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EVALUATION OF IN-VITRO ANTIOXIDANT POTENTIAL OF DIETHYL PHTHALATE ISOLATED FROM DECALEPIS HAMILTONII WIGHT & ARN

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ABSTRACT

Antioxidants are substances that inhibit or delay the oxidation processes. Therefore, they are able to protect the human body, foods and drugs from oxidative damages. It acts as free radical scavengers, reducing agents, quenchers of singlet oxygen molecule and activators for antioxidative enzyme to suppress the damage induced by free radicals in biological system. Free radicals play an important role in cell's life and death. It is normally balanced by endogenous antioxidant system. Imbalances in redox status may develop cellular oxidative stress. If the endogenous antioxidants fail to overcome the reactive metabolites production, then exogenous antioxidants would be necessary to balance redox status. Dietary sources, including plants, herbs, spices, vitamins and herbal extracts, play an important role in this regard. Decalepis hamiltonii Wight and Arn is the sole species of plant in the genus Decalepis belonging to the family Asclepiadaceae. It is endemic and endangered species of Peninsular India is commonly known as Magali Kizhangu in Tamil. Structural elucidation of the compound isolated from ethanol extract of Decalepis hamiltonii Wight & Arn seed was accomplished by GC-MS, UV, IR, ^{1}H- NMR and ^{13}C- NMR spectroscopic methods. In the present study, the antioxidative and radicalscavenging activities of the Diethyl phthalate and Methanolic extract were studied using Ferric Thiocyanate (FTC) and Hydrogen peroxide method . MEDEP of the plant exhibited strong antioxidant activity. The results indicate that MEDH contain significant amounts of phytoconstituents such as flavonoids, saponins,

phenolic compounds. Based on the findings, Methanolic extract was commonly found to have synergistically higher antioxidant activity.

KEYWORDS: Asclepiadaceae, Decalepis hamiltonii, Diethyl phthalate, Reactive oxygen species, Reactive nitrogen species.

INTRODUCTION

Antioxidants are substances that inhibit or delay the oxidation processes. Therefore, they are able to protect the human body, foods, and drugs from oxidative damages. Due to the benefits of antioxidants, food, and pharmaceutical products are normally enriched with synthetic antioxidants such as BHA, BHT, *etc*. However, most of the compounds have side effects . Hence, strong restrictions have been mandated for their application and there is also a trend to the development of more effective and safer antioxidants, especially from natural origins. Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals.[1] A free radical is a highly reactive molecule or molecular fragment that contains one or more unpaired electrons in its outer orbit and is capable of independent existence. Free radicals are generally of two types: "reactive oxygen species," (ROS) and "reactive nitrogen species" (RNS). Under certain conditions oxygen becomes much more active, forming ROS, such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and non free-radical species, such as H_2O_2 and singlet oxygen ($^1O_2$).The important RNS are nitric oxide radical ($NO^{\cdot}$) and peroxynitrite anion (ONOO–).[2] The sources of these free radicals in living body are respiratory chain, phagocytes, Arachidonic acid metabolism, cytophosphokinase , non enzymatic reaction of oxygen etc. These radicals sometimes working together with common metals like copper, iron and cobalt attack an important group of tissue constituent and results in oxidative stress. During normal physiologic processes in the body ROS are continuously generated and subsequently removed by the antioxidant defence mechanisms of the body.[3] Biological combustion involved in various processes produces harmful products or intermediates called reactive oxygen species or free radicals. Excess of free radicals in living beings has been known to cause various problems like asthma, cancer, cardiovascular diseases, liver diseases, muscular degeneration, and other inflammatory processes, resulting in the so-called oxidative stress. Oxidative stress is defined as imbalance between oxidants and antioxidants and causes damage in all types of biomolecules like protein, nucleic acid, DNA and RNA.[4] Hence, the

balance between reactive species or free radicals and antioxidants is believed to be a critical concept for maintaining a good biological system. Antioxidants act as free radical scavengers, reducing agents, quenchers of singlet oxygen molecule, and activators for antioxidative enzyme to suppress the damage induced by free radicals in biological system.[5] During the last decade, there has been an escalation of interest in the role of antioxidants in health care and diseases.[6] Antioxidants act as free radical scavengers and are thus found to play significant protective role against oxidative stress in a variety of diseases such as liver cirrhosis, inflammation, atherosclerosis , diabetes, cancer, neurodegenerative disease, nephro toxicity and also the aging process.[7] Antioxidants have the potential to prevent these oxidative damages and thereby minimize the homeostatic disturbances, by interfering with the oxidation process by reacting with free radicals, chelating catalytic metals or by acting as oxygen scavengers. Plants have significant antioxidant activities due to the presence of different compounds like polyphenols, flavonoids, terpenoids, *etc* . For example, various plant essential oils (such as clove, oregano, rosemary, sage, and lavender) have been reported to exhibit strong antioxidant and lipid protection properties. Generally, essential oils are widely used as food flavors and preservatives and extend the shelf life of dishes and processed food products.[8] Many species of fruits, vegetables, herbs, cereals, sprouts and seeds have been investigated for antioxidant activity during the past decade . Natural antioxidants are being extensively studied for their ability to protect organisms and cells from damage caused by oxidative stress which is considered as a cause of ageing and degenerative diseases.[9] Herbs and spices are, in general, harmless sources for obtaining natural antioxidants. The antioxidant capacity of plants is clearly associated with the activity of ‘‘free radical scavenging enzymes’’ (superoxide dismutase, catalase, peroxidase, etc.) and the contents of antioxidant substances mainly phenolic compounds, carotenoids, tocopherol and ascorbic acid There is an increasing demand to evaluate the antioxidant properties of plant extracts and in recent years, attention has been focused on antioxidant products from natural sources.Therefore there is a growing interest in finding naturally occurring potential antioxidants, especially from plant origin. Studies to date have shown that various common fruits and vegetables contain different promising antioxidant compounds such as Vitamin E, Vitamin C, β-Carotenoids as well as flavonoids, tannins and other polyphenolic constituents.[10]

Decalepis hamiltonii (Wight & Arn) is the sole species of plant in the genus Decalepis belonging to the family Asclepiadaceae. It is endemic and endangered species of Peninsular

India is commonly known as Magali Kizhangu in Tamil. It prefers to grow along rocky slopes, big rock boulders and rocky crevices and small mounds where there is thick vegetation at an altitude from 300 to 1200 m. Morphologically as well as chemically the plant resembles African liane called Mondia whitei Skeels. Milky latex is present in the entire plant. It has good medicinal importance and used in wide drug preparations.[11,12] It is one of the important plants in Ayurvedic system of medicine in India and are used in curing various diseases like stomach disorders, gastric ulcers ,stimulate appetite and as a general tonic, demulcent, diaphoretic, diuretic and tonic. It is useful in the loss of appetite, fever, skin disease, diarrhoea, nutrition disorders, blood purifier and flavouring principle. It is used as a food and health drinks.[13,14] Phytochemistry, pharmacology and conservation is required. The tubers have reported antimicrobial, antipyretic, antiulcer, antidiabetic, antioxidant, anti-inflammatory, chemoprotective, cytoprotective, insecticidal, neuroprotective and hepatoprotective activities. This plant has been used in the preparation of several herbal drugs like Amrutamataka taila, Drakshadi churna, Shatavari rasayana and Yeshtimadhu taila. A number of phytochemical compounds have been isolated from the seed this plant; of these, Ethyl linoleate and 4-tert-butylcyclohexyl acetate were bioactive compound with greater antidiabetic and antioxidant activity respectively.[15, 16] GC-MS analysis of Methanolic extract of Decalepis hamiltonii Wight and Arn seed revealed the presence of 13 compounds. The major constituent is Diethyl phthalate, which has antioxidant activity. Information on Decalepis hamiltonii Wight and Arn seed is scanty in available literatures thus suggesting that not much work has been done on the antioxidant potentials of this Bioactive constituent. The structure of the isolated compound was elucidated by GC-MS, UV, IR, ^{1}H- NMR and ^{13}C- NMR spectroscopic methods. In the present study, therefore isolate the phytoconstituent and evaluate its Antioxidant potential.

MATERIALS AND METHODS

Plant Materials

The Decalepis hamiltonii Wight and Arn seed were collected from Veeracholapuram of Ariyalur Distict , Tamil Nadu state, India. They were identified and authenticated by Prof. Dr. N.Ramakrishnan, Head and Associate Professor and voucher specimens (Department of Botany) and voucher specimens (GACBOT-168) were deposited at the Herbarium of the Department of Botany, Government Arts College (Autonomous), Kumbakonam, Bharathidasan University, India.

Extraction and Isolation: The important stage in the experimental work includes first the isolation of chemical substances from the chosen plant and secondly, the characterization of those isolated compounds. Dried Decalepis hamiltonii Wight &Arn seeds(1.5kg) were ground to a fine powder and extracted with MeOH at 37°C. The extract was concentrated and the solvent recovered by simple distillation. The isolation of the MeOH extract was dried by vacuum evaporation and then subjected to column chromatography over silica gel (60-120 mesh, Merck, India) as the stationary phase. The charged column was then eluted with mixture of EtOH, EtOAc and $CHCl_3$ (60:20:20). The fractions were monitored by TLC. First Five fractions indicated presence of viscous liquid along with some unidentified compounds . First Five fractions were further purified by re-column chromatography obtained as pure transparent colourless liquid (12g). The fractions were grouped together and dried by vacuum evaporation and had taken up in acetone and dried by vacuum evaporation when a colourless liquid separated, Boiling point 295°C.[17] The synthesized molecule show bioactivity as already reported. Structural elucidation of the compound isolated from Methanol extract of Decalepis hamiltonii Wight &Arn seed was accomplished GC-MS, UV, IR, ^{1}H- NMR and ^{13}C- NMR spectroscopic methods.

Structural Identification: GC-MS was recorded using Perkin Elmer Clarus 500. UV spectral analyse was recorded using UV- Visible Spectrophotometer Lambda 35 from Perkin Elmer, UV(EtOH) λ_{max} 204, 227, 281 and 312 nm . IR spectrum was recorded with a Perkin Elmer RXI FT-IR spectrometer as a thin film on KBr plate. IR spectra were recorded on a Perkin Elmer spectrum on spectrometer using KBr disc given in cm^{-1} 851, 873, 1015, 1066, 1136, 1363 , 1453,1733,2855,2924 and 2963. Supporting evidence for the structure of the compound is provided by the ^{1}H (DMSO, 300 MHz) and ^{13}C- NMR (100 MHz, DMSO) spectra were recorded on a Bruker AMX 300 NMR spectrometer.

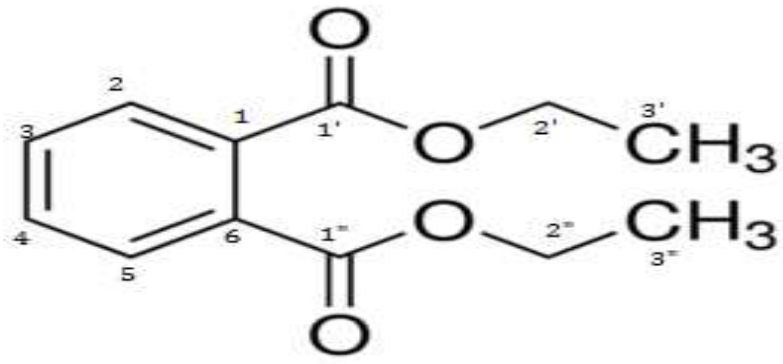

Figure.1 Structure of Diethyl phthalate

EVALUATION OF IN VITRO ANTIOXIDANT ACTIVITY

Hydrogen peroxide scavenging activity: The scavenging capacity for hydrogen peroxide was measured according to the method.[18] Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the fractions (25-400 µg/ml) in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging ability (in triplicate) was calculated by the formula: % scavenging = (1 – Ae / Ao) × 100 where Ao is the absorbance without sample, and Ae is absorbance with sample.

The percentage scavenging activity at different concentrations of the fractions was determined and the IC_{50} values were compared with the standard, α-tocopherol.

Table 1: Hydrogen peroxide scavenging activity of Diethyl phthalate(DEP) and Methanolic extract of Decalepis hamiltonii Wight &Arn seed.

Concentration (µg/ml)	% Percentage of Inhibition		
	α -Tocopheral	DEP	MEDH
0.5	26	24*	38
1.0	37	35*	41
1.5	40	39*	45
2.0	45	46*	53
IC_{50} (µg)	2.5	2.3	1.25

Values are average of triplicate experiment and are represented as Mean The p-values <0.05 are regarded as significant.

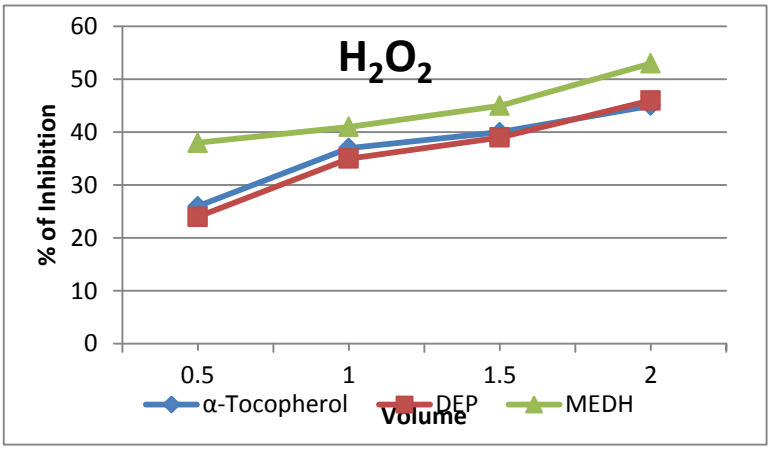

Figure 2: Hydrogen peroxide scavenging activity of Diethyl phthalate and Methanolic extract of Decalepis hamiltonii Wight &Arn seed.

Ferric thiocyanate (FTC) method: The antioxidant activities of plant extracts were determined according to the FTC method[19] with slightly modifications. The peroxy radical scavenging activity was determined by Thiocyanate method using α- tocopherol (50-800 µg/ml) as standard. Increasing concentration of the fractions (50-800 µg/ml) in 0.5 ml of distilled water was mixed with 2.5 ml of 0.02 M Linoleic acid emulsion (in 0.04 M phosphate buffer pH 7.0) and 2 ml phosphate buffer (0.04M, pH 7) in a test tube and incubated in darkness at 37°C. At intervals during incubation, the amount of peroxide formed was determined by reading the absorbance of the red colour developed at 500 nm by the addition of 0.1 ml of 30% ammonium thiocyanate solution and 0.1 ml of 20 mM Ferrous chloride in 3.5% hydrochloric acid to the reaction mixture. % Inhibition = 100 - [(A_1/A_0) × 100]

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample extracts. The percentage scavenging activity was calculated and the IC_{50} values of the fractions were compared with the standard, α-tocopherol.

Table 2: Ferric thiocyanate scavenging activity of Diethyl phthalate and Methanolic extract of Decalepis hamiltonii Wight &Arn seed

Concentration (µg/ml)	% Percentage of Inhibition		
	α -Tocopheral	DEP	MEDH
0.5	45	66	68
1.0	40	58	58
1.5	38	30*	36*
2.0	26	8	32
IC_{50} (µg)	2.52	1.30	0.88

Values are average of triplicate experiment and are represented as Mean.

The p-values <0.05 are regarded as significant.

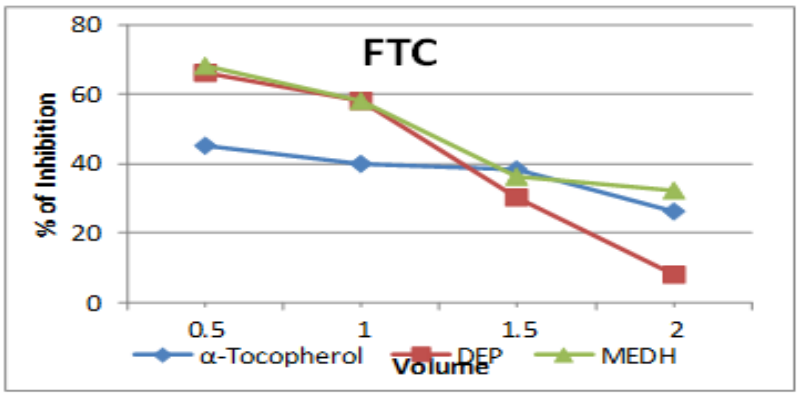

Figure 3: Ferric thiocyanate scavenging activity of Diethyl phthalate and Methanolic extract of Decalepis hamiltonii Wight &Arn seed.

Calculation of 50% Inhibitory Concentration (IC_{50}): The concentration (mg/ml) of the fractions that was required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the fractions. Percentage of inhibition (%I) was calculated using the formula,

% I = (Ac-As) / Ac x 100

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

Statistical Analysis: All experimental measurements were carried out in triplicate and were expressed as average of three analyses. Statistical analyses were performed by p-value was done by one way ANOVA. The p-values <0.05 were regarded as significant.

RESULT AND DISCUSSION

Structure of Diethyl phthalate: The Compound isolated as a colourless transparent liquid. It exhibits a molecular ion peak at *m/z* 223 $[M+1]^+$ which is a base peak. Typical fragment at 148.9 *m/z* (64*%)* is noted for phthalate moiety, which is in accordance with the molecular formula $C_{12}H_{14}O_4$. The IR spectrum displays a characteristic absorption frequency at 1733 cm^{-1} for ester carbonyl groups. The absorption bands at 2963 cm^{-1}, 2924 cm^{-1} and 2855 cm^{-1} are noticed for C-H stretching frequency of ethyl group. The absorption bands at 1458 cm^{-1} and 1383 cm^{-1} are noticed for aromatic stretching frequency. Bands at 1286 cm^{-1}, 1135 cm^{-1} (C-O Stretching of -COO-) and 1066 cm^{-1} (C-O Stretching of O-CH_2) are noticed for -C-O stretching and at 851 cm^{-1} (out of plane of bending) represents ortho disubstitution of benzene ring. ^{1}H NMR spectrum, has displayed a downfield triplet at δ1.33 (t, *J* = 7 *Hz*, 6H) for (H 3' and H 3") methyl protons which are attach to oxy methylene group. A downfield quartet at δ 4.37 (q, *J* = 7 *Hz*, 4H) is noticed for (H 2' & H2") protons. The doublet of doublets at δ 7.52 (dd, *J* = 8 & 2 *Hz*, 2H) and δ 7.72 (dd, *J* = 8 & 2 *Hz*, 2H) are indicated for (H 2 & H 5) and (H 3 & H 4) aromatic protons respectively.^{13}C NMR spectrum, displays presence of six signals. This is in accordance with twelve carbon atoms, indicating two sets of identical and equivalent carbon atoms. Each signal corresponds two carbon atoms. A quartet at δ 14.06 is assigned to (C 3' and C 3") methyl carbon atoms. The triplet at δ 61.57 is noticed for (C 2' & C 2") for ethoxy methylene carbon atoms. The downfield doublets at δ 128.83 and δ 130.09 are observed for (C2 & C5) and (C3 &C4) aromatic carbon atoms. A singlet at δ 132.27 is indicated for (C1 and C6) tetra substituted aromatic carbon atoms. The most downfield singlet at δ 167.59 is assigned for ester carbonyl carbons(C 1' & C 1").[20] GC-MS, UV, IR, ^{1}H- NMR and ^{13}C- NMR spectral analyses showed that the active molecule

isolated from Decalepis hamiltonii Wight & Arn seed was Diethyl phthalate ($C_{12}H_{14}O_4$) as in Figure -1.

Hydrogen peroxide scavenging activity: Table -1(Figure-2) shows hydrogen peroxide scavenging activity of the DEP ,MEDH and standard. MEDH caused a strong dose-dependent inhibition of hydrogen peroxide. The MEDH showed good scavenging ability compared to the standard compound. The IC_{50} values for the extract were found to be 1.25 µg /ml compared to standard ascorbic acid 2.5 µg /ml. At a concentration of 2 µg /ml, the scavenging percentages were 46 and 53 for MEDH and standard respectively.

Ferric thiocyanate (FTC) method: From the analysis, it shows that all samples exhibited good effect in inhibiting linoleic acid oxidation compared to control (α -Tocopheral) ($p<0.05$). The result in Table-2 and Figure- 3 indicated that the inhibition percentage of lipid peroxidation of DEP (8 %) was significantly lower ($p<0.05$) compared to reference compound α -Tocopheral (26 %). Also, MEDH inhibition percentage of lipid peroxidation had greater than that DEP ($p<0.05$). The most likely mechanism of antioxidant protection is direct interaction of the extract (or compounds) and the hydrogen peroxide rather than altering the cell membranes and limiting damage . The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging . The results indicate that MEDH contain significant amounts of phytoconstituents such as flavonoids, saponins and phenolic compounds. Flavonoids and phenolic compounds have good antioxidant potentials and mechanism of action of flavonoids is through scavenging or chelation[21], while phenolic compound are important because of their hydroxyl groups which confer scavenging ability.

CONCLUSION

This study affirms the *in vitro* antioxidant potential of Diethyl phthalate and Methanol extract of *Decalepis hamiltonii*.Results are compared with ascorbic acid. To our knowledge, this is the first report demonstrating that Diethyl phthalate and Methanol extract of *Decalepis hamiltonii* have antioxidant activity as seen in the hydroxyl radical scavenging assay and FTC. From the two samples, methanol extract shows high antioxidant properties than Diethyl phthalate. But further studies are required to clarify the *in vivo* potential of this plant. Polyphenolic compounds are known to have antioxidant activity. This activity is believed to

be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. The results strongly suggest that phenolics are important components of this plant and some of its pharmacological effects could be attributed to the presence of these valuable constituents.[22] People who eat fruits and vegetables have a lower risk of heart disease and some neurological diseases and there is evidence that some types of vegetables, and fruits in general, protect against some cancers Since fruits and vegetables happen to be good sources of antioxidants, this suggested that antioxidants might prevent some types of diseases.. This suggests that these health benefits come from other substances in fruits and vegetables or come from a complex mix of substances. The replacement of synthetic with natural antioxidants (because of implications for human health) may be advantageous. The obtained results might be considered sufficient to further studies for the isolation and identification of some other bioactive principles responsible for the antioxidant activity.

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