Journal of Medicinal Plants Research Vol. 7(12), pp. 706-712, 25 March, 2013
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.446
ISSN 1996-0875 ©2013 Academic Journals

Full Length Research Paper

Antioxidant potential and secondary metabolites in *Ocimum sanctum* L. at various habitats

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Accepted 3 May, 2011

In this study, the secondary metabolites and antioxidant property of holy basil (*Ocimum sanctum* Linn.) Plant at various habitats was compared with those of respective callus cultures induced from each explants in *in-vitro*. The callus cultures were successfully initiated on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) (1 mg/L) combined with different concentrations (0.1 to 1.0 mg/L) of kinetin as plant growth regulators. The distribution of flavonoids and phenolic compounds in the plant extracts were analyzed by using Aluminium (III) chloride colorimetric assay with standards. Flavonoids were found in all callus extracts in comparison with wild plant parts at various habitats. In this study, the antioxidant activity of the extracts was evaluated i*n vitro* antioxidant-testing systems. The secondary metabolites of flavonoid and phenolic acid contents of the *O. sanctum* were studied in different habitats and *in-vitro* callus culture extract. Among these studies hills and wet land habit plants showed maximum secondary metabolites than the other habitats. The antioxidant potential was studied by dot blot assay. Among these results, we concluded that, the environmental stress factors such as dryness, temperature, salt and soil pH is an essential factor for release of secondary metabolites antioxidant potent of *O. sanctum* L.

Key words: *Ocimum sanctum*, callus culture, secondary metabolites, antioxidant activity.

INTRODUCTION

Natural products are important sources for biologically active drugs. There has been an increasing interest in the study of medicinal plants as natural products in different parts of the world (Gazzaneo et al., 2005). Medicinal plants containing active chemical constituents with high antioxidant property play an important role in the prevention of various degenerative diseases (Lukmanul et al., 2008) and have potential benefits to the society. The medicinal value of these plants depends on bioactive phytochemical constituents that produce definite physiological action in the human body. Some of the most important bioactive phytochemical constituents include alkaloids, flavonoids, phenolics, essential oils, tannins and saponins (Krishnaiah et al., 2009). Phenolics are commonly found in medicinal plants and have been reported to have multiple biological effects, including antioxidant activity. Potential sources of antioxidants have been found in leaves, oilseeds, barks and roots (Marija et al., 1999). Natural antioxidants from plant sources are potent and safe due to their harmless nature,

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wild herbs have been investigated for their antioxidant properties (LeeKoon et al., 2004).

Among the various medicinal and culinary herbs, some endemic species are of particular interest because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et al., 2002; Jaleel et al., 2006). The preservative effect of many plant species and herbs suggests the presence of oxidative and antimicrobial constituents in their tissues (Jaleel et al., 2007a- e). Many medicinal plants contain large amounts of antioxidants other than vitamin C, vitamin E and carotenoids (Jaleel et al., 2008). Most of the antioxidative potential of herbs and spices is due to the redox properties of their phenolic compounds, which permit them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Caragay et al., 1992). Plant phenolic compounds are mostly secondary metabolites possessing high antioxidant activity and are widespread in the species of Lamiaceae (Chang et al., 1977; Gang et al., 2001). Advances in the area of tissue culture for the production of secondary metabolites by callus culture have made it possible for the increased yield of a wide variety of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenolics, and flavonoids (Ramachandra et al., 2002).

The plants of genus *Ocimum* belonging to family Lamiaceae are rich in phenolic compounds and are very useful for their therapeutic potentials. They are widely used in traditional systems of medicine (Prakash et al., 2005). *Ocimum sanctum L.* are examples of species of genus *Ocimum,* which grow in different parts of the world and are known to have medicinal properties (Maria et al., 2008). *O. sanctum L.,* known as 'Tulsi' in Hindi and 'Holy Basil' in English, is an aromatic herb found throughout India. In traditional systems of medicine, different parts (leaves, stem, flower, root, seeds and even whole plant) of *O. sanctum* have been recommended for the treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, chronic fever and insect bite etc. *O. sanctum* L. has also been suggested to possess anti-fertility, anticancer, anti-diabetic, anti-fungal, anti-microbial, hepatoprotective, cardioprotective, analgesic and adaptogenic properties. In Ayurveda, *O. sanctum* has been well documented for its therapeutic potentials (Prakash et al., 2005). *O. sanctum* to be used in the pharmaceutical industry to produce drugs that are beneficial, one outstanding obstacle, which is the standardization of quality and quantity of the compounds extracted from the plant itself, has to be overcome. As discovered by various researches, even close members of the same genus (*Ocimum*) do not possess the same chemical constituents (Singh and Sehgal, 1999).

Razdan (2003) revealed that as *O. sanctum* is able to cross-pollinate with other plants of the similar genus, certain plants would not be true-to-type, and if there are genetic variations in the plant, the chemical constituents would be different. This is where plant tissue culture could be applied and it helps to solve the problem, as plant tissue culture produces offspring that are identical to the parent plant. In consideration of the role that callus plays in micropropagation, as well as to estimate the potential of the usage of callus to extract secondary metabolites, hence the present study was carried out to identify the best treatment for callus induction from the leaf explants of *O. sanctum*. Apart from that, the total flavonoids content in leaf-derived callus and *in vivo* leaf tissues of *O. sanctum* was also compared.

Establishment of protocols for cultivation of medical plants using different growth regulators to enhance the production of bioactive compounds is required for comercial and research application. Bioactive compounds were found to be accumulating in culture cells at higher level than those in natural plants though optimization of culture conditions (Mulabagal et al., 2004). Cytokinins are plant growth regulators used for stimulating cell division, as well as for the formation and growth of auxiliary and shoots. This group consisted of the naturally occurring cytokinins which include zeatin and adenine and another type is synthetic cytokinins that consist of substituted purine, 6-benzylamino-purine and kinetin. The concentration of macro-and micro elements of culture medium without or with activated charcoal (AC) remarkably affected rhixogensis behaviour as showed by Sakr et al. (1999). The callus has potential to show metabolic activity and can be compared in this respect with mother plants. For callus induction and growth, an exogenous auxin supply of regulators is often recommended to initiate callus formation from explant. Particularly, auxins effect growth, callus formation Gamborag et al. (1995). It was found that naphthalene acetic acid (NAA) and 2,4-D was the most active compounds.

Antioxidants are important in the prevention of human diseases. Antioxidant compounds may function as free radical scavengers, complexing agents for pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation (Ric-Evans et al., 1997). Thus, every part of the plant has useful application; even today people use different parts of this plant for treatment of various ailments based on traditional knowledge. However, in the modern scientific world such claims warrant scientific proof and validation. Although the ancient traditional claims about medicinal properties of *Tulsi* are being investigated scientifically, majority of these studies are only limited to *in-vitro* and experimental animal models only. Studies on human subjects are very few. Therefore, an effort has been made to review various scientific studies that have considerably contributed on various

Table 1. Collection of plant (*Ocimum sanctum)* sample.

Sampling area	Location
Hill area	Yercaud
Dry land	Dharmapuri
Wet land	Erode

aspects of the plant *O. sanctum* (Linn) and described under specific headings.

Although antioxidant properties of ethanol extract of holy basil leaves have already been reported (Juntachote et al., *2005*), In the present study, we have attempted for the first time to compare the antioxidant properties between holy basil plant parts (leaves, stem, and inflorescence) and *in vitro* callus cultures induced from each explants including total phenolic content (TPC) (qualitative–quantitative composition), antioxidant activity. We have found significantly higher levels of antioxidant activity in the callus extracts than in the field-grown plant parts of holy basil. Since holy basil extract is one of the important herbal formulations in ayurvedic medicine as a tonic for treating many diseases, this study adds further value for the possible use of this plant, especially the *in vitro*-induced callus extract, as a food additive.

MATERIALS AND METHODS

The young leaf, shoot tip, node, internodes of *O. sanctum* was collected from different locations like hills, dry land, and wet land for this study. These samples were brought to the laboratory and kept in refrigerator for callus induction and secondary metabolite studies (Table 1).

In-vitro culture-callus formation

Media preparation

The most popular medium of MS media was used for study the chemical used for this experiment were obtained from Hi-media laboratory Pvt. Ltd, Mumbai in India. The major and minor nutrients were added in 500 ml double distilled water in 1 L standard flask. All the hormones were used this study obtained from Hi-media laboratories Pvt. Ltd, Mumbai, India. Various auxins and cytokines were used in this study as hormone sources 2,4-D, where used auxins and BAP was used as cytokine. The auxins were added to culture medium at a concentration range varying from 0.1 mg l^{-1} to 1 mg l^{-1}. The auxins were added for the regulation of cell elongation, tissue swelling and cell division particularly in callus formation.

Surface sterilization

Young leaves were placed in a clean beaker and were rinsed under running tap water for 30 min before the initiation of surface sterilization. The young leaves were immersed in 25% (v/v) clorox containing three drops of Tween 20 for 10 min. The young leaves were then rinsed with sterile distilled water several times until all traces of clorox were eliminated. The sterilized young leaves were cut into 5 × 5 mm in size and were transferred to the medium with sterile forceps.

Basal medium

Murashige and Skoog (1962) MS medium was used as the basal medium. Sucrose at 3% (w/v) was added into the mixture. The pH of the medium was adjusted to 5.7±0.1 with 0.1 M HCl or 0.1 M NaOH followed by addition of 0.8% (w/v) agar. The medium was then autoclaved at 121°C, 15 psi for 15 min. After autoclaving, a total of 25 ml of the sterile medium was poured into culture tubes in the laminar flow, and was allowed to solidify. The culture tubes were then sealed prior to the initiation of treatments (Zi Xiong et al., 2009).

Callus induction in single auxin treatments

In order to study the effects of various concentrations of different auxins on callus induction from the leaf explants, the MS medium was supplemented with different auxins concentrations. The auxins treated were 2, 4-Dichlorophenoxyacetic acid (2, 4 –D) and Indole-butyric acid (IBA), at the concentrations of 0.1 to 0.5 mg/L. MS medium devoid of plant growth regulator was used as the control.

Callus induction in combination treatments

At first the auxin alone used for experimental studies, then 1 mg/L of 2, 4-D was further combined with kinetin in order to study the effects of combination of auxin and cytokinin on callus induction. The concentrations of cytokinins examined were 0, 0.1, 0.5, 1.0 and 2.0 mg/L. The control for the experiment was MS medium lacked of plant growth regulator.

Culture conditions

All the cultures were maintained in the culture room at 25±1°C, under photoperiod of 16 h light, 8 h dark provided by white fluorescent tubes with the intensity of 1000 lux. The cultures were incubated for 30 days and daily observations were made to monitor the day of initial callus formation.

Data collection

The day of initial callus formation, the morphology and colour of the callus were recorded. At the end the observation period, percentage of the explants forming callus as well as the degree of callus formation was measured.

Extraction procedure for field-grown plant parts

Leaves and stems of *O. sanctum* were harvested from field grown plants and shade-dried for 7 days. The dried materials were powdered using a mechanical grinder. From these 10 g of each powdered material was extracted twice with 500 ml of methanol continuously. Thereafter, the resulting methanolic extract was

reduced in vacuum (40°C), freeze-dried and stored at 4°C until further use in the experiment.

Callus extraction procedure

Callus induced on MS medium was harvested and dried, and the dry weight was determined (Szabo et al., 1999). One gram (dry weight) of callus was soaked in 10 ml of 80% methanol for 3 h and sonicated in an ultrasonic sonicator at 20 pulses for 20 min. The extract was centrifuged at 10000 rpm for 10 min. Then the supernatant was concentrated under vacuum (40°C), freeze-dried, and stored at 4°C until further use in the experiment.

Analysis of secondary metabolites in callus culture and fresh leafs

Estimation of total flavonoids content

In order to compare the total flavonoid contents between leaf-derived calli and *in vivo* leaves of *O. sanctum*, aluminum (III) chloride colorimetric assay was carried out. The samples or the biochemical tests were *in vivo* young leaves, and a month-old calli induced and maintained in MS medium supplemented with 1 mg/L of 2, 4-Dichlorophenoxyacetic acid (2, 4 –D). Total of 0.5 g of samples were weighed and extracted with 50 ml of 80% (v/v) methanol. The mixtures were then ultrasonicated for 20 min followed by centrifugation at 12,000 revolutions per minute (rpm). Using a pipette, 1 ml of the supernatant as collected into a test tube, and 4 ml of deionized water as added. After that, 0.3 ml of 10% (w/v) $NaNO_2$ were added to the test tubes, and was left to react for 5 min. Then, 0.3 ml of 10% (w/v) $AlCl_3$ was added and was left for 1 min to exact. Lastly, 2 ml of 1 M NaOH was added and the mixtures were shaken. A total of 2 ml of the mixtures were transferred to a cuvette, and the absorbance values of both types of samples were measured using spectrophotometer at 510 nm. A mixture of 1 ml of 80% v/v) methanol, 4 ml of deionized water, 0.3 ml of 10% w/v) $NaNO_2$, 0.3 ml of 10% (w/v) $AlCl_3$ and 2 ml of M NaOH were prepared as the blank. Catechin is assured as a standard in determining the total flavonoids content. From a catechin tock concentration of 100 mg/L, several dilutions were added to prepare a series of concentrations at 0, 10, 20, 40, 0, 80 and 100 mg/L. A standard curve was constructed with the optical density at 510 nm against the concentrations of catechin. The total flavonoids content of he samples were then estimated from the standard curve and further expressed in milligram of catechin equivalent per gram of sample fresh mass (mg /g).

Estimation of total phenols

Total phenol was estimated by the method of Malick and Singh (1980). 500 mg of fresh plant tissue was ground in a pestle and mortar with 10 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was evaporated to dryness. The residue was dissolved with 5 ml of distilled water and used as extract.

To 2 ml of the extract, 0.5 ml of Folin- Ciocalteau reagent was added. After 3 min, 2 ml of 20% NaCO solution was mixed thoroughly. The mixture was 2 3 kept in boiling water for exactly one minute and after cooling the absorbance was read at 650 nm. The total phenol was determined using a standard curve prepared with different concentration of gallic acid. The results were expressed in milligrams per gram fresh weight.

Dot-plot rapid screening method

According to Soler-Rivas et al. (2000), the Dot-blot test is easy, fast and reliable way to compare radial scavenging capacity of various plant extracts. 3 µl of aliquots of fresh and sunshade dried powder sample extract of leafs and *in-vitro* callus of *O. sanctum* were spotted on the TLC plate and allowed to air dry. The TLC plate bearing the dry spots was placed upside down for 10 s in a solution of DPPH (0.1 mM/l) in methanol. The spot exhibiting radial scavenging antioxidant activity showed up as yellow spot against a purple background.

RESULTS

Callus induction

Most naturally occurring antioxidants are secondary metabolites, which include flavonoids, and phenolic acids. The flavanoids, phenolic compounds and anti-oxidant activity of these compounds were studied from *O. sanctum* plants in their leaf and callus culture. The plants were collected from different locations like Hills, dry and wet lands (Figures 1; a, b and c).

Callus induction from *Ocimum sanctum* L using various concentrations auxins

In the present investigation, the percentage of callus induction from leaves and stems was depend on the concentration of 2,4-D, IBA used (Table 2). Leaf explants of *O. sanctum* on MS medium supplemented with 2,4-D (1 mg/L), IBA. 1 mg/L) showed the maximum callus induction, but the same hormone was insufficient for the induction of callus tissues from internodes explants of this plant (Table 3).

Similarly the holy basil leaf explants showed maximum (99%) culture response in the cultures supplemented with the combination of 2,4-D (1 mg/L) and kinetin 0.1 (mg/L), but the same combination was insufficient for maximum culture response from the stem explants. In contrast to leaf explants, the stem explants of holy basil showed the maximum (100 and 99%) culture response on MS medium supplemented with the combination of 2,4-D (1 mg/L) and kinetin (0.5 mg/L) (Table 4). In this study, the total flavonoids content of *O. sanctum* were successfully estimated using aluminium (III) chloride colorimetricassay where by a total of 55 mg/g of total flavonoids in the leaf from hills and the total flavonoids content then the other samples. The leaf in hills showed higher amount of flavonoids than others and they were expressed in Figure 2. In this study, we evaluated the TPC of methanolic extract of *O. Sanctum* (holy basil) leaves and the callus

Table 2. Effect of various concentrations of auxins on callus induction - using leaf, stem explants of *O. sanctum.*

Hormone concentration (mg/l)		Culture response (%)		Callus formation (Day)	
2,4-D	IBA	Leaf	Stem	Leaf	Stem
0.1	0.1	99	85	8	10
0.5	0.5	100	100	7	9
1.0	1.0	100	100	7	9

Table 3. Callus induction from the leaf explants *O. sanctum* after 4 weeks of culture in MS medium supplemented with 1 mg/L of 2,4-D and kinetin at different concentration.

Hormone concentration (mg/L)	Culture response (%)		Callus formation (Day)	
2,4-D+kinitin	Leaf	Stem	Leaf	Stem
1+0.1	99	85	8	10
1+0.5	100	100	7	9
1+1.0	100	100	7	9

Table 4. Total flavonoids analysis.

Secondary metabolite	Plant material	Habitat			
		Hills	Dry land	Wet land	Callus
Flavonoid (mg/g)	Fresh leaf	55	40	38	36

cultures in various habitations (Table 5). The phenolic content of leaves in hills habitats was 60 mg/g and callus culture possess 55 mg/g of extract. Among these results the callus in hills habitat showed maximum yield of phenolic compound than others. The comparative representation was expressed in Figure 3.

Antioxidant activity (Dot-blot rapid screening method)

The result of the dot –blot assay showed colored spots where the aliquots of different extract were placed in rows. The purple area on the plate indicates no free radical scavenging (Antioxidant) activity and yellow areas indicates free radial scavenger or antioxidant activity. Too more intense of the yellow colour indicates the greater the antioxidant activity. The diameter of the colour change was measured (cm) and represented in Table 6.

From this dot blot assay the methanolic extract showed maximum antioxidant potential in leaf extract obtained from hills and dry land. But wet land and callus extract showed lesser activity. Other two extracts (acetone and distilled water) showed lesser activity than the methanolic extract (Figure 4).

DISCUSSION

The antioxidant property of plant products includes their secondary metabolites such as: flavonoids and phenolic acids. The release secondary metabolites from plants that may vary from plant to plant and species to species. Among the species it is also varied according to the environmental influences. So that, this work highlights the sample were collected from the different habitats of the same species. They were also compared with *in-vitro* callus culture cultivated in the laboratory.

From this reports the release of secondary metabolites of flavonoids and phenolic acids showed maximum production in the hills area and dry land plant leaf of *O. Sanctum*. From this report it strongly shows that the plant samples which were collected from hill area posses more secondary metabolites then the dry and wet land. Because, increase in high elevated temperature or low water availability, and brought conditions often are cor-related with high concentrations in the soil. The effects of drought and salt stress on the accumulation of secondary metabolites plant products can be made. In a whole array of experiments it could be shown the plants which are exposed to drought stress indeed produce higher amount of secondary metabolites. This result is similar to earlier

Table 5. Quantitative analysis of total phenolic content.

Secondary metabolite	Plant material	Habitat			
		Hill	Dry land	Wet land	Callus
Phenolic content (mg/g)	Fresh leaf	60	55	50	52

Table 6. Dot blot assay of different extracts.

Solvent	Hill area (cm)	Dry land (cm)	Wet land (cm)	Callus (cm)
Methanol	1.8	1.0	1.0	1.4
Acetone	1.0	0.8	0.7	1.0
Distilled water	1.2	0.5	0.5	1.0

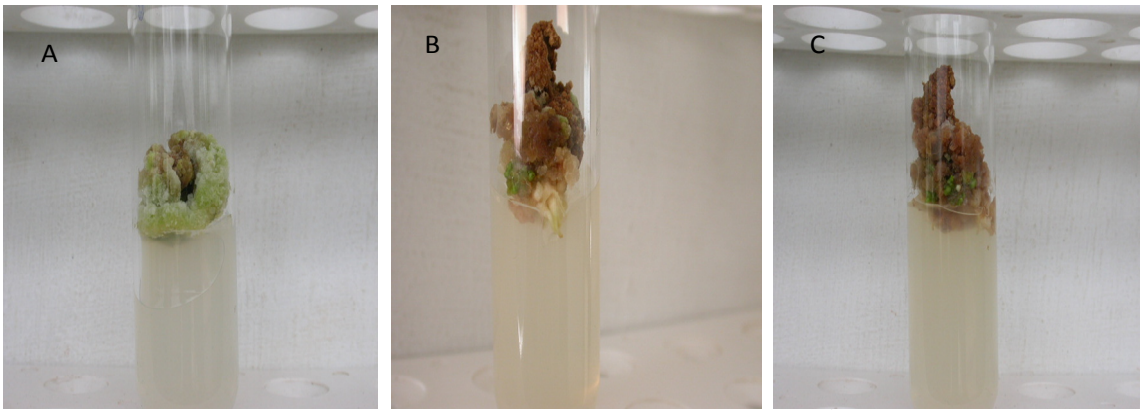

Figure 1. Callus induction from *O. sanctum* L using various concentrations auxins.
(a) Hill area callus, (b) Dry land callus, (c) Wet land callus.

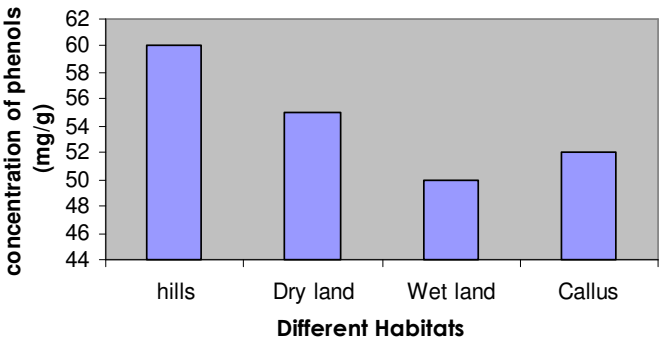

Figure 2. Estimation of phenolic content at various habitats.

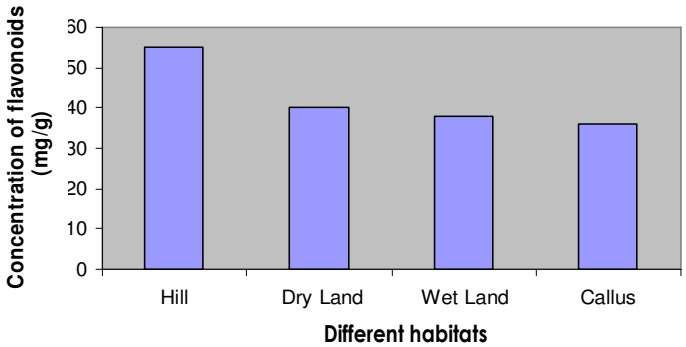

Figure 3. Estimation of flavonoids at various habitats.

work in phenols and terpenoids as well as for nitrogen containing substances, such as: Alkaloids, flavonoids, steroids, these is no doubt that application of drought stress enhances the concentration of secondary metabolites posses more in *O. Sanctum* fresh leaf sample (Lako et al., 2007).

The antioxidant property of the *O. Sanctum* plant was stained by dot blot assay method. From this result, methanolic extracts showed maximum antioxidant potential than the other extracts. This dot blot assay was confirmed these plants having the potential antioxidant property. This work also supported by Lukmanul et al.

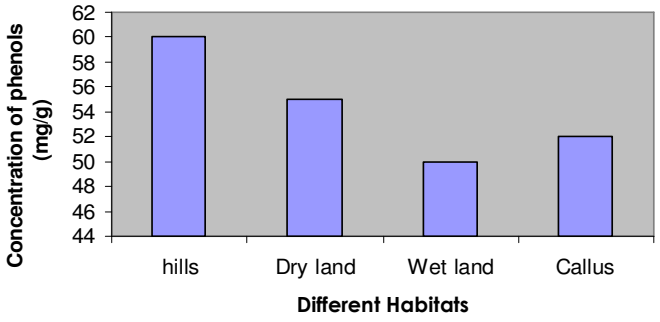

Figure 4. Estimation of phenolic content at various habitats.

(2007). They did the same experiment to conform antioxidant property in *O. sanctum* L. they also compared the antioxidant potential of *in-vitro* grown callus and field grown plants.

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