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Evaluation of *In Vitro* Antioxidant Activity and Phytochemical Screening of *Croton macrostachyus* Hochst. by using Different Solvent Extracts

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ABSTRACT

The present study was to evaluate preliminary photochemical analysis and *in vitro* antioxidant activities of leaves, fruit and stem extracts of *Croton macrostachyus* Hochst. (Family: Euphorbiaceae) by using different solvents like bdenzene, methanol, carbon tetra chloride and hexane. Phytochemical analysis showed the presence of alkaloids, aminoacids and proteins, flavonoids, saponins, steroids, tannins and triterpenoids. The leaves and fruits extracts were further investigated for its potential antioxidant activity by using readical scavenging DPPH (2, 2-Diphenyl-2-picryl-hydrazyl) technique. Reducing power of *C. macrostachyus* extracts were also screened and ascorbic acid was used as standard. Methanol extract of leaves and benzene and methanol extracts of fruits were exhibited a noteworthy DPPH radical scavenging activity compared to standard. The results were concluded that extracts have a more secondary metabolites and potential source of antioxidants, which is warranty to evaluate further *in vivo* pharmacological studies.

Keywords: *Croton macrostachyus* L, phytochemical analysis, *in vitro* Antioxidant activity, traditional medicine

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INTRODUCTION

Now a days, there has been increasing attention in the free radical causing diseases and antioxidants. The main objectives of the interest is the shield of cells, organelles, metabolic diseases, oxygen free radicals and their reactive oxygen species (ROS) [1]. These harmful free radicals normally scavenge by natural antioxidants present in the plants. Free radicals are any species competent of self-regulating existence that include one or more unpaired electrons, that attack other neighbouring molecules by captivating or giving electrons causing many pathological situation[2]. Eventually it may cause a variety of physiological processes such as diabetes, cardiovascular and cerebrovascular disorders, ageing, and cancer. Many researchers have confirmed that plant(s) manufacture potent numerous antioxidants, that eradicate disease causing ROS [3.] The generation of ROS such as superoxide (O_2-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO-) radicals, and reactive hydroxyl (OH-) radicals) and reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite anion (ONOO) are more detrimental effect to the human beings [4]. Under normal circumstances, the generation of ROS and RNS are detoxified by the antioxidants usually provided by the plants or synthesis of antioxidant enzymes. On the other hand, due to oxidative stress, the over production of ROS cannot nulled by antioxidant provided by the diet. Based on the failure of equilibrium, the ROS readily persuade oxidative damage to a choice of biomolecules such as DNA, proteins, lipids and carbohydrates [5] Based on growing attention on free radical biology and shortage of successful therapies for important chronic diseases, the plant derived antioxidants are essential to compact against these diseases.

Clinical research studies exhibited that the utilization of antioxidants such as vitamin C, A and E reduce the menace of cancer and a variety of heart diseases[6.] These antioxidants play as intracellular protection from free radical damage and helps to prevent extensive lysis of the cell. However, the scavenging and reducing the generation of ROS are not completely efficient. The consumption of these antioxidant vitamins taken as supplements are also may be diminish oxidative damages, perhaps may not over come of all free radical generations. At present the existing synthetic drugs do have potential unfavourable reactions and which can be reduced to a larger extent usage of natural products. There are still many traditional drugs which has not been investigated scientifically [7, 8.]

Croton macrostachyus Hochst. (Family: Euphorbiaceae) is well known tree grow upto 16 m tall found in forested savanna, Eastern Africa. It is native to Eastern African countries including Ethiopia, Eritrea, Nigeria, Kenya, Tanzania and Uganda. There are eight Croton Gentre found in

Ethiopia [9] and used for treatment of pain killer, diabetes [10,] malaria [11,] dysentery, stomachache, ascariasis and taeniasis, abdominal pain [12,] gonorrhoeae, wounds, ringworm infestation, hemorrhoids [13,] venereal diseases, cough[14,] and rheumatism[15.] It is also used to impede bleeding in child birth. The seeds are consumed as abortifacient. In Ethiopia, the plant has traditional medicinal uses as purgative, dermatitis, management of helminthes and sexually transmitted diseases[16.]

Based upon ethanobotanical survey of Ethiopian indigenous medicinal plants, *C.macrostachyus* has been choosed to prove scientifically having phytoactive compounds and antioxidant activity on *in vitro* studies. The phytochemicals generated data from four different extracts of these plants may be used as tools for quality control of drugs in the future, for the healing of a multiplicity of diseases.

MATERIALS AND METHOD

Chemicals

Ascorbic acid, Ferric chloride, HCl, Dragendorff 's reagent, hexane, methanol, carbon tetrachloride, gallic acid, chloroform, H_2SO_4, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl and glacial acetic acid, were all purchased from Chemico Glass & Scientific Company, Erode, Tamilnadu, India. All the chemicals used in the present study were of analytical grade.

Collection and authentication of plant material

Croton macrostachyus was collected from Jimma University Garden, Jimma, South West Ethiopia in the month of October-2014. The plant has been taxonomically identified and authenticated by the Jimma University Botanist Dr. Ramesh Moochikkal and kept in Jimma University Botanical Science and Herbarium for future references.

Preparation of various extracts of *C.macrostachyus*

The leaves, fruits and stem of *C.macrostachyus* were obtained and air dried under shadow and followed by almost pulverized separately by the way of mechanical mixer. The powder was conceded through sieve and stock up in an sealed container for the solvent extraction.

Benzene extract of leaves, fruit and stem of *C.macrostachyus*

The shadow dehydrated roughly powdered of leaves, fruits and stem of *C.macrostachyus* was engrossed and haul out with benzene for 72hrs. After finishing point, the defatted solutions were sieved by filter paper Whatmann No.1 to eliminate any contamination. The extract was intended by vaccum dessicator to reduce the degree; the concentrated samples were relocated to another

beaker and the residual solvent was further vaporized. Finally the dark greenish yellow coloured extract was formed and again it was kept in a vaccum dessicator to get rid of unnecessary wetness. Dehydrated extract was stored in sealed container for phytochemical screening studies.

Methanol, CCl_4, and hexane extracts of leaves, fruit and stem of *C.macrostachyus*

The resudues left after benzene extraction was dehydrated and then engrossed separately with methanol, CCl_4 and hexane respectively upto 3days. After finishing point of extraction, the organic solvents were eliminated by vaccum dessicator. Dark greenish yellow colour extracts were formed and then stored in a sealed container for further studies.

Preliminary phytochemical studies [17, 18]

The extracts obtained (benzene, methanol, carbon tetrachloride, and hexane) were employed to the subsequent phytochemical screening.

Test for Alkaloids

a) Dragendorff's test:

Take 1ml of the solvent extract, add equal volume of distilled water followed by 1ml of 2molar solution of HCl added until acidification reaction take place. To add this 1ml of Dragendorff's reagent. Orange or red colour is formed, indicated that the occurrence of alkaloids.

b) Hagger's Test: Take 1ml of the solvent extract in a cleaned test tube, add 1ml of Hager's reagent. Yellow precipitate is formed, indicated that the occurrence of alkaloids.

c) Wagners Test: Take 1ml of solvent extract acidified with 1ml of 1.5 % v/v of HCl and add 1ml of wagners reagent. Formation of yellow or brown precipitate, which indicated that the occurrence of alkaloids.

d) Mayers Test: Take 1ml of Mayers reagent, add 1ml of solvent extract. White or pale yellow precipitate is formed, indicated that the occurrence of alkaloids.

Test for Carbohydrates

a) Anthrone Test: Take 1ml of solvent extract and 10ml of distilled water in a test tube, shaken vigorously and filtered. To this filtrate, add 1ml of anthrone reagent and mixed. Green or blue color is formed, indicated that the occurrence of carbohydrates.

b) Benedicts Test: Take 1ml of solvent extract and 10ml of water in a test tube, shaken vigourously and filtered. To this filtrate, add 3ml of Benedicts reagent and kept in a boiling water bath for 5min. Appearance of reddish brown colour indicated that the occurrence of reducing sugar.

c) Fehlings Test: Take 1ml of solvent extract and 10ml of water in a test tube, shaken vigorously and filtered. To this filtrate, add 1ml of Fehlings solution A and 1ml of Fehlings solution B and

kept in a boiling water bath for 5min. Appearance of reddish brown colour indicated that the occurrence of reducing sugar.

d) Molischs Test: Take 1ml of solvent extract and 10ml of water in a test tube, shaken vigourously and filtered. To this filtrate, 2 drops of Molisch reagent added followed by few drops of Conc. H_2SO_4 added in the side of the test tube. Development of two junction, which indicates the occurrence of carbohydrates.

Test for flavonoids.

a) Shinods test: Take 1ml of solvent extract diluted with 3ml of ethanol followed by 2ml dilute HCl and pinch of Mg in a test tube, shaken gently. Appearance of pink or brown precipitate indicated that the occurrence of flavonoids.

b) With Con. H_2SO_4 test: when treated with Con. H_2SO_4, appearance of the following colour like yellow colour (anthocyanins), yellow colour change to orange (flavones); orange colour change to crimson (flavonones) respectively.

Test for Glycosides

Molisch Test: Take 1ml of solvent extract and 10ml of water in a test tube, shaken vigorously and filtered. To this filtrate, 1ml of Molisch reagent added followed by few drops of Conc. H_2SO_4 added in the side of the test tube. Development of two junction indicated that the occurrence of glycosides.

Test for proteins and free amino acids

1. Millions test- Take 1ml of solvent extract with 1ml of Millions reagent, shake gently. Appearance of cherry red color pointed out that the occurrence of free amino acid.

2. Ninhydrin test- Take 1ml of solvent extract with 1ml of Ninhydrin reagent, shake gently Formation of violet color indicated that the occurrence of free amino acids.

3. Biuret test: Take 1ml of solvent extract with 1ml of 10% NaOH and 1ml of 1% copper sulphate in a test tube, shake gently. Appearance of purple color pointed out that the occurrence of proteins.

Test for gums and mucilage

With 95% alcohol: Take 1ml of solvent extract with 25 ml of 95% alcohol in a test tube, shake gently and filtered. The residue was air dried and examined for its bulging property. It indicated that the occurrence of gums and mucilages.

Test for anthraquinones

Take 2ml of the solvent extracts acid hydrolyzed with Conc. H_2SO_4 followed by extracted with benzene. Add 2ml of dilute ammonia. Formation of pink color pointed out that the occurrence of anthraquinones.

Test for Saponins

Foam test : Take 5ml of solvent extracts in a test tubes add a drop of sodium bicarbonate, shaken vigorously and kept it stand for 3min. Formation of cloudy white precipitate pointed out that the occurrence of saponins.

Test for Sterols

a) Liebermann-Buchards test: Take 1ml of solvent extract in a test tube and add acetic anhydride and kept in a boiling water bath for 5min, then cooled followed by 1ml of Con. H_2SO_4 added along the sides of the test tube. Appearance of green color indicated that the occurrence of steroids.

b) Salkowski reaction: Add 1ml of solvent extract diluted with chloroform and followed by 1ml of Con. H_2SO_4 added along the sides of the test tube. Appearance of two junction/layer indicated that the occurrence of steroids.

Test for fixed oils

Spot test: Take 0.5ml of solvent extract and pressed in between the two filter papers. Formation of oil stains on the paper indicated the existence of fixed oil.

Add 1ml of 0.5N alcoholic KOH and 1ml of solvent extract along with a single drop of phenolphthalein in a test tube. The residues were kept in a boiling water bath for 20min. Appearance of soap or incomplete neutralization of alkali indicated that the occurrence of fixed oils.

Test for triterpenoids

Add 2ml of solvent extract and 1 ml of $CHCl_3$ followed by 1 ml of acetic anhydride in a test tube and shake gently. Add 1ml of Con. H_2SO_4 added along the sides of the test tube. Appearance of two junction/layer indicated that the occurrence of triterpenoids.

Test for phenolic compounds and tannins

About 5ml of solvent extracts and equal volume of water added and perform the following reagent for confirmation of phenolic compounds and tannins.

Ferric chloride reagents-It gives a violet color

Gelatin containing sodium chloride- It gives a white precipitate.

Lead acetate solution- It gives a white precipitate

***In vitro* antioxidant activity estimated by method of DPPH**

In vitro antioxidant activity of *C.macrostachyus* extract was estimated by using a method of DPPH free radical scavenging assay. The DPPH free radical scavenging assay of leaves, fruit and stem extracts of *C.macrostachyus* was carried out based to the technique of Alekhya et al.[19] with smaller alteration. About 1ml of solvent extract diluted with 1ml of ethanol taken in a test tube and added 4

ml of 0.1M ethanol solution of DPPH and keep shake strongly. Then the tubes were kept in a dark room at room temperature incubation for 15 min. Blank was arranged without solvent extract and ethanol. Absorbance read at 517 nm using UV-visible spectrophotometer. The percentage reduce in the absorbance and reduce DPPH were calculated for individual concentration. DPPH method was calculated as the inhibition percentage and was estimated by following formula:

$$\text{Radical scavenging activity (\%)} = [(A0 - A1 / A0) \times 100]$$

Where A0 - control absorbance (blank, without extracts)

A1 was the Absorbance of solvent extracts.

Radical scavenging activity of vitamin C was also calculated and evaluated with the diverse solvent extracts.

RESULTS AND DISCUSSION

In the study, preliminary phytochemical investigation showed in the four extracts (benzene, methanol, carbon tetrachloride and hexane) of *C.macrostachyus* leaves fruits and stems showed the presence of phytochemical constituents namely alkaloids, aminoacids, saponins, flavonoids, steroids, triterpenoids and tannins and absence of anthoquonones and glycosides described in Table 1.

Table 1: Phytochemical investigation of leaves, stem and fruits of Croton macrostachyus L.using Benzene, methanol, CCl4 and hexane solvents.

Analysis	Croton macrostachyus L.											
	Leaves				Stem				Fruits			
	Benzene	Methanol	CCl_4	Hexane	Benzene	Methanol	CCl_4	Hexane	Benzene	Methanol	CCl_4	Hexane
Alkaloids	++	-	++	++	++	-	+++	++	+++	+	++++	+++
Protein and aminoacids	-	+++	-	-	-	-	-	-	++	+	-	-
Anthraquinones	-	-	-	-	-	-	-	-	+	-	-	-
Flavonoids	-	++	-	-	-	+	+	-	+++	+++	-	-
Glycosides	-	-	-	-	-	-	-	-	-	-	-	-
Saponins	-	+	-	-	-	-	++	++	+	+++	-	+++
Steroids	-	-	-	-	-	-	-	-	+++	-	-	+
Total phenols and Tannins	-	++	-	-	+	+	+	-	-	+++	-	-
Triterpenoids	-	++	-	-	-	-	-	-	+++	+++	-	-

+++ = appreciable amount (positive within 5 mins.); ++ = moderate amount (positive after 5 mins. but within 10 mins); + = trace amount (positive after 10 mins. but within 15 mins); - = completely absent.

Figure 1 and 2 illustrated *in vitro* antioxidant assay of the *C.macrostachyus* leaves and fruit extracts which has significant antioxidant potential compared with standard ascorbic acids. The proportion inhibition of lipid peroxide at the first phase of oxidation showed antioxidant activity of different solvent leaves extract of *C.macrostachyus* of methanol *(*84%), benzene (78%) CCl4 (46%), and hexane (42%) compared to those of ascorbic acid (97%) respectively. The proportion inhibition of lipid peroxide at the first phase of oxidation showed antioxidant activity of different solvent fruits extract of *C.macrostachyus* of methanol *(*78%), benzene (68%) CCl4 (56%), and hexane (58%) compared to those of ascorbic acid (97%) respectively.

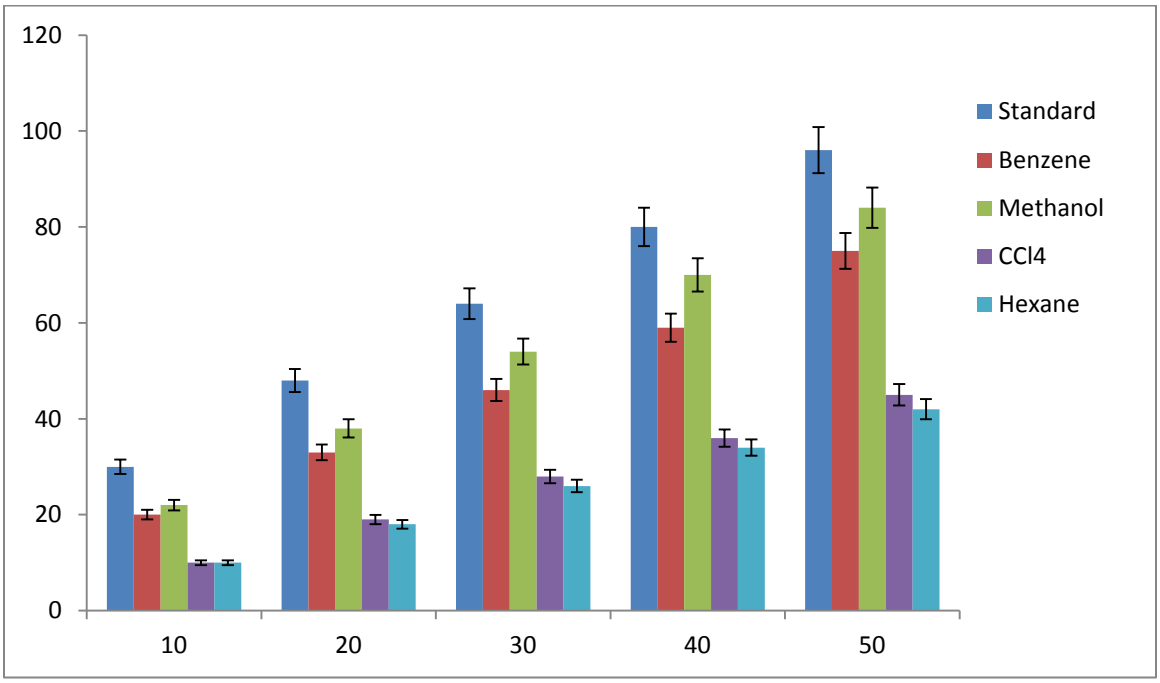

Figure 1: In vitro antioxidant properties of different solvent leaves extracts of *C.macrostachyus* compared with Ascorbic acid as standard measured by DPPH radical scavenging method.

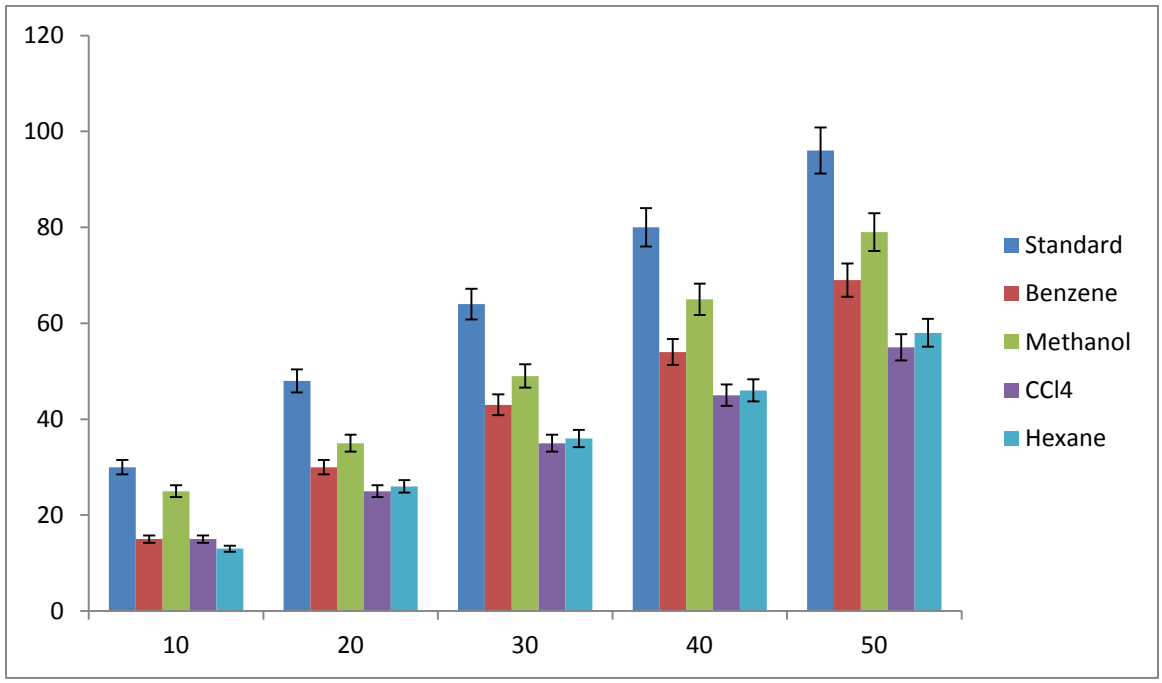

Figure 2: In vitro antioxidant properties of different solvent fruit extracts of *C.macrostachyus* compared with Ascorbic acid as standard measured by DPPH radical scavenging method.

The phytochemical analysis of leaves, fruits and stem extracts of *C.macrostachyus* revealed the presence of alkaloids, saponins, flavonoids, tannins, and triterpenoids. Tannins are recognized to be helpful in the treatment of chronic inflammation in tissues and they have notable activity on anticancer [20.] Thus, *C.macrostachyus* containing these chemical compounds may provide as active principle in the treatment of various cancer.

Flavonoids are one of the significant phenolic compounds that are acting as principal antioxidants or free radical scavengers and give out as health promoting compound [20.] Since these phenolic compounds were originated to be present in the extracts, it might be accountable for the potent antioxidant capacity of *C.macrostachyus*. These phytochemicals of medicinal plants have primarily reported for their medicinal value, which can be valuable folklore remedies in the treatment of cold, headache, acne, malaria and bacterial diseses [21.] The plant containing phenolic compounds contributed to their antioxidative properties and thus the value of the plants are in folklore medicine. Phenols have been practicing in the preparation of some antimicrobial agents such as dettol and cresol. Both plants are widely used regularly among many tribes in Africa for the treatment of various diseases. For instance, saponins proved as hypotensive and cardiodepressant properties [22,] which are helpful for the management of heart failure and cardiac myopathy[23] The occurrence of saponins in *C.macrostachyus* might play a role in the

cardioprotective potential. alkaloids have the potential of anti-hyperglycaemic and anti-inflammatory activities [24.]

The result of DPPH radical scavenging activity analysis indicates that both leaves and fruits of *C.macrostachyus* was potentially antioxidant properties. These results recommend that the plant extracts contain compounds that are potential to donate hydrogen atom to a free radical and makes them unstable. The capacity of these plant extracts to scavenge DPPH may possibly reproduce and prevent the generation of free radicals. The radical scavenging activity of these plant extracts were found to be significant; this shows that *C.macrostachyus* may be useful for treating radical associated pathological tissue injury [25.]

CONCLUSION

Based on several studies, ROS creates various pathophysiological disease conditions and lessen endogenous defense mechanisms. An increasing concentration of antioxidant can reduce oxidative stress and especially plant derived constituents could assist to reduce ROS by scavenging activity. Quality and amount of the plant based antioxidant compounds will really aid to create a new drug candidate for antioxidant rehabilitation. Medicinal herbs are the chief sources for getting natural antioxidants for a variety of therapeutic uses such as diabetes, inflammation, aging, cancer and diseases associated to radical actions. The present study is to investigate the antioxidant potential derived from natural herbs and it is an instance to explore conventional medicinal data which helps to fight against ROS.

REFERENCES

1. Tiwari AK. Imbalance in antioxidant defense and human diseases: multiple approach of natural antioxidants therapy. *Curr Sci* 2001; 81: 1179–1187.
2. Madhavi DL, Deshpande SS, Salunkhe DK. *Food antioxidants: Technological, Toxicological. Health perspectives*, Marcel Dekker, New York, 1996; 267-359.
3. Khlifi S, Hachimi YE, Khalil A, Es-Safi N, Belahyan A, Tellal R, Abbouyi E. I*n vitro* antioxidant properties of *Salvia verbenaca* L. hydromethanolic extract. *Indian J Pharmacol* 2006; 38: 276-280.
4. Joyce DA. Oxygen radicals in disease. *Adv Drug React Bull* 1987; 127: 476–479.
5. Farber JL. Mechanisms of cell injury by activated oxygen species. *Env Health Perspec* 1994; 102: 17-24.
6. Marchioli R, Schweiger C, Levantesi G, Tavazzi L, Valagussa F. Antioxidant vitamins and prevention of cardiovascular disease. *Epidemiol clin trial data Lipid* 2001; 36: 53-63.

7. Devaki T, Ravikumar V, Shivashangari KS, Govindaraju P. Effect of Boerhaavia diffusa on tissue anti-oxidant defense system during ethanol-induced hepatotoxicity in rats. *J Nat Rem* 2005; 5: 102-107.
8. Hamzah Z, Peter C, Mashhor M. *Gardens' Bulletin Singapore* 2012; 64: 523–527.
9. Gilbert MG. Euphorbiaceae. In: Flora of Ethiopia and Eritrea. Canellaceae to Euphorbiaceae.(Edwards S, Tadesse M, Hedberg I, Eds.), Vol. 2, part 2, Addis Ababa, Ethiopia, Uppsala, Sweden 1995.
10. Moshi MJ, Uiso FC, Mahunnah RLA, Mbwambo ZH, Kapingu MC. A survey of plants used by traditional healers in the management of noninsulin dependent diabetes mellitus. East Centr Afri J Pharmarceut Sci 2000; 3:30-39.
11. Mesfin F, Sebsebe D, Tilahun T. An ethno botanical study of medicinal plants in Wonago Woreda, SNNPR, Ethiopia. J Ethnobiol Ethnomed 2009; 5:28.
12. Yirga G, Teferi M, Kasaye M. Survey of medicinal plants used to treat human ailments in Hawzen district, Northern Ethiopia. Inter. J Biodivers Conserv 2011; 3:709-714.
13. Geyid A. A study of surface proteins, other adhesions and iron acquiring mechanisms in the pathogenesis of *Neisseria gonorrhoeae* strains isolated from STD patients in Ethiopia. J Ethiop Med Pract 2002; 4:14-20.
14. Mazzanti G, Bolle P, Martinoli L, Piccinelli D, Grgurina I, Animati F. *Croton macrostachys*, a plant used in traditional medicine: purgative and inflammatory activity. J Ethnopharmacol 1987; 19:213-219.
15. Kloos H. Preliminary studies of medicinal plants and plant products in markets of central Ethiopia. Ethnomed 1977; 4:63-104
16. Schmelzer GH, Gurib-Fakim A. Plant resources of tropical Africa medicinal plants I. Buckhuys publishers, Wageningen, Netherlands, 2008, 203- 206.
17. Basset J, Denny J, Jeffery JH, Mendham J. Vogel's textbook of quantitative inorganic analysis, 4thedition, ELBS-Longman, Essex, UK, 1985; 196.
18. Kokate CK, Purohit AP, Gokhle SB. Textbook of pharmacognosy, 1st edition, Nerali Prakasan, Pune,1990; 123.
19. Alekhya V, Deepan T, Shaktiprasanna Sahoo and Dhanaraju MD. Preliminary Phytochemical Screening and Evaluation of In vitro Antioxidant Activity of *Anthocephalous cadamba* by Using Solvent Extracts. Eur J Biol Sci 2013; 5 (1): 34-37.

20. Aiyegoro OA, Okoh AI. Preliminary phytochemical screening and *In vitro* antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. BMC Compl Alt Med 2010; 10:21
21. Fergusion LR. Role of plant polyphenols in genomic stability. Mutat Res 2001: 475: 89-111.
22. Olaleye MT. Cytotoxicity and antibacterial activity of methanolic extract of Hibiscus sabdariffa. J Med Plants Res 2007; 1: 9–13.
23. Brian FH, Thomas-Bigger J, Goodman G. The Pharmacological Basis of Therapeutics.7th Macmillan, New York: NY, USA, 1985.
24. Augusti KT, Cherian S. Insulin sparing action of leucopelargonidin derivative isolated from *Ficus bengalesis* Linn. Indian J Exp Biol 2008; 33: 608-611.
25. Wang M, Li J, Rangarajan M, Shao Y, La Voie EJ, Huang T, Ho C. Antioxidative phenolic compounds from sage (*Salvia officinalis*). J Agric Food Chem 1998; 46: 4869-4873.