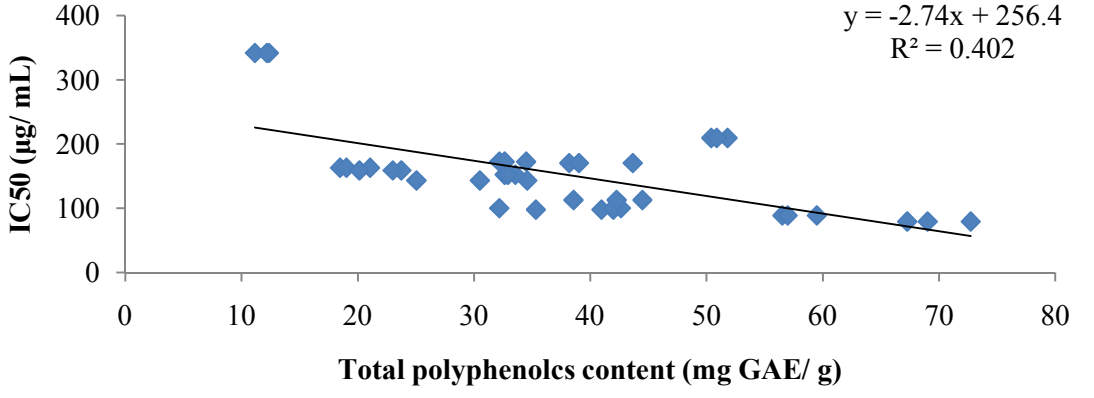


Figure 10. Regression of IC_{50} against total polyphenolics content



CHAPTER FIVE: DISCUSSION

5.1. Preliminary phytochemical screening

The preliminary phytochemical screening on selected orchids showed the similar result with previous studies. Their ethanol extracts were with the presence of tested phytochemicals – alkaloids, flavonoids, saponins, steroids and terpenoids, and tannins. However, some extracts were not detected with certain phytochemicals.

Tannins are polyphenolic compounds in plants which are responsible for antioxidant activity. As shown in the Table. 1., the presence of tannins in all the extracts is similar with other orchids viz., *Papilionanthe teres* (Mazumder *et al.* 2010), *Acampe premorsa* (Gurucharan *et al.* 2012), *Dendrobium macrostachyum* (Nimisa *et al.* 2012), *Eria pseudoclavicaulis* (Sahaya *et al.* 2012), *Cymbidium aloifolium* (Radhika *et al.* 2013), *Flikingeria nodosa* (Nagananda *et al.* 2013).

Alkaloids are presence in all the extracts except *Gastrochilus distichus* whole plant and *Rhynchostylis retusa* leaf. The presence of alkaloids is similar with the extacts of *Vanda tessellata* (Ahmed *et al.* 2001; Nayak *et al.* 2005), *Vanilla planifolia* (Shanmugavalli *et al.* 2009), *Dendrobium macrostachyum* (Nimisha *et al.* 2012), *Eria pseudoclavicaulis* (Sahaya *et al.* 2012), and *Eulophia nuda* (Kurapa *et al.* 2012).

Flavonoids – a group of compounds performing antioxidant activity – are also presence in all the tested extracts except *Luisia trichorhiza* root. Since, flavonoids are found in most of the species in previous studies with few exceptions present study gave similar result.

Saponins were detected in all the extracts of *Gastrochilus acutifolius* and *G. distichus* in the present study; similar results were obtained in previous studies of Sahaya *et al*. (2012), and Mazumder *et al*. (2010). However, saponins were absent other extracts which is similar with *Satyrium nepalense* (Mishra *et al*. 2012).

Detection of Steroids and terpenoids was positive in all the extracts except *Trudelia cristata* leaf, *Otochilus albus* pseudobulbs, and *Eria graminifolia* pseudobulbs. In previous studies, steroids and terpenoids were found in extracts of *Dendrobium aqueum,*

D. barbatulum, *D. didon*, *Gastrochilus acaulis*, *and Luisia zeylanica* (Maridass *et al.* 2008), *Vanilla planifolia* (Shanmugavalli *et al.* 2009), and *Cymbidium aloifolium* (Radhika *et al.* 2013). *Papilionanthe uniflora* in this study was not detected with steroids and terpenoids but *P. teres* was detected with these phytochemical classes (Mazumder *et al*. 2010).

Thus, ethanol extract of selected orchids in present study have all the tested phytochemicals which may be attributed to the medicinal activities.

5.2. Total flavonoids content

The total flavonoids content in ethanol extract of selected orchids in this investigation has been found as similar or different amount with various extracts of medicinal plants, and orchids in previous studies. Ethanol extract of *Gastrochilus distichus*, *G. acutfolius* root, *Otochilus albus* pseudobulbs, and *Pholidota articulata* pseudobulbs were found with higher total flavonoids contents than the extract of *Withania somnifera* leaf (13.53 ± 0.85 mg QE/g) (Ghimire *et al.* 2011) and sepals of *Rhododendron arboreum* (Bhandari *et al.* 2014). As previously mentioned the total flavonoids content in methanol extracts of *Rhododendron arboreum* stem and bark were found with 45.0±2.54 mg and 51.3±6.49 mg QE/g (Bhandari *et al.* 2014) were comparable with ethanol extract of *Luisia trichorhiza* leaf in this assessment. The total flavonoids content in methanol extaract of *Rhododendron arboreum* sepals and *Ageratum conizoides* whole plant (12.7±2.24 mg QE/g) (Ghimire *et al.* 2011) in previous study is considerably lower content than in all the ethanol extracts of *Gastrochilus acutifoilius* and *G. distichus* of this study while the previous finding in *Rhododendron arboreum* twigs (67.5±2.50) and androecium and gynoecium (53.4±2.04 mg QE/g) by Bhandari *et al*. 2014 is not higher than in *Trudelia cristata* leaf - the fourth highest content - in present investigation.

The total flavonoids content in methanol extract of *Dendrobium speciosum* leaf and stem is fewer than in all the extracts of present investigation. The total flavonoids content in a terrestrial orchid *Calanthe triplicata* plant (57.70±0.02 mg QE/ g of extract) in a study by Mythill *et al.*2014 was higher than in all the extracts except *Rhynchostylis retusa* leaf,

Pholidota articulata leaf, *Papilionanthe uniflora* whole plant, and *Trudelia cristata* leaf of this study.

The total flavonoids content in medicinal plants reported from Jhapa and Ilam district, Nepal *Rauvolfia serpentina* (15.63 ± 0.91 mg QE/g extract), *Drymaria cordata* (15.50 ± 2.26 mg QE/g extract), *Achyranthes aspera* (16.83 ± 0.31 mg QE/g extract), *Sapium insigne* (20.07 ± 1.62 mg QE/g extract) were estimated by Ghimire *et al.* 2011 with fewer content than in *Gastrochilus acutifolius* root which is lowest content in this study.

The *Luisia trichorhiza* root extract was estimated with 32.87 ± 2.31 mg QE/g of extract of total flavonoids content but it was not detected with flavonoids during preliminary phytochemical screening. This contradiction may be due to very less intense pink tomato colouration after the reaction between flavonoids and reagents or such colouration was dominated by preoccupied brownish colour of sample itself.

Thus, total flavonoids content in ethanol extracts of selected orchids in this study are in near about contents with different medicinal plant extracts of previous studies.

5.3. Total polyphenols content

Different medicinal plants have varied amount of total polyphenolics content. Present study also revealed the significantly variation of total polyphenolics content in ethanol extract of selected orchids.

Orchids selected for the present study were with fewer, and moderately higher total polyphenols content than medicinal plants in previous studies; comparable with other plant extracts. The highest total polyphenols content in *Trudelia cristata* stem (69.68±2.78 mg GAE/g) of present study was very low content than methanolic extract of some medicinal plants viz., *Terminaliabellirica* fruit (237.827 ±10.130 mg GAE/g), *Terminalia chebula* fruit (149.690 ±6.088 mg GAE/g), *Phyllanthus emblica* fruit (197.371 ±4.244 mg GAE/g), *Bergenia ciliata* rhizome (304.00 ±18.180 mg GAE/g), *Adhatoda vasica* leaves (109.512 ±9.589 mg GAE/g), and *Vitex negundo* leaves (151.618 ±6.3886 mg GAE/g). Total polyphenols content in *Gastrochilus acutifolius* leaf (11.89±0.46 g GAE/g), the lowest content in this study was very high content than in *Fritillaria*

delavayi whole plant extract (0.4 GAE/100g DW) (Maharjan *et al.* 2013). Total polyphenols content in methanol extracts of *Calanthe triplicata* whole plant (23.53± 0.25 mg GAE/g) (Mythill *et al*. 2014), a terrestrial orchid was comparable with *Gastrochilus distichus* whole plant in this assessment. Except highest and lowest content in our study, other total polyphenols content in selected orchids were comparable with methanolic extract of *Ipoemea carnea* (47.0±3.93 mg GAE/g) (Pandey *et al.* 2014), Withania somnifera (28.97 ± 2.36 mg GAE/g extract), Sapium insigne (34.30 ± 0.40 mg GAE/g extract), Rauvolfia serpentine (29.43 ± 0.76 mg GAE/g extract), Persea odoratissima (54.00 ± 1.00 mg GAE/g extract), Drymaria cordata (23.80 ± 1.11 mg GAE/g extract), Cassia fistula (36.43 ± 1.55 mg GAE/g extract), Asparagus racemosus (45.37 ± 1.07 mg GAE/g extract) (Ghimire *et al*. 2011).

So, it can be noticed that the total polyphenols content in ethanol extract of selected orchids in present study are comparable with or relatively higher or lower content than different medicinal as well as other plant extracts of previous studies.

5.4. Antioxidant activity

It is established that the extracts of different plants and other plant products show higher antioxidant activity with variation among them. Moreover, in DPPH free radical assay, the food, fruit, and medicinal plants showing low half effective concentration (EC_{50}) or half inhibition concentration (IC_{50}) have high radical scavenging activity; thus, high antioxidant activity and vice-versa. In this study, ethanol extracts of selected orchids showed antioxidant activity. The variation of DPPH free radical scavenging activity among orchid extracts was significant as in previous studies. The variation occurred with highest antioxidant activity in extracts of *Trudelia cristata* to lowest activity in extract of *Gastrochilus acutifolius*.

Results of present assessment were similar with other medicinal plants of previous studies with minor differences. Antioxidant activity of *Rhododendron arboreum* bark (65.45 µg/ml), stem (67.83 µg/ml) and twigs (46.02 µg/ml) extracts in previous study (Bhandari *et* al. 2014) were with nearest IC_{50} values of *Trudelia cristata* stem*, Pholidota articulata* pseudobulbs of this study. *Trudelia cristata* stem extract (extract with lowest

IC_{50} value) in this study was with very high IC_{50} value than a *Rhododendron arboreum* leaves (8.34 µg/ml), flowers (25.15 µg/ml) and petals (16.83 µg/ml). However, leaf stem extract of *Dendrobium speciosum* (1.054±0.047 mg/ml) (Moretti *et al.* 2013), hot successive extract of water of *Flikingeria nodosa* pseudobulbs (1083.88 µg/ml) (Nagananda *et al.* 2013), *Terminalia chebula* fruits (109.78 µg/ml), and *T. bellirica* fruits (213.11 µg/ml) were with significantly higher IC_{50} value than *Trudelia cristata* stem extract. Extract of *Gastrochilus acutifolius* root, *Gastrochilus distichus* whole plant, *Otochilus albus* pseudobulbs, *Papillionanthe uniflora* whole plant and *Eri*a *graminifolia* pseudobulbs had IC_{50} value nearer to *Ficus benghalensis* leaves (135.00 µg/ml) (Pandey *et al.* 2014) and aqueous extract of *Coelogyne nervosa* leaf (126 µg/ml). Methanlic extract of *Dendrobium speciosum* (a medicinal orchid) leaf had below three times lower IC_{50} (IC_{50} 0.026±0.004 mg/ml) than lowest IC_{50} of our study but its stem extract (IC_{50} 1.15±0.10 mg/ml) (Moretti *et al*. 2013) had well above the two folds of *Gastrochilus acutifolius* leaf (with highest IC_{50}). Antioxidant activity of *Luisia trichorhiza* root was with IC_{50} nearest to ethanol extract of *Coelogyne nervosa* leaf (IC_{50} 206 µg/ ml) of previous study (Sahaya *et al*. 2013) but all extracts of other orchids which are not recorded as medicinal viz., *Eria graminifolia*, *Gastrochilus acutifolius* root*, Gastrochilus distichus* whole plant, *Papilloinanthe uniflora* whole plant had IC_{50} well below it. The IC_{50} of *Rhynchostylis retusa* leaf and *Luisia trichorhiza* leaf in this study are close to *Terminalia chebula* fruit (109.78µg/ml) of previous study (Genwali *et al.* 2013). *Papillionanthe uniflora* whole plant and *Pholidota articulata* leaf extracts were well below the IC_{50} value than that of *Terminalia bellirica* Fruit (213.11 µg/ml) (Genwali *et* al. 2013).

Plant extract with lower IC_{50} of radical scavenging assay has higher antioxidant activity. The higher antioxidant activity of plant extract is attributed to biological and medicinal property. Ethanol extracts of orchids - especially those are recorded as medicinal - in this study had higher or lower antioxidant activity with considerable margin than medicinal plants and orchids of previous studies. So, the medicinal properties of these orchids may be attributable to their higher antioxidant activity. Extracts of orchids those are not recorded with medicinal property have comparable antioxidant activity with some medicinal plants of previous studies. These orchids too may have medicinal property.

5.5. Relationship of antioxidant activity with total polyphenolics and flavonoids content

In this study, IC_{50} of ethanol extract of selected orchids are negatively correlated with their total polyphenols and flavonoids content. Since a lower IC_{50} value is associated with higher DPPH radical scavenging activity (Shukla *et al.* 2012) the negative correlation of former with total polyphenolics and flavonoids content interprets positively correlation of antioxidant activity with them.

Slightly negative association of IC_{50} value with total flavonoids content (Regression equation $y = -0.4247x + 174.71$; $R^2 = 0.0405$) in this study implies the similar result with previous studies regarding antioxidant activity. However, the extent of negative association of IC_{50} and total flavonoids content in previous studies are higher than this study. Bhandari *et al.* 2014 found higher negative association ($y = -0.58x + 90.884$; $R2 = 0.9653$) of IC_{50} value and total flavonoids content in different extracts of *Rhododendron arboreum*. Also, Ghimire *et al*. 2011 observed positive association of the total flavonoids content with the percentage inhibition of DPPH radical (regression equation $y = 0.6327x + 38.869$; $R^2 = 0.4294$). This means the antioxidant activity is positively related with the total flavonoids content as in previous studies.

Present study observed the moderately negative association of IC_{50} value with total polyphenolics content with regression equation $y = -2.74x + 256.43$; $R^2 = 0.4022$. This negative association of IC_{50} i.e., positive association of antioxidant activity with total polyphenolics content is similar with previous studies with varying degree of strongness. Genwali *et al.* 2013 found negative association of IC_{50} value with total phenol content (regression equation $y = -0.771x + 295.6$; $R^2 = 0.999$) in extracts of different medicinal plants. Pandey *et al.* 2014 also witnessed negative association of IC_{50} with total phenolic content (regression equation $y = 0.897x + 286.7$; $R^2 = 0.931$) in extracts of some medicinal plants.

CHAPTER SIX: CONCLUSION

The variation in antioxidant activity along with the phytochemical constituents, total polyphenols and flavonoids content of ethanol extract of selected wild orchids of Nepal has been assessed. The orchids of present study were detected with phytochemicals that have biological as well as medicinal property. The antioxidant activity significantly varied with highest in *Trudelia cristata* stem (IC_{50} 79.69 μg/ml) and lowest activity in *Gastrochilus acutifolius* leaf (IC_{50} 341.79 μg/ml). Moreover, the total flavonoids content was varying with highest content in *Rhynchostylis retusa* leaf (110.68 ± 4.52 mg QE/g) and lowest content in *Gastrochilus acutifolius* root (22.32 ± 1.10 mg QE/g). Again, the total polyphenols contents varied with highest content in *Trudelia cristata* stem (69.68±2.78 mg GAE/g) and lowest content in *Gastrochilus acutifolius* leaf (11.89±0.64 mg GAE/g). Furthermore, the relationship of antioxidant activity to total flavonoids and total polyphenols content was positively associated. Hence, it can be claimed that the ethanol extract of selected wild orchids of Nepal show significantly varying antioxidant activity. Also, their medicinal property is attributed to antioxidant activity.

CHAPTER SEVEN: RECOMMENDATIONS

Since the ethanol extracts of medicinal orchids among the selected orchids have higher total polyphenolics and flavonoids content and antioxidant activity further pharmacological and clinical studies for these species is recommended. Other orchids in this study viz., *Eria graminifolia*, *Gastrochilus acutifolius*; *Gastrochilus distichus* have total polyphenols and flavonoids content and antioxidant activity. Phytochemical and pharmacognostic researches such as isolation of biologically active compounds are suggested to carry out in future. Further, it is recommended to assess them on the basis of chemotaxonomy; their total polyphenols and flavonoids contents, and antioxidant activity considering ecological aspects and physiological stresses.

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ANNEX I

Table.2. Dry powder yield (DPY) and dry extract yield (DEYF and DEYP) from different organs of selected orchids.

S. N.	Name of Orchid	Part Used	Code	FW(g)	DP(g)	DE(g)	DPY(%)	DEYF(%)	DEYP(%)
1	*Eria graminifolia*	Pseudobulbs	Egp	250	25	3	10	1.2	12
2	*Gastrochilus acutifolius*	Leaf	Gal	200	20	2.5	10	1.25	12.5
		Root	Gar	300	25	4	8.33	1.33	16
3	*Gastrochilus distichus*	Whole plant	Gdw	200	25	3.5	12.5	1.75	14
4	*Luisia trichorhiza*	Leaf	Ltl	400	40	5	10	1.25	12.5
		Root	Ltr	200	22	2.5	11	1.25	11.36
5	*Otochilus albus*	Pseudobulbs	Oap	1000	50	5.5	5	0.55	11
6	*Papillionanthe uniflora*	Whole plant	Puw	250	20	2.5	8	1	12.5
7	*Pholidota articulata*	Leaf	Pal	150	15	2	10	1.33	13.33
		Pseudobulbs	Pap	350	30	3.5	8.57	1	11.67
8	*Rhynchostylis retusa*	Leaf	Rrl	250	20	2	8	0.8	10
9	*Trudelia cristata*	Leaf	Tcl	250	20	2	8	0.8	10
		Stem	Tcs	100	15	2	15	2	13.33

Here, **FW** = Fresh Weight of material; **DP** = Dry Powder; **DE** = Dried Extract; **DPY** = Dry Powder Yield; **DEYF** = Dried Extract Yield from Fresh material; **DEYP** = Dried Extract Yield from Powder.

Table.3. Total flavonoids content in ethanol extracts of selected orchids

S. N.	Name of Orchid	Part Used	Code	TFC [1]	(Mean ± SD) mg QE/g
1.	*Eria graminifolia*	pseudobulbs	Egp	42.680	40.63 ± 1.85 [d]
				39.104	
				41.099	
2.	*Gastrochilus acutifolius*	Leaves	Gal	31.343	29.09 ± 1.99 [e]
				27.562	
				28.358	
		Roots	Gar	23.582	22.32 ± 1.1 [f]
				21.592	
				21.791	
3.	*Gastrochilus distichus*	Whole plant	Gdw	26.766	22.99 ± 3.28 [f]
				21.194	
				20.996	
4.	*Luisia trichirhiza*	Leaves	Ltl	53.432	48.19 ± 4.57 [c]
				45.074	
				46.069	
		Roots	Ltr	35.522	32.87 ± 2.31 [e]
				31.741	
				31.343	
5.	*Otochilus albus*	Pseudobulbs	Oap	23.383	23.05 ± 0.3 [f]
				22.786	
				22.985	
6.	*Papillionanthe uniflora*	Whole plant	Puw	93.830	90.58 ± 2.82 [b]
				89.054	
				88.855	
7.	*Pholidota articulata*	Leaves	Pal	95.422	95.09 ± 0.94 [b]
				94.029	
				95.820	
8.	*Rhynchostylis retusa*	Pseudobulbs	Pap	25.572	24.84 ± 0.7 [f]
				24.179	
				24776	
		Leaves	Rrl	115.522	110.68 ± 4.52 [a]
				106.567	
				109.950	
9.	*Trudelia cristata*	Leaves	Tcl	86.069	83.48 ± 2.41 [b]
				81.293	
				83.084	
		Stem	Tcs	29.552	27.96 ± 1.7 [e]
				26.169	
				28.159	

Table.4. Total polyphenolics content in ethanol extract of selected orchids.

S. N.	Name of Orchid	Part Used	Code	TPC [2]	(Mean±SD) mg GAE/g
1.	*Eria graminifolia*	Pseudobulbs	Egp	34.583	30.04±4.78 [d]
				30.509	
				25.046	
2.	*Gastrochilus acutifolius*	Leaves	Gal	11.157	11.89±0.64 [e]
				12.361	
				12.175	
		Roots	Gar	18.472	19.52±1.36 [e]
				19.027	
				21.064	
3.	*Gastrochilus distichus*	Whole plant	Gdw	23.750	22.29±1.90 [e]
				20.138	
				23.009	
4.	*Luisia trichirhiza*	Leaves	Ltl	42.268	41.77±2.99 [c]
				38.564	
				44.490	
		Roots	Ltr	51.805	51.03±0.70 [b]
				50.416	
				50.879	
5.	*Otochilus albus*	Pseudobulbs	Oap	32.638	33.04±0.47 [d]
				33.564	
				32.916	
6.	*Papillionanthe uniflora*	Whole plant	Puw	38.194	40.29±2.94 [cd]
				39.027	
				43.657	
7.	*Pholidota articulata*	Leaves	Pal	32.175	33.10±1.22 [d]
				32.638	
				34.490	
		Pseudobulbs	Pap	59.490	57.66±1.59 [b]
				56.527	
				56.990	
8.	*Rhynchostylis retusa*	Leaves	Rrl	32.175	38.93±5.86 [cd]
				42.638	
				41.990	
9.	*Trudelia cristata*	Leaves	Tcl	35.324	39.42±3.59 [cd]
				40.972	
				41.990	
		Stem	Tcs	72.731	69.68±2.78 [a]
				69.027	
				67.268	

Here, 1 = $^{a-f}$ TFC (Mean±SD) with different superscript differ at P = 0.05.

2 = $^{a-e}$ TPC (Mean±SD) with different superscript differ at P = 0.05.

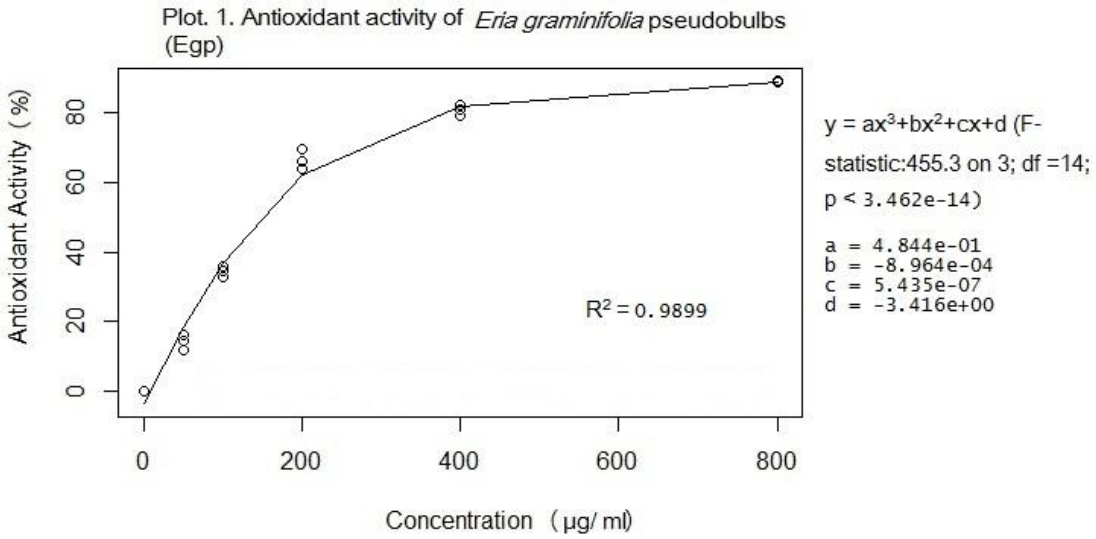
Table.5. One-Sample Test values for total polyphenolics and flavonoids content

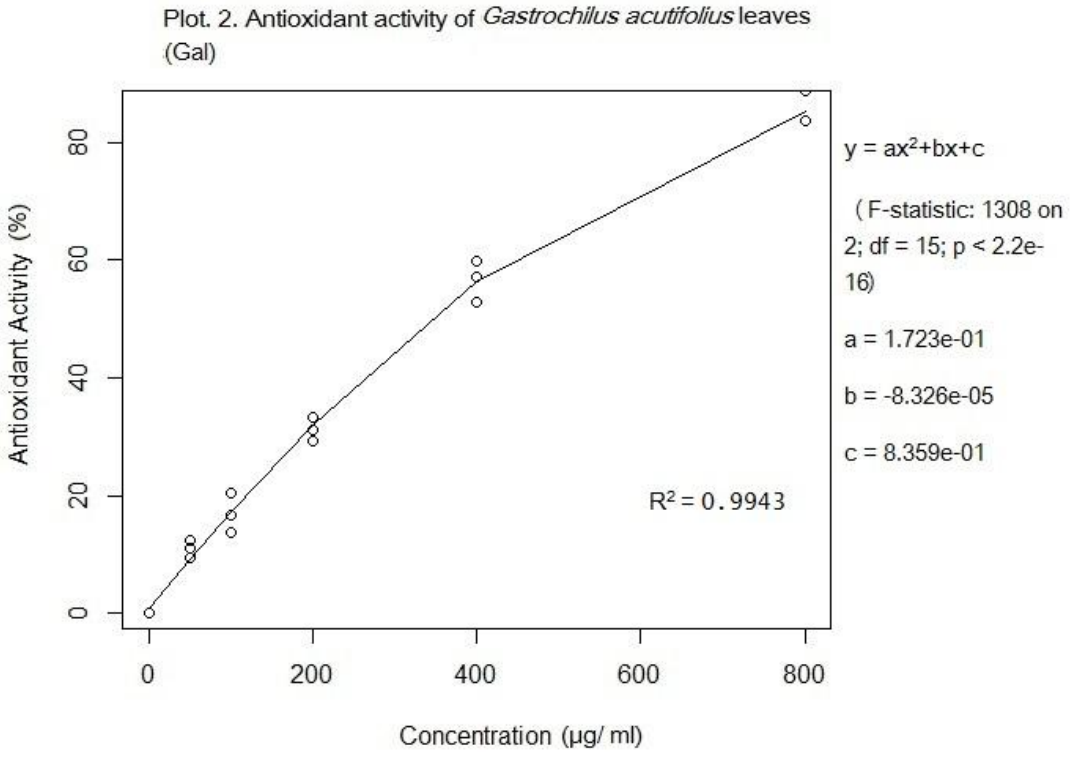
	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
tfc	9.888	38	.000	50.13650	39.8715	60.4015
tpc	15.174	38	.000	37.59378	32.5782	42.6094

Table.6. Percentage antioxidant activity of orchid extracts at different concentrations

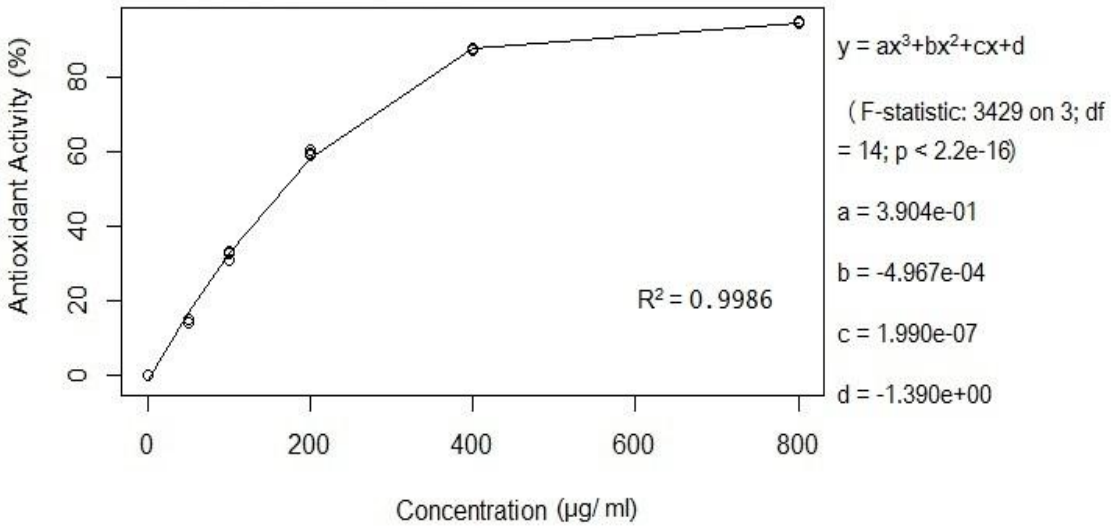
Concentration (µg/ ml)	Antioxidant activity of extracts (%)													
	Egp	**Gar**	**Gdw**	**Tcl**	**Ltr**	**Rrl**	**Pal**	**Tcs**	**Ltl**	**Gal**	**Oap**	**Puw**	**Pap**	**Qrctn**
800	89.22	94.74	93.75	90.16	88.62	92.57	83.33	92.16	93.23	83.65	87.02	92.82	86.59	98.43
800	88.59	94.74	93.44	91.56	88.02	92.41	85.15	91.91	93.23	83.65	86.94	92.91	85.81	98.53
800	88.75	95.34	94.22	92.5	88.20	93.48	83.42	92.16	93.89	88.67	88.58	92.65	86.33	96.53
400	80.63	87.65	90.16	91.88	66.81	91.42	63.28	88.70	90.43	57.27	80.45	85.90	84.17	93.04
400	78.91	87.85	82.97	92.03	66.47	93.23	64.03	89.93	89.36	52.94	82.09	85.38	84.08	96.04
400	81.88	87.25	90.47	91.41	63.90	92.08	61.14	90.35	88.04	59.95	84.69	82.96	83.22	97.04
200	63.59	59.11	65.16	75.47	47.47	76.90	48.02	77.72	78.38	29.32	58.48	59.86	61.25	95.05
200	69.38	60.73	57.19	73.91	45.51	65.43	48.60	77.56	63.20	31.14	62.37	48.18	78.98	96.05
200	65.94	59.72	61.09	78.91	52.61	73.51	46.86	80.61	60.56	33.30	61.94	55.80	85.64	92.05
100	35.94	31.17	32.97	51.41	28.31	47.52	40.92	58.25	48.35	16.52	35.90	38.32	58.56	92.99
100	32.66	32.99	31.56	50.47	28.66	47.28	39.27	58.25	44.14	20.33	33.13	37.98	54.41	93.99
100	34.53	33.40	32.66	54.06	28.40	47.61	35.89	63.37	45.13	13.75	34.95	36.42	50.78	89.99
50	14.69	15.18	15.62	30	20.44	40.59	26.24	44.97	30.94	10.90	22.23	27.42	42.82	90.43
50	12.03	14.37	17.97	30.94	19.07	42.00	26.82	44.14	34.49	9.34	18.60	26.38	37.63	91.43
50	16.09	14.98	16.72	32.97	18.65	41.17	34.32	44.06	36.63	12.37	19.46	26.64	37.28	87.43
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Plots : Antioxidant activity (%) against concentration of orchid extracts for the calculation of IC_{50} value

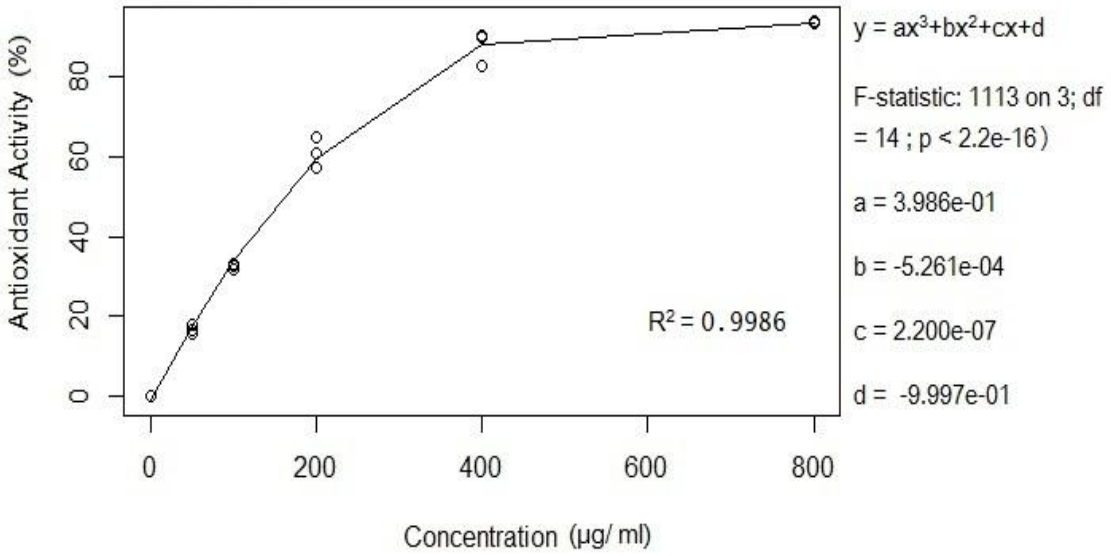




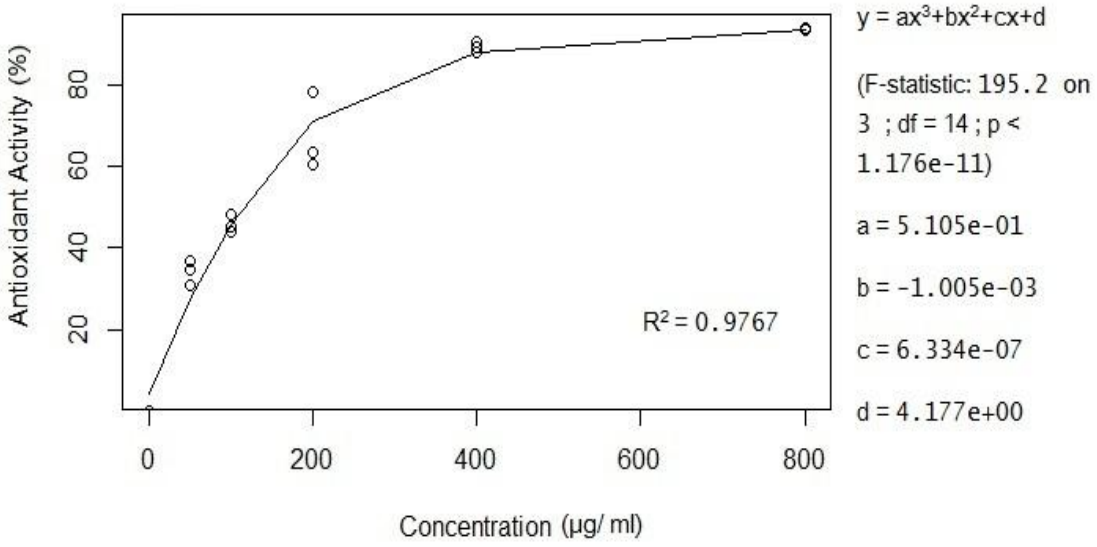
Plot. 3. Antioxidant activity of *Gastrochilus acutifolius* root (Gar)



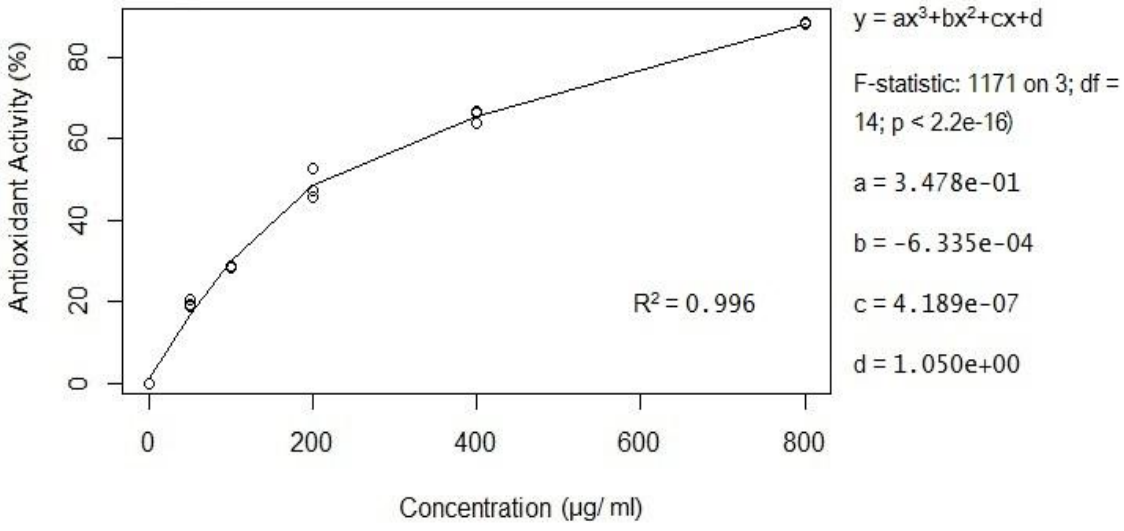
Plot. 4. Antioxidant acitivity of *Gastrochilus distichus* whole plant (Gdw)



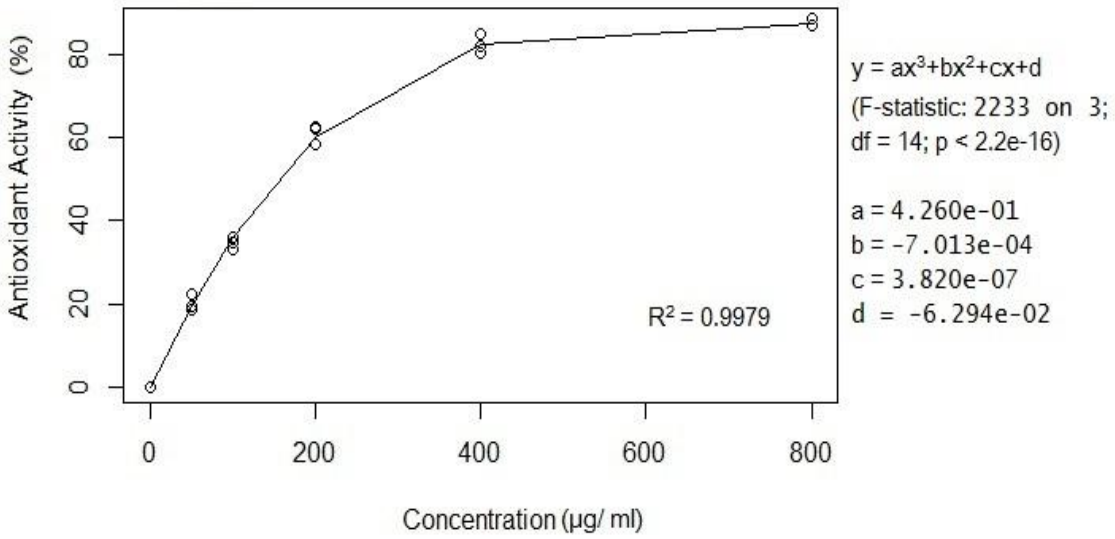
Plot. 5. Antioxidant activity of *Luisia trichorhiza* leaf (Ltl)



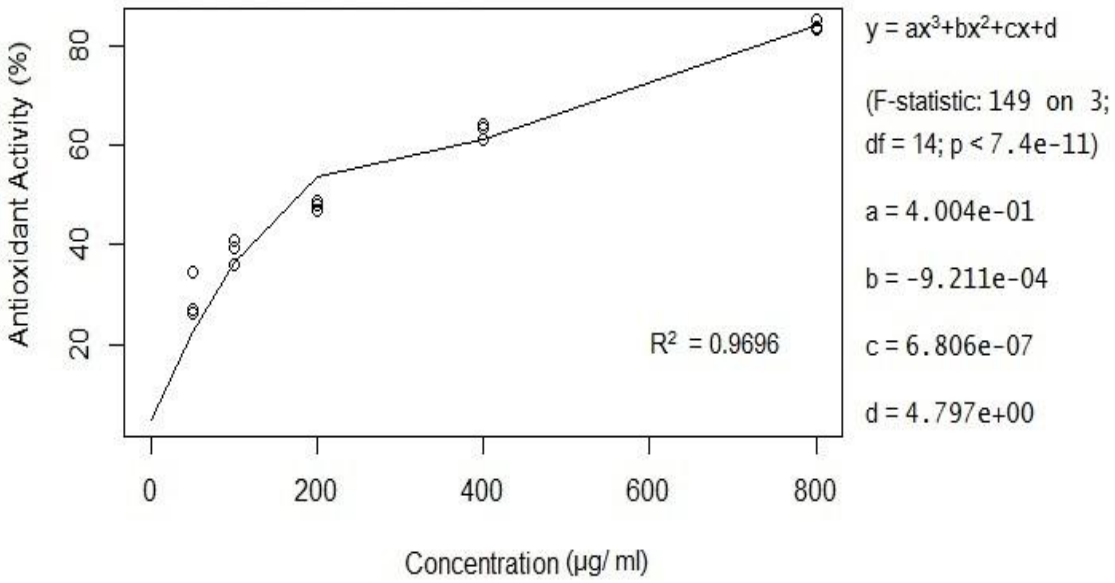
Plot. 6. Antioxidant activity of *Luisia trichorhiza* roots (Ltr)



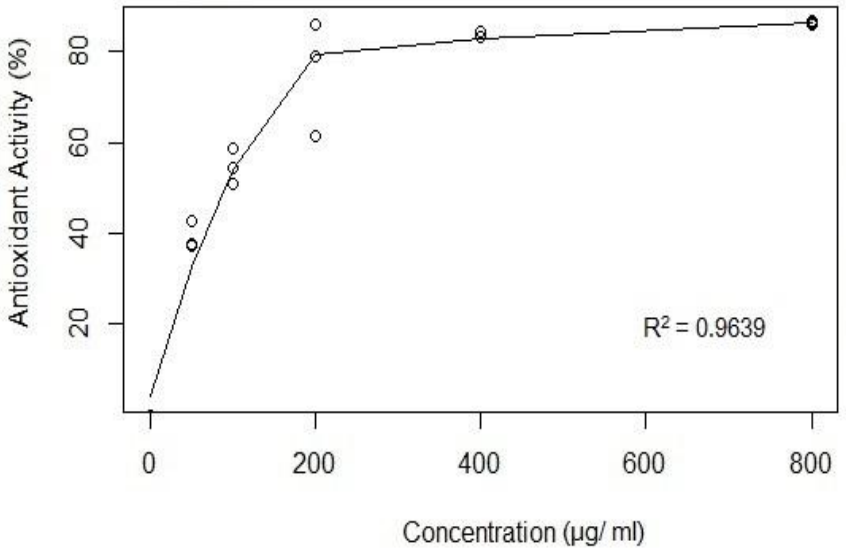
Plot. 7. Antioxidant activity of *Otochilus albus* pseudobulbs (Oap)



Plot. 8. Antioxidant activity of *Pholidota articulata* leaves (Pal)



Plot. 9. Antioxidant activity of *Pholidota articulata* pseudobulbs (Pap)

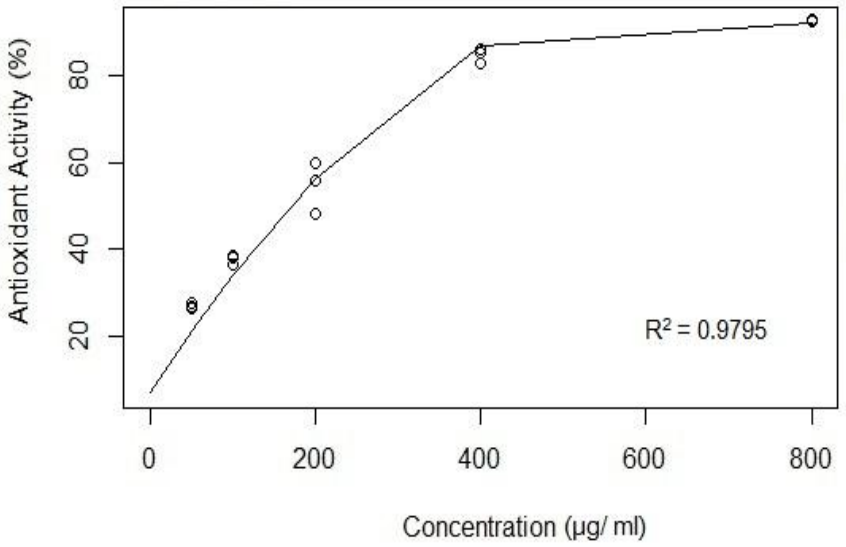


$y = ax^3+bx^2+cx+d$

(F-statistic: 124.6 on 3; df = 14; p < 2.475e-10)

a = 6.440e-01
b = -1.564e-03
c = 1.109e-06
d = 4.220e+00

Plot. 10. Antioxidant activity of *Papilionanthe uniflora* whole plant (Puw)

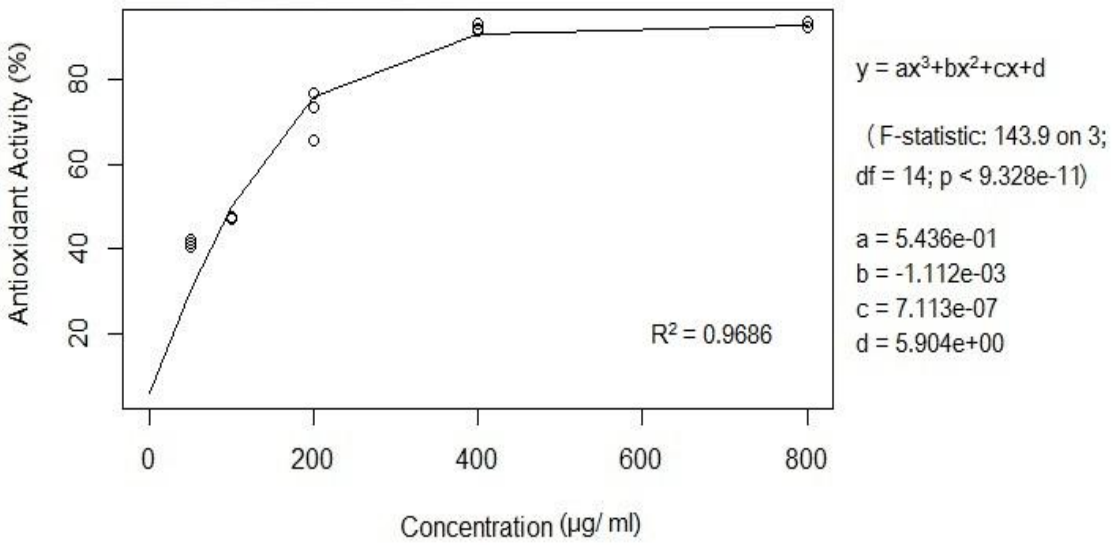


$y = ax^2+bx+c$

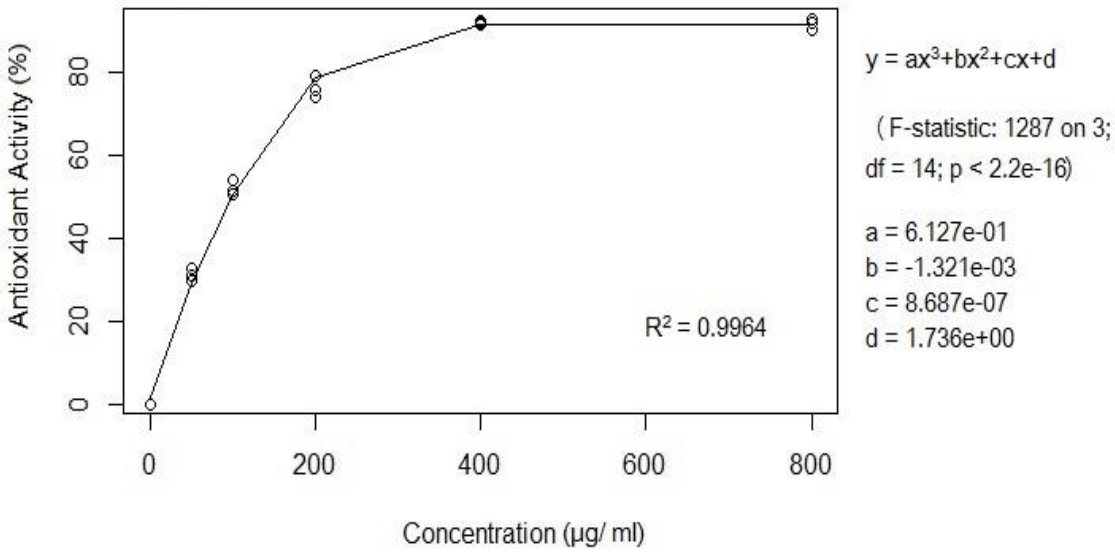
(F-statistic: 358.7 on 2; df = 15; p < 2.164e-13)

a = 2.926e-01
b = -2.322e-04
c = 6.826e+00

Plot. 11. Antioxidant activity of *Rhynchostylis retusa* leaves (Rrl)



Plot. 12. Antioxidant activity of *Trudelia cristata* leaves (Tcl)



Plot. 13. Antioxidant activity of *Trudelia cristata* stem (Tcs)

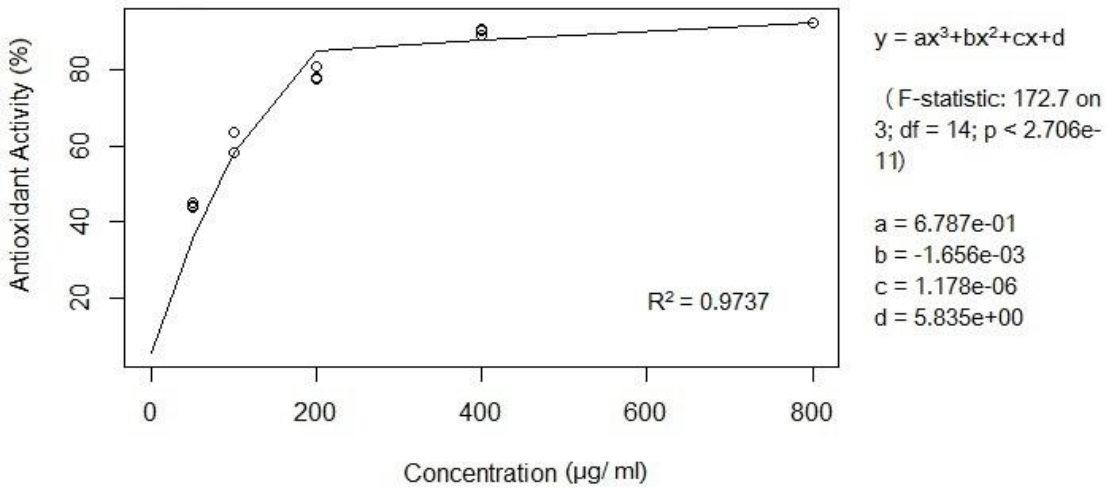


Table.7. IC_{50} of ethanol extract of selected orchids and quercetin

S.N.	Name of orchid/ compound	Part Used	Code	IC_{50} (µg/ml)
1.	*Eria graminifolia*	pseudobulbs	Egp	143.6
2.	*Gastrochilus acutifolius*	Leaves	Gal	341.79
		Roots	Gar	163.37
3.	*Gastrochilus distichus*	Whole plant	Gdw	159.15
4.	*Luisia trichirhiza*	Leaves	Ltl	113.18
		Roots	Ltr	209.78
5.	*Otochilus albus*	pseudobulbs	Oap	152.57
6.	*Papillionanthe uniflora*	Whole plant	Puw	170.67
7.	*Pholidota articulata*	Leaves	Pal	172.84
		pseudobulbs	Pap	89.18
8.	*Rhynchostylis retusa*	Leaves	Rrl	100.42
9.	*Trudelia cristata*	Leaves	Tcl	98.23
		Stem	Tcs	79.69
10.	Quercetin		Qrctn	32.90

ANNEX II

Photo plate I: Selected wild orchids

Plate.1. *Eria graminifolia*

Plate.2. *Gastrochilus acutifolius*

Plate.3. *Gastrochilus distichus*

Plate.4. *Luisia trichorhiza*

Plate.5. *Otocilus albus*

Plate.6. *Papillionanthe uniflora*

Plate.7. *Pholidota articulata*

Plate.8. *Rhynchostylis retusa*

Plate.9. *Trudelia cristata*

Photo plate II: Instruments

Plate.1. Electric blender

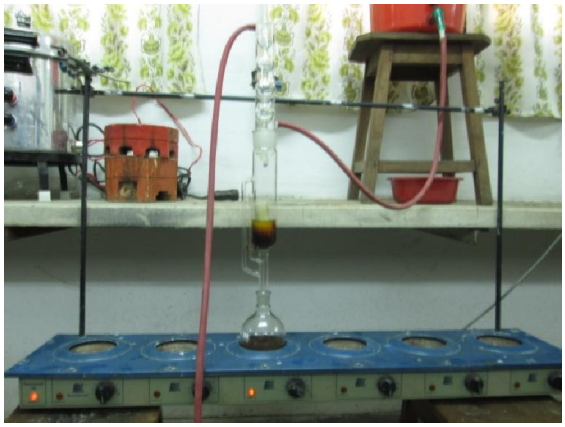


Plate.2. Soxhlet apparatus

Plate.3. Rotavapor

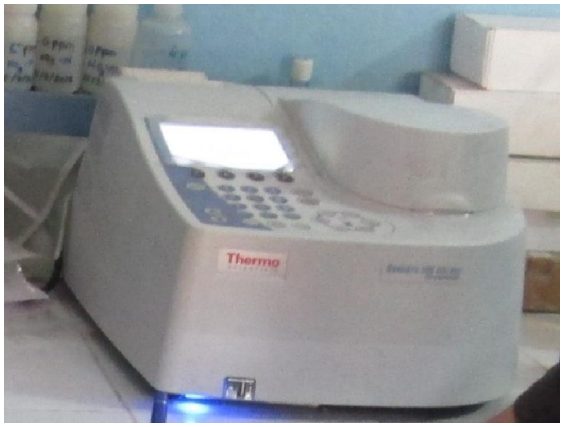


Plate.4. UV-Visible Spectrophotometer

Photo plate III: Grinded powder of selected orchids

Plate.1. *Gastrochilus acutifolius* roots

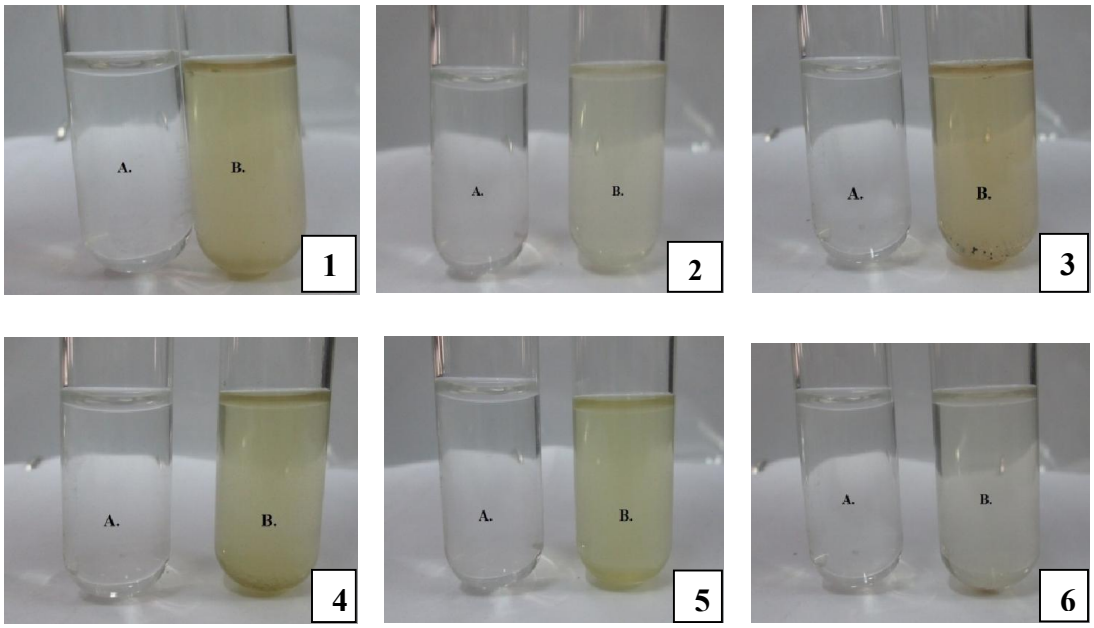
Plate.2. *Otochilus albus* pseudobulbs

Plate.3. *Rhynchostylis retusa* leaves

Plate.4. *Trudelia cristata* stem

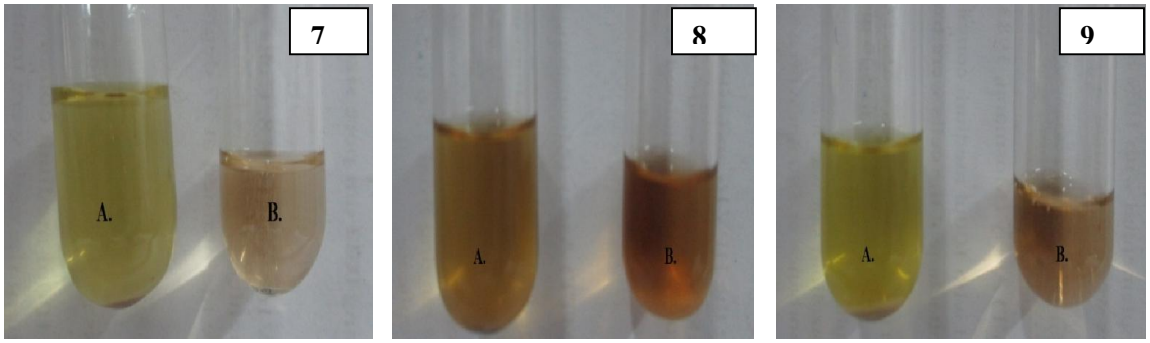
Photo plate IV: Preliminary phytochemical screening

Plate.1-6: Presence of Alkaloids in selected orchids



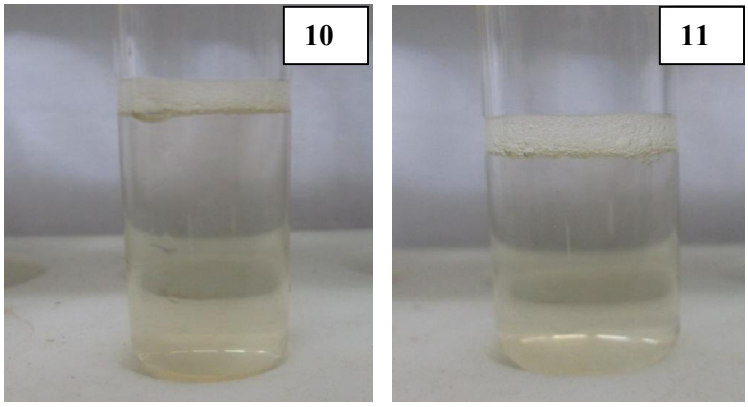
Here, **1.** *Gastrochilus acutifolius* root; **2.** *Luisia trichorhiza* root; **3.** *Pholidota articulata* leaf; **4.** *Rhynchostylis retusa* leaf; **5.** *Trudelia cristata* leaf; **6.** *Trudelia cristata* stem. **A** = 'Extract only'; **B** = 'Extract and reagent only".

Plate.7-9: Presence of Flavonoids in selected orchids



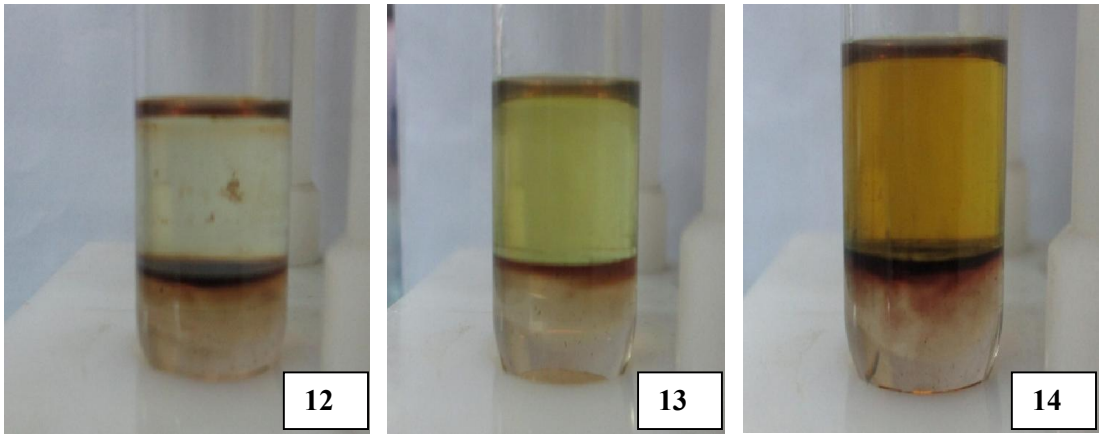
Here, **7.** *Eria graminifolia* pseudobulbs; **8.** *Gastrochilus acutifolius* leaf; **9.** *Luisia trichorhiza* leaf; **A** = "Extract only"; **B** = "Extract and reagent complex"

Plate.10-11: Presence of Saponins in selected orchids



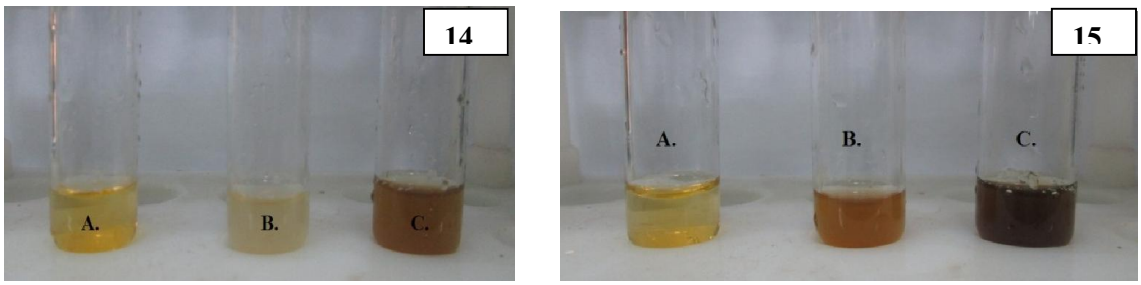
Here, **10.** *Gastrochilus acutifolius* roots; **11.** *Gastrochilus distichus* whole plant.

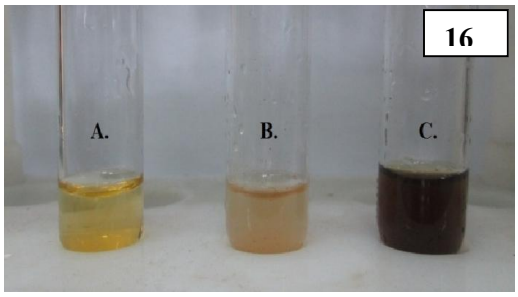
Plate.12-14: Presence of Steroids and terpenoids in selected orchids

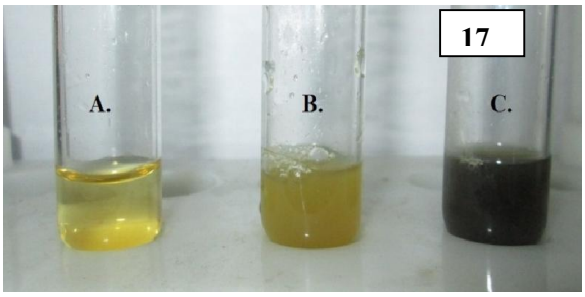


Here, **12.** *Gastrochilus distichus* whole plant; **13.** *Luisia trichorhiza* leaf; **14.** *Pholidota articulata* pseudobulbs;

Plate.14-24: Presence of Tannins in selected orchids







Here, **14.** *Luisia trichorhiza* root; **15.** *Pholidota articulata* leaf; **16.** *Pholidota articulata* pseudobulbs; **17.** *Trudelia cristata* leaf; **A**= "Reagents only", **B** = "Extract only"; **C** = "Extract and reagent complex"

Photo plate V: Total polyphenolics and flavonoids content estimation and DPPH free radical scavenging activity

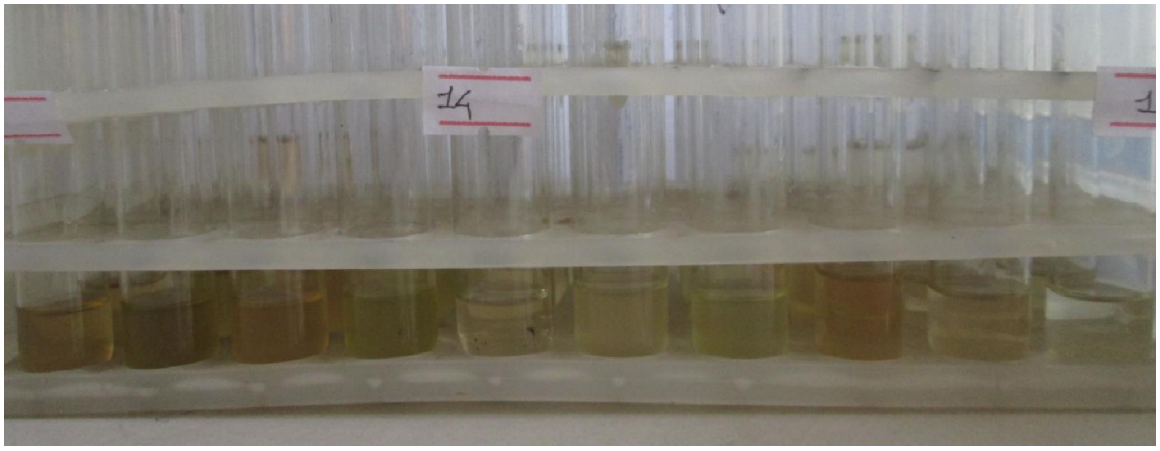


Plate.1. Orchid extracts-aluminium chloride complex after an hour of incubation during total flavonoids content estimation

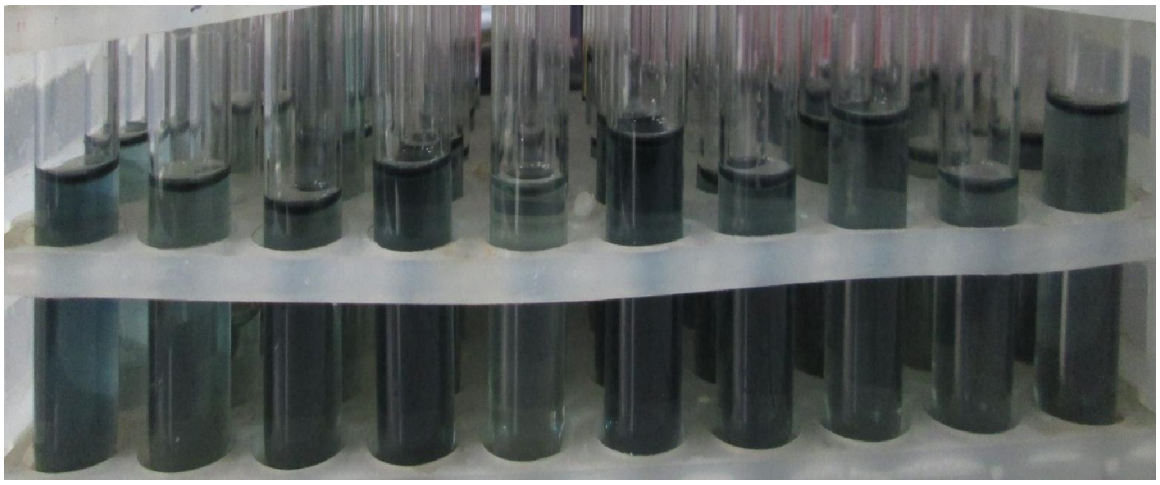


Plate.2. Orchid extracts-Folin-Ciocalteu reagent complex after 45 minutes of incubation during polyphenolics content estimation

Presented Papers

Chand M B., Paudel M R., and Pant B. 2014. Estimation of Total Polyphenol and Flavonoid Contents of In-vitro Grown Selected Medicinal Orchids of Nepal. National Conference on Biotechnology (22-23 Nov. 2014), Kathmandu, Nepal.

Paudel M R., **Chand M B**., Pradhan S., and Pant B. 2014. Medicinal Orchids of Daman: Diversity, Conservation and Phytochemical Analysis. National seminar on The Importance of Research in Medicinal Orchid Conservation and its Linkage with Local Livelihoods (9-10 June 2014), Kathmandu, Nepal.