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Research Article

IN VITRO AND *IN VIVO* DETERMINATION OF PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITY OF DESERT PLANTS OF APOCYNACEAE FAMILY

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ABSTRACT

Antioxidants are compounds that can stop the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidizing chain reactions, which can thus prevent damage done to the body's cells by oxygen. It plays an important role in protection against disorders caused by oxidation damage. The methanolic, ethanolic, acetone and aqueous crude extracts of *Nerium oleander* and *Thevetia peruviana* were screened for their free radical scavenging properties using ascorbic acid as standard antioxidant. Free radical scavenging activity was evaluated using di (phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) assay. The maximum amount of polyphenolic content was also observed in *Nerium oleander* (48.94 mg/100gm), while lower amount was recorded in aqueous extract of callus (223.71mg/100gm). However, polyphenol was higher in the methanol extract of *Thevetia peruviana* (32.64 mg/100gm) and lower in aqueous extract of callus (188.14 mg/100gm), respectively. The antioxidant activity in *Nerium oleander* (48.94±0.41µg/ml) and *Thevetia peruviana* (32.64±0.06 µg/ml) was found to be maximum in *in vitro* methanolic extract, respectively. Hence, a positive correlation was found between polyphenolic activity and antioxidant activity in *in vitro* methanolic extract of both the plant species. The *in vitro* extraction exhibited more antioxidant activity and polyphenolic content as compared to *in vivo* extract. Hence, the need exists for safe, economic antioxidants with high activity from natural sources to replace synthetic chemicals.

Key words: DPPH, *Nerium oleander, Thevetia peruviana,* ascorbic acid, lipids, polyphenols, antioxidants, oxidation.

INTRODUCTION

Nature has always been playing a balancing role in human life. When man is increasing complexities for himself by adding free radicals, nature is helping him out by bestowing natural antioxidants which inactivate the free radicals and stop the detrimental action. These antioxidants stop unwanted oxidation in the body, which involve the formation of free radicals and which further deteriorate the physiological condition of the body [1]. Antioxidants are specific and necessary nutrients that protect the body from cellular damage by oxidation. "Oxidation is the addition of oxygen or the removal of hydrogen and it can be caused by free radical". An antioxidant can slow down or even stop the chain reaction of oxidation by giving an electron without changing its stability. Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various human ailments because they contain the components of therapeutic value [2, 3]. In recent decades, phytochemicals such as essential oils, flavonoids and anthocyanins have been of great interest as the sources of natural antioxidants not only in health promotion but also in cosmetics, because they are safer and environment friendly than synthetic antioxidants. Numerous natural antioxidants have already been isolated from different varieties of plant material such as leafy vegetables, fruits, seeds, cereals and algae [4]. Most popularly used di (phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) is a stable radical that has been used widely to evaluate the antioxidant activity of various natural products [5] .There are many synthetic antioxidants which are in use. However, it is reported that they have several side effects such as risk of liver damage and carcinogenesis in laboratory animals [6,7,8,9]. Therefore, there is a need for more effective, less toxic and cost effective antioxidants. Most of the plants including medicinal plants appear to have these desired advantages of antioxidants. Hence, the interest in natural antioxidants is growing rapidly which we can find out from plants. Plants being the store house of these antioxidants in natural system, they absorb the sun's radiation and generate high levels of oxygen as a product of photosynthesis. Plants produce various antioxidative compounds to counteract these ROS in order to survive [10].Thus, medicinal plants are most important source of antioxidants [11]. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk to certain diseases such as cancer, heart diseases and stroke [12]. The secondary metabolic products like phenolic compound and flavonoids from plants have been reported to have free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark [13].

Keeping in view the importance of the antioxidants derived from the plants, we have chosen two medicinally potent plants such as *Nerium oleander* L. and *Thevetia peruviana* (Pers.) Schum for the present study.

The plant *Nerium oleander* L. has important pharmacological activities like anti-inflammatory, antibacterial, anticancer, antinociceptive and CNS depressant activity [14]. Similarly, *Thevetia peruviana* (Pers.) Schum. contains flavonol glycoside in the leaves and shows inhibitory effects against HIV-1 reverse transcriptase and HIV-1 Integrase [15]. Recently, many researchers have been done to find out the antioxidant activity of medicinally important plants like in *Newbouldia Laevis*[16]; *Lippia Alba* [17]; C*assytha filiformis* [18] etc.. Therefore, it is of great interest to evaluate the phenolic compounds and antioxidant activity of both the plants viz. *Nerium oleander* L. and *Thevetia peruviana* (Pers.) Schum. grown in *in vivo* and in *in vitro* conditions.

MATERIALS AND METHODS

The explants from *Nerium oleander* L. and *Thevetia peruviana* (Pers). Schum were procured from *in vivo* grown plants at Nursery of University of Rajasthan and authenticated by Herbarium, Department of Botany, University of Rajasthan, Jaipur, India.

The leaves of the respective plants were dried and powdered (each 50g) were extracted separately with double distilled water, ethanol, acetone and methanol (each 400ml.) for 10-12 hours using a Soxhlet apparatus. Then crude extracts were filtered through Whatt man No-1 filter paper. The extracts were evaporated to dryness at 60ºC by Rotary vacuum evaporator to obtain the respective extracts and stored in a refrigerator until further analysis.

CHEMICALS AND GLASSWARE

All the chemicals and growth regulators were of analytical grade and purchased from Hi Media Pvt. Ltd. Mumbai, India. At the same time, glassware used during the experiments were procured from Borosil, India.

CALLUS INDUCTION

Leaf explants were surface sterilized by 1 % Teepol for 15 min followed by immersion in 70 % ethanol for 1 min and in 0.1 % mercuric chloride for 2 min, and then rinsed thoroughly with sterile distilled water. The leaf explants of *Nerium oleander* inoculated on the MS medium [19] fortified with different concentrations (0.5 to 5.0 mg/l) of 2, 4-D and BAP to stimulate callus production. Whereas, in

case of *Thevetia peruviana,* leaf segments inoculated on 0.5 to 5.0 mg/l of 2, 4-D and Kn separately as well as in combination incorporated in MS medium. The pH of the media was adjusted to 5.8± 2 before autoclaving. All media were autoclaved at 1.06 kg cm^{-2} and 121ºC for 15 min. The cultures were incubated in growth room at temperature 25 ± 2 °C and 16/8 hours photoperiod. 28 replicate were taken and each experiment was repeated at least thrice and the cultures were observed at regular intervals.

It was observed that *Nerium oleander* at 2,4-D + BAP (4.5mg/l + 2.5mg/l) and *Thevetia peruviana* at 2,4-D+Kn (2.5mg/l+1.2mg/l) concentration gave maximum green callus after four weeks of inoculation in *Nerium oleander* and *Thevetia peruviana,* was proved to be the best combination and concentration for callus induction. This *in vitro* grown callus obtained was then taken for further study.

TOTAL PHENOLS DETERMINATION:

Total phenolic content was determined according to the Folin-Ciocalteu method [20] using Catechol as standards. 500 mg of test sample was taken and ground in 10- times volume of 80% ethanol. The homogenate Centrifuged at 10,000 rpm for 20 min., supernatant was saved. Re- extracted the residue with 50- times the volume of 80% ethanol, centrifuged and supernatant was pooled. The supernatant was then evaporated to dryness. After that the residue was dissolved in a known volume of distilled water (5 ml). Pipette out aliquots (0.2ml) into the test tubes. Make up the volume in each tube to 3 ml with water. 0.5 ml of Folin- Ciocalteu reagent was added. After 3 min, 2 ml of 20% Na_2CO_3 solution was also added to each tube. The tube was placed in boiling water for exactly one minute, cooled and measured the absorbance at 650 nm against a reagent blank. A standard curve was prepared using different concentrations of Catechol.

DPPH ASSAY

The antioxidant activities were determined using di (phenyl)-(2, 4, 6-trinitrophenyl) iminoazanium (DPPH) as a free radical. Experiments were initiated by preparing a 0.2 mM solution of DPPH and 1mg/ml solution of different plant parts extracts in methanol was prepared. Different concentrations (10, 50, 100, 200, 400, 600, 800, 1000 µg/ml) of test plant samples were mixed with 2ml of DPPH. After 30 minutes of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm. DPPH is a purple colored stable free radical; when reduced it become yellow-colored di (phenyl)-(2,4,6-trinitrophenyl) iminoazanium. The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517 nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH Solution is decolorized as the color change from deep violet to light yellow. The degree of reduction in absorbance measured is an indicative of the radical scavenging (antioxidant) power of extract. Initially, absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured as control [21] Ascorbic acid (Merck; M.W. 176.13) was used as standard. The experiment was carried out in triplicate.

The inhibitory effect of DPPH was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

The antioxidant activity of each sample was expressed in terms of IC50. (µg /ml concentration required to inhibit DPPH radical formation by 50%, calculated from the inhibition curve [22,23,24,25].

RESULT

The amount of total polyphenol was higher in *in vitro* extract of methanol (48.94 mg/100gm) of *Nerium oleander* while lower amount was recorded in *in vitro* aqueous solution (223.71mg/100gm); however, in *Thevetia peruviana* the polyphenol was higher in *in vitro* extract of methanol (32.64 mg/100gm) and lower in aqueous extract in *in vitro* (188.14 mg/100gm); respectively (Table 1). The free radical scavenging activity in the different extracts decreased in the following order:

Nerium oleander in *in vitro* Methanol (48.94 ±0.41µg/ml) > *Nerium oleander* in *in vitro* Ethanol (83.42±0.30µg/ml*)* > *Nerium oleander* in *in vitro* Acetone (121.24±0.71 µg/ml) > *Nerium oleander* in *in vitro* Aqueous (223.71±0.11µg/ml).

Nerium oleander in *in vivo* Methanol (78.52±0.37 µg/ml) > *Nerium oleander* in *in vivo* Aqueous (89.35±0.26µg/ml) >*Nerium oleander* in *in vivo* Ethanol (107.56±0.52 µg/ml) > *Nerium oleander* in *in vivo* Acetone (127.52 ±0.71µg/ml).

Whereas in,

Thevetia peruviana in *in vitro* Methanol (32.64±0.06 µg/ml) > *Thevetia peruviana* in *in vitro* Acetone (78.52±0.32µg/ml).> *Thevetia peruviana* in *in vitro* Ethanol (92.48±0.78µg/ml) > *Thevetia peruviana* in *in vitro* Aqueous (188.14±0.63 µg/ml).

Thevetia peruviana in *in vivo* Methanol (69.79 ±0.12µg/ml) > *Thevetia peruviana* in *in vivo* Ethanol (109.61±0.42µg/ml) > *Thevetia peruviana* in *in vivo* Acetone (123.02±0.21µg/ml) > *Thevetia peruviana* in *in vivo* Aqueous (129.72±0.56µg/ml).

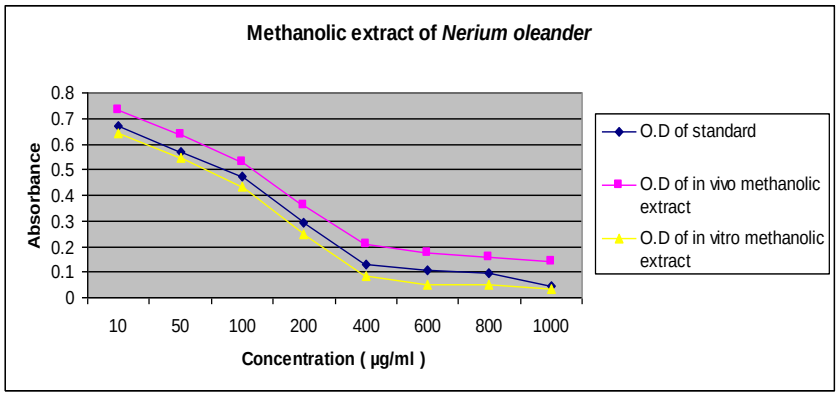
Table 1: Antioxidant Activity of Different Extracts Of *nerium oleander* l. and *thevetia peruviana* (pers.) schum.

Sample extraction	Phenol content mg/100gm		IC50% (µg/ml)	
	Nerium oleander	***Thevetia peruviana***	***Nerium oleander***	***Thevetia peruviana***
Methanol *in vivo*	254.89±0.13	233.68±0.12	78.52±0.37	69.79±0.12
Methanol *in vitro*	258.06±0.21	234.67±0.52	48.94±0.41	32.64±0.06
Ethanol *in vivo*	241.04±0.56	220.52±0.36	107.56±0.52	109.61±0.42
Ethanol *in vitro*	250.56±0.68	228.32±0.78	83.42±0.30	92.48±0.78
Acetone *in vivo*	236.61±0.15	218.63±0.04	127.52±0.71	129.72±0.56
Acetone *in vitro*	239.67±0.78	231.65±0.17	121.24±0.12	78.52±0.32
Aqueous *in vivo*	244.75±0.27	214.59±0.38	89.35±0.26	123.02±0.21
Aqueous *in vitro*	228.54±0.11	210.02±0.21	223.71±0.11	188.14±0.63

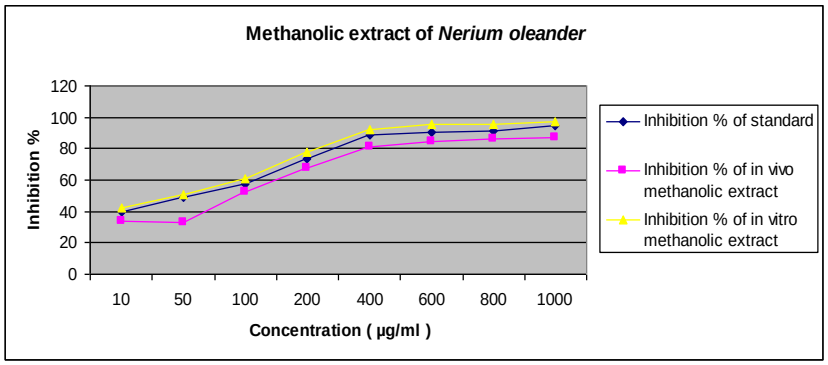
DISCUSSION

Phenols are very important plant constituents with multiple biological functions including antioxidant activity because of their radical scavenging ability due to their OH groups. Many reports are available showing the relative correlation between phenol and antioxidant activity viz., *Newbouldia Laevis* [16], *Achillea millefolium, Arctium lappa* and *Betula pendula* [26.] DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [27]. Scavenging activity for free radicals of di (phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. The result of the present study showed that the *in vitro* methanolic extract of *Nerium oleander* L *.and Thevetia peruviana* (Pers.) Schum. contained highest amount of phenolic compounds and exhibited the maximum antioxidant activity. The high scavenging property of methanolic extract in *in vitro Nerium oleander* L. and *Thevetia peruviana* (pers.) Schum. may be due to hydroxyl groups existing in the phenolic compounds, chemical structure that can provide the necessary component as a radical scavenger [27]. The results obtained above are in consonance with other researches which have also shown a directly proportional relationship between Phenol and antioxidant activity [28].

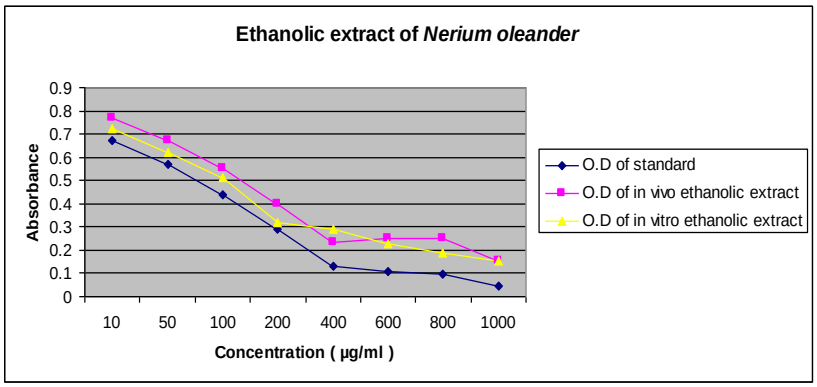
Graphs: Antioxidant activity of different extracts of *Nerium oleander* L. and *Thevetia peruviana* (Pers.) Schum.



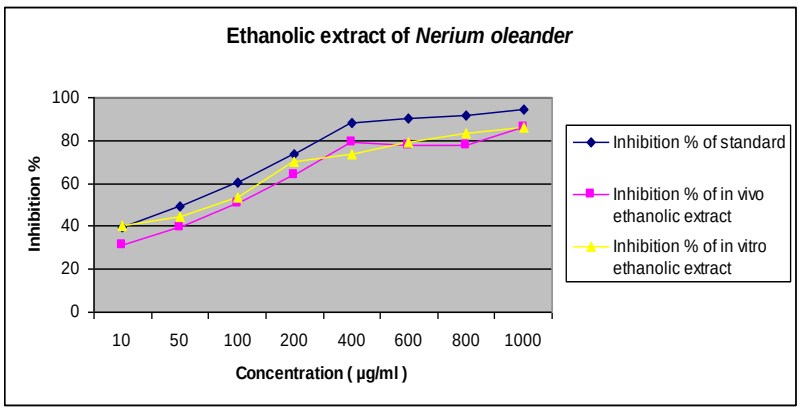
Graph A: DPPH scavenging assay of the *in vitro* and *in vivo* methanolic extract of *Nerium oleander* compared with standard ascorbic acid.



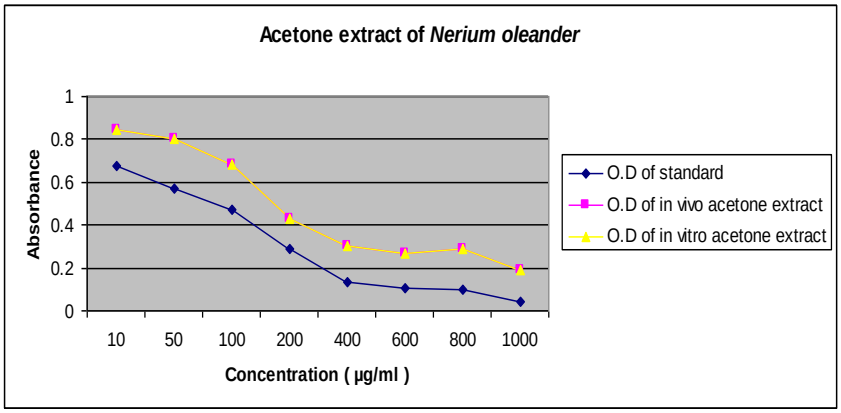
Graph B: Evaluation of IC 50% of the *in vivo* and *in vitro* methanolic extract of *Nerium oleander* and the standard ascorbic acid.



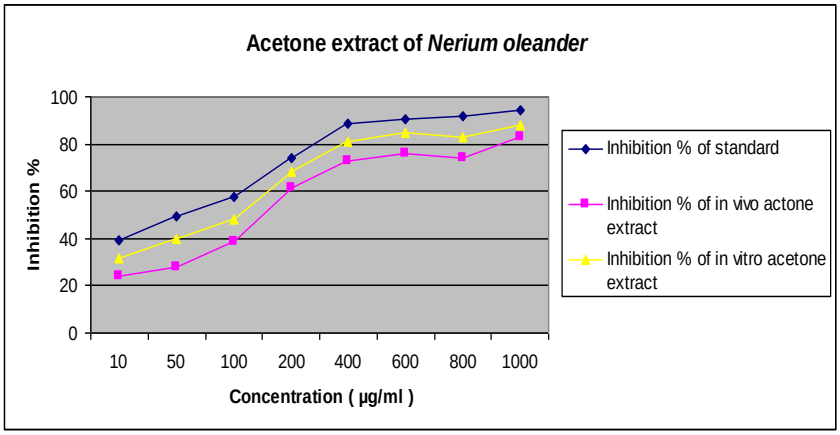
Graph C: DPPH scavenging assay of the *in vitro* and *in vivo* ethanolic extract of *Nerium oleander* compared with standard ascorbic acid.



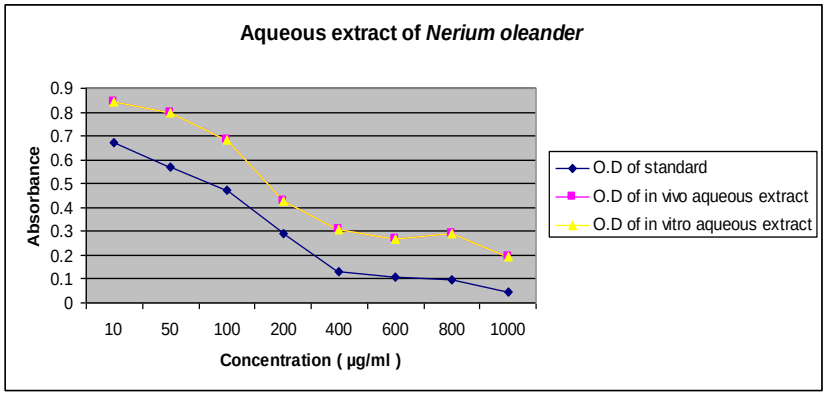
Graph D: Evaluation of IC 50% of the *in vivo* and *in vitro* ethanolic extract of *Nerium oleander* and the standard ascorbic acid.



Graph E: DPPH scavenging assay of the *in vitro* and *in vivo* acetone extract of *Nerium oleander* compared with standard ascorbic acid.



Graph F: Evaluation of IC 50% of the *in vivo* and *in vitro* acetone extract of *Nerium oleander* and the standard ascorbic acid.



Graph G: : DPPH scavenging assay of the *in vitro* and *in vivo* aqueous extract of *Nerium oleander* compared with standard ascorbic acid.

Type of extraction also plays an important role in determining the antioxidant activity. The above experiments reveal that in both the plants viz., *Nerium oleander* and *Thevetia peruviana* the methanolic extracts contain maximum antioxidant activity. Similarly Vinayagam and Sudha, 2011 have also reported in *Nerium indicum,* [29,] Species whereas [30]reported in *Oenanthe javanica*, *Centella asiatica, Murraya koenigii and Cosmos caudatus* .This might be due to that both the plant belongs to the same family and hence the antioxidant mechanism of both the plant extracts is more or less the same in both the plant species. This study of antioxidant activity with various extraction has also been reported by many scientist in different plants like [18] in *Cassytha filiformis*. He has taken the extracts in hexane, ethyl acetate and methanol on the basis of Radical Scavenging activity using DPPH assay in which methanolic extract was found to be more active as a radical scavenger than Hexane and ethyl acetate extract. Further more than one extraction system is recommendable for detailed assessment of the antioxidant properties of medicinal plants [31, 32, 27].

Many other reports show the co- relation between *in vivo* and *in vitro* plants over the years [27, 33]. The present study also reveals that in both the plant species *in vitro* extract possesses more Antioxidant activity with IC 50% value is 48.94µg/ml in *Nerium oleander* and 32.64µg/ml in *Thevetia peruviana* respectively (Graph B and J).

The improved DPPH method described by [34] was successfully used in this study to systematically assess the total antioxidant capacity of the medicinal plant extracts on a large scale, being simple, fast, reliable and inexpensive. This efficient and effective method can be used for systematic screening of medicinal and dietary plants for their relative antioxidant content. Several studies have revealed that intake of natural antioxidants is correlated with low incidence of cancer, heart diseases, diabetes, and other diseases associated with ageing [35, 36].

Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy [37,38,39,40]. The plants may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms [41, 42].

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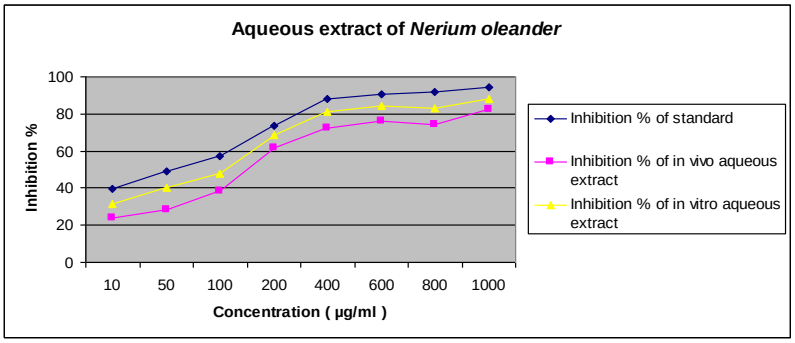
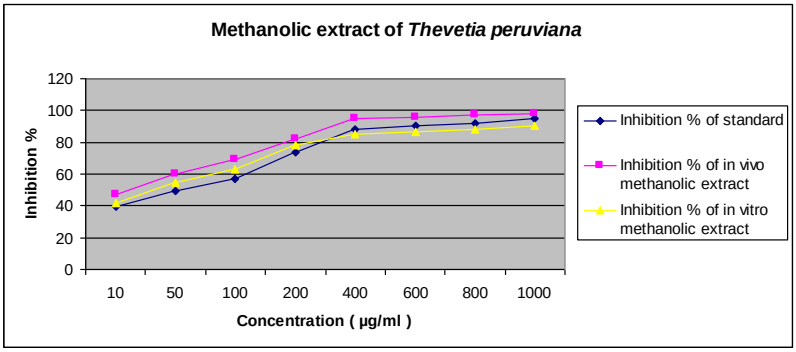
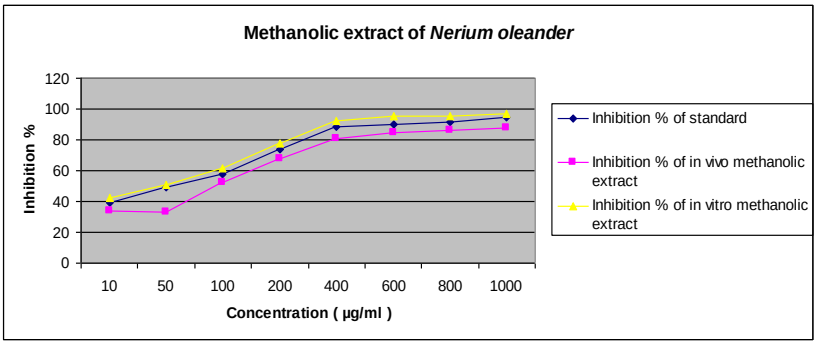


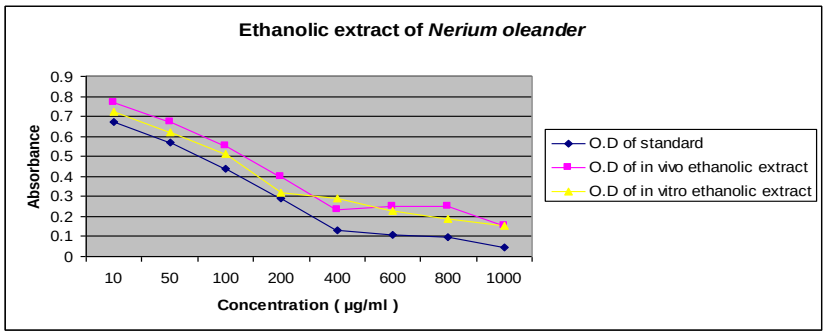
Graph H: Evaluation of IC 50% of the *in vivo* and *in vitro* aqueous extract of *Nerium oleander* and the standard ascorbic acid.



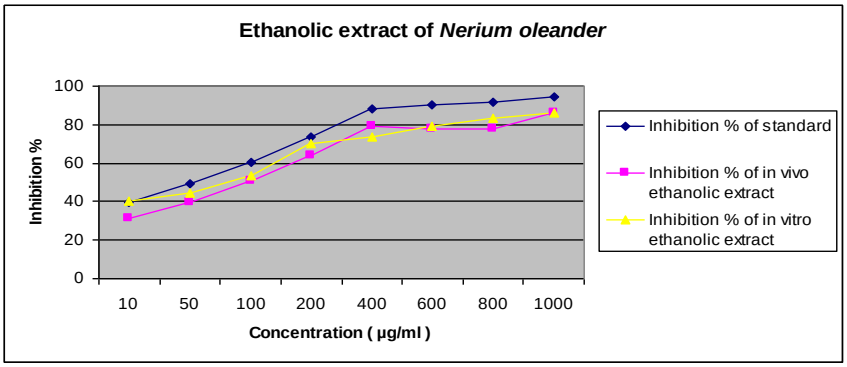
Graph I: DPPH scavenging assay of the *in vitro* and *in vivo* methanolic extract of *Thevetia peruviana* compared with standard ascorbic acid.



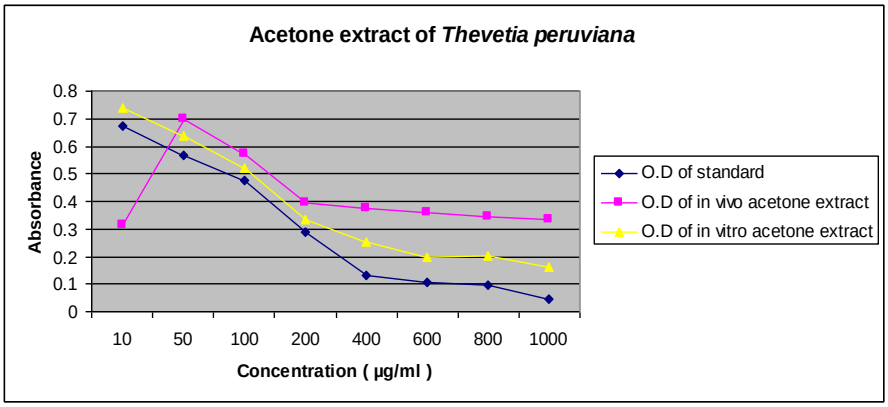
Graph J: Evaluation of Ic 50% of the *in vivo* and *in vitro* methanolic extract of *Thevetia peruviana* and the standard ascorbic acid.



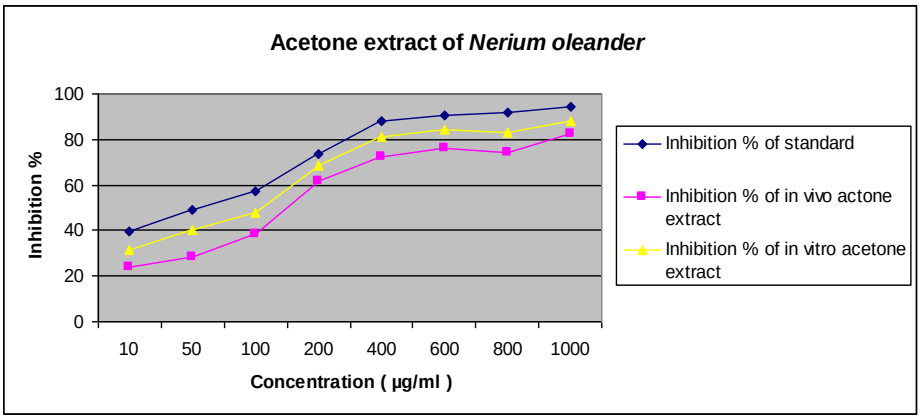
Graph K: DPPH scavenging assay of the *in vitro* and *in vivo* ethanolic extract of *Thevetia peruviana* compared with standard ascorbic acid.



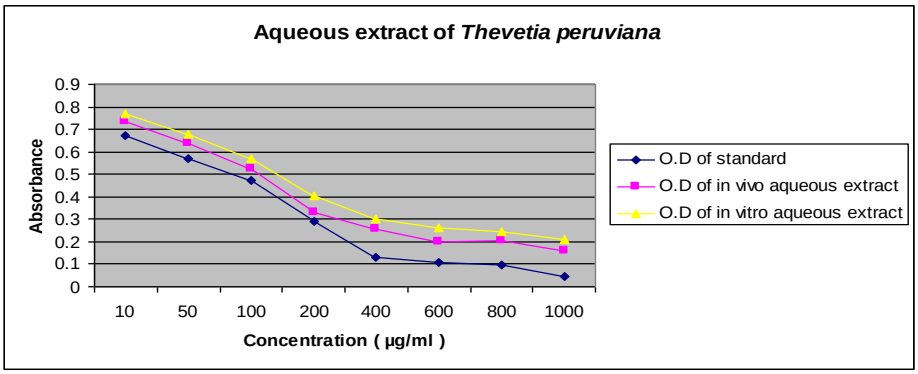
Graph L: Evaluation of Ic 50% of the *in vivo* and *in vitro* ethanolic extract of *Thevetia peruviana* and the standard ascorbic acid.



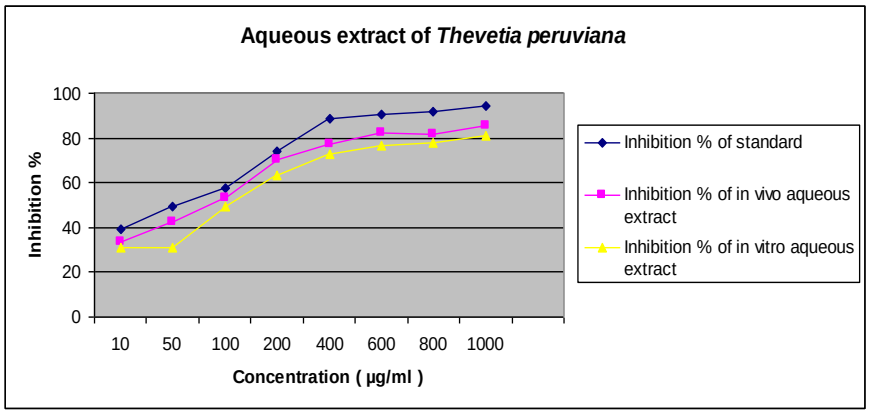
Graph M: DPPH scavenging assay of the *in vitro* and *in vivo* acetone extract of *Thevetia peruviana* compared with standard ascorbic acid.



Graph N: Evaluation of IC 50% of the *in vivo* and *in vitro* acetone extract of *Thevetia peruviana* and the standard ascorbic acid.



Graph O: DPPH scavenging assay of the *in vitro* and *in vivo* aqueous extract of *Thevetia peruviana* compared with standard ascorbic acid.



Graph P: Evaluation of IC 50% of the *in vivo* and *in vitro* aqueous extract of *Thevetia peruviana* and the standard ascorbic acid.

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