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Original Article

Determination of antioxidant activity of the selected extracts of *Mathuka Neriifolia* (Moon) H.J. Lam, isolation and characterization of phytoconstituents of the ethanol extract by column chromatography.

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ABSTRACT

The present study involves the determination of antioxidant activity of the selected extracts of *Mathuka Neriifolia* (Moon) H.J. Lam, isolation and characterization of phytoconstituents of the ethanol extract by column chromatography.The antioxidant potential of plant stem extracts were investigated by hydroxyl radical scavenging activity, super oxide free radical scavenging activity, DPPH assay, nitric oxide radical scavenging activity, total antioxidant activity. Among the three extracts ethanol extract was having maximum antioxidentactivity. So the ethanol extract was subjected to column chromatography,by means of gradient elution technique.The fractions 260 – 300 and 301 – 344 ,gave two compounds A 1 and A 2.Later by spectral analysis they were found to be Quercetin and Luteolin 7 – glucoside.

KEYWORDS : *Mathuka Neriifolia* (Moon) H.J. Lam.,soxhlet extractor, hydroxyl radical scavenging activity, super oxide free radical scavenging activity, DPPH assay, nitric oxide radical scavenging activity, total antioxidant activity, Quercetin and Luteolin 7 – glucoside.

INTRODUCTION

There are a number of higher plant species on this planet of which 250,000 are estimated 2[-3]. Out of these, only about 6% have been screened for biologic activity and 5% have been evaluated phytochemically[4].It was estimated in 1991 in United States that for every 10,000 pure compound that are biologically evaluated, 20 would be tested in animal models,and 10 of these would be clinically evaluated, and one would reach U.S. Food and Drug Administration approval for marketing.The time required for this process was estimated at 10 years at a cost of $231 million[5].

Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide play a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson's disease. The free radicals in the human body are generated through aerobic respiration or from exogenous sources [6]. Some of the invivo free radicals play a positive role in phagocytosis, energy production and regulation of cell growth, etc. However, free radicals may also be dangerous. Free radicals produced in the body react with various biological molecules namely lipids, proteins and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants. Even though our body is safeguarded by natural antioxidant defense, there is always a demand for antioxidants from natural sources [7]. Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals [8]. They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers [9]

Antioxidants from plant materials terminate the action of free radicals, thereby protecting the body from various diseases [10]. There is a growing interest all over the world for discovering the untapped reservoir of medicinal plants. Hence, the present study was aimed at measuring the antioxidant activity and the compounds responsible for the activity in *Mathuka Neriifolia* (Moon) H.J. Lam.

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Madhuca neriifolia (Moon) H.J. Lam belongs to the family Sapotaceae, this is a medium sized tree,bark dark brown. Leaves simple, crowded at the tips of branchlets,linear-oblong or oblanceolate, acute or obtuse, 7.5-25x 2.5-6 cm. Flowers yellowish white, in clusters of 4- 10,axillary or from the scars of fallen leaves. Fruits ellipsoid, about 2.5 cm long. Flowers are used in the treatment of kidney complaints. Fruits are recommended in cases of rheumatism, biliousness, asthma and worm trouble. Oil from seeds are used to treat rheumatism and for improved growth of hair.[12]

From the literature it was found that no other study regarding the antioxidant activity of the plant *Madhuca neriifolia* (Moon) H.J. Lam, has been conducted and as the plant was us to treat rheumatism and other ailments which is a caused due to free radical activity we decide to go for antioxidant study [17] .

MATERIALS AND METHODS

Plant material and preparation of the extract: Fresh stem of ***Mathuka Neriifolia*** (Moon) H.J. Lam. was collected from the outskirts of Kerala, authenticated and identified by Dr.St. Tessy Joseph,H.O.D ,Dept of Botany,Nirmala College of pharmacy, Kerala. Shadow dried and powdered. Powdered material was passed through sieve No.60. Then extracted separately using hexane, petroleum ether, chloroform,ethyl acetate, ethanol by the soxhlet extraction method. The hot percolation method was employed for water for 48 hrs. The extracts were concentrated using a rotary vacuum evaporator. Dried extracts were stored in an airtight container and placed in refrigerator.[11-13]

Antioxidant assays

Hydroxyl Radical Scavenging Activity: This assay is based on the quantification of the degradation product of 2 deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} - ascorbate- EDTA –H_2O_2 system (The Fenton reaction). The reaction mixture contained in the final volume of 1 mL-2 deoxy 2 ribose (2.8mM) KH_2PO_4—KOH buffer (20 mM pH 7.4), FeCl3 (100µM), EDTA (100µM), H_2O_2 (1.0mM), ascorbic acid (100µm) and various concentrations (0-200µg/ml)of the extracts. After incubation for 1hour at 37°C, 0.5 ml of the reaction mixture was added to 1ml of 2.8% TCA, then 1ml aqueous TBA was added and the mixture was incubated at 90°C for 15 minutes to develop the color. After cooling the absorbance was measured at 532nm against a blank solution.

Super oxide free radical scavenging activity: Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion is generated in riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. Different concentrations of extracts (125-2000µg/ml), 0.05ml of Riboflavin solution (0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of the solution was measured at 560nm using DMSO as blank after illumination for 5 min and difference in OD was determined after 30 minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560 nm on UV visible spectrophotometer.

DPPH assay: The radical scavenging activity of different extracts was determined by using DPPH assay according to. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm .Ascorbic acid (10mg/ml DMSO) was used as reference.1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as, DPPH + [H-A] →DPPH-H + (A).Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (1.25-10µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm.3ml of DPPH was taken as control.

Nitric Oxide Radical Scavenging Activity: Nitric oxide (NO) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite the possible beneficial effects of NO., its contribution to oxidative damage is also reported. This is due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potent oxidant that can decompose to produce OH and NO.. The procedure is based on the principle that, sodium nitro prusside in aqueous

solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO. may lead to tissue damage. Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL^{-1})in phosphate buffered saline pH 7.4,was mixed with different concentration of the extract (62.5-2000µg mL^{-1})prepared in methanol and incubated at 25°C for 30minutes .A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide,2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl ethylene diamine dihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard.

Total Antioxidant Activity: 0.3ml extract was obtained with 3ml of reagent solution (0.6ml H_2SO_4,28mM sodium phosphate and 4mM ammonium molybdate).The tube containing the reaction solutions were incubated at 95c for 90 minutes.The absorbance of the solution was measured at 695nm against blank after cooling to room temperature (Methanol 0.3ml) in the place of extract was used as blank.The antioxidant activity is expressed as number of gram equivalent of ascorbic acid.

Isolation and characterization of phytoconstituents from ethanolic extract of *Mathuka Neriifolia* (Moon) H.J. Lam whole plant

Elution of column

A gradient elution technique was followed , the ratios are given in the following table:

Fraction Number	Solvent ratio for column elution	NO. of spots	Rf value
1-4	100% P.E	-Nil-	-Nil-
4-6	P.E 90% : B 10%	-Nil-	-Nil-
6-15	P.E 80%: B 20%	-Nil-	-Nil-
15-30	P.E 70% : B 30%	Three	0.65,0.7,0.4
30-50	P.E 60% :B 40%	-Nil-	-Nil-
50-70	P.E 50% : B 50 %	-Nil-	-Nil-
70-100	P.E 30% : B 70 %	-Nil-	-Nil-
100-120	100% B	-Nil-	-Nil-
121-130	B 90% :C 10%	Three	0.5,0.6,0.9
131-135	B 80%:C 20%	-Nil-	-Nil-
136 – 145	B 50%:C 50%	-Nil-	-Nil-
146 – 166	B 20%:C 80%	-Nil-	-Nil-
167 – 180	B 10%: C 90%	-Nil-	-Nil-
181 – 190	100% C	-Nil-	-Nil-
191 – 195	C99% :M1%	-Nil-	-Nil-
196 – 200	C98%:M2%	-Nil-	-Nil-
201 – 210	C97%:M3%	Three	0.8,0.6,0.4
211 – 230	C96%:M4%	Three	0.8,0.6,0.4
231 – 259	C95.5%:M4.5%	Three	0.8,0.6,0.4
260 – 300	C95%:M5%	One	0.6
301 – 344	C93%:M7%	One	0.7

P.E.: Petroleum ether, B:Benzene, C:Chloroform, M:Metanol

Evaporation of fraction: During the column elution process, the fractions 260 – 300 and 301 – 344 has a single banding pattern which was confirmed by TLC study.So the fractions are combined and kept for evaporation to dryness in room temperature.After drying the dried residue was scrapped off once again checked for its purity and named as A1 for first fraction and A 2 for second.The remaining fractions were not worked out because of lower yield as well as impure.The compounds were sent for spectral analysis i.e., FTIR,LC-MS,C13 NMR and HNMR for structural elucidation.

RESULT AND DISCUSSION

Several techniques have been used to determine the antioxidant activity in vitro in order to allow rapid screening of the extracts of ***Mathuka Neriifolia* (Moon) H.J. Lam** . Free radicals are known to

play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. The electron donation ability of natural products can be measured by 2,20 -diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching [18]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [19]. In the present study among all the fractions tested, ethanol extract showed significantly higher inhibition percentage. Results of this study suggest that the plant extract contains phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage and showed the presence of phenols. Superoxide radical is considered a major biological source of reactive oxygen species [20]. The results of our study revealed satisfactory with the ethanol extract and showed the presence of flavanoids.

The antioxidant capacity of the fractions was also performed. The results were satisfactory and showed the presence of both phenols and flavanids.Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [21,22]. The hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and contributes to carcinogenesis, mutagenesis and cytotoxicity [23]. Hydroxyl radicals were produced by the reaction of H2O2 and the ferrous that would react with 2-deoxyribose. The reaction was stopped by adding TBA reagent that would give a red color if the malonaldehyde was formed as the result of the reaction between the radical and 2-deoxyribose. Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour [24]. All fractions of ***Mathuka Neriifolia*** **(Moon) H.J. Lam** when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose. Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food [23]. H2O2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage [25]. Ethanol fraction of ***Mathuka Neriifolia*** **(Moon) H.J. Lam** efficiently scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it into water.

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, and inhibition of platelet aggregation and regulation of cell mediated toxicity [26]. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [27,28]. NO is known to be a ubiquitous free-radical moiety, which is distributed in tissues or organ systems and is supposed to have a vital role in neuromodulation or as a neurotransmitter in the CNS [29]. In the previous study, all the three extracts of ***Mathuka Neriifolia*** **(Moon) H.J. Lam** showed significant decrease in the NO radical due to the scavenging ability . This was due to the presence of numerous flavonoids, including quercetin, kaempferol, catechin, rutin, and naringin[30].

Determination of Invitro antioxidant activity of extracts of stem of *Mathuka Neriifolia* (Moon) H.J. Lam. : The in vitro antioxidant activity by hydroxyl radical scavenging activity, super oxide free radical scavenging activity, DPPH assay, nitric oxide radical scavenging activity, total antioxidant activity were conducted. Control and the stem chloroform ethanol and water extracts of ***Mathuka Neriifolia*** **(Moon) H.J. Lam.** (Test) were used. The test results are presented in Table 1-5 and Figure 1-5. Antioxidant activity of plant extracts was carried at different concentrations to determine the IC50 (50% growth inhibition) . The high antioxidant activity was with ethnic extract of ***Mathuka Neriifolia*** **(Moon) H.J. Lam** in Table 6. It was found that, the percentage of growth inhibition is increasing with increasing concentration of test compounds.

Isolation and characterization of phytoconstituents from *Mathuka Neriifolia* (Moon) H.J. Lam: *Mathuka Neriifolia* (Moon) H.J. Lam. was extracted by the successive solvent extraction by means of a soxhlet extractor, using hexane, petroleum ether,chloroform, ethylacetate, ethanol and water. The extracts were concentrated and were subjected to a preliminary phytochemical screening .The ethanol, chloroform and water extracts were having maximum number of constituents.Classicaly the plant was used as an anticancer agent in various parts of Kerala.So these extracts were subjected to antioxidentactivity. Among the three extracts ethanol extract was having maximum antioxidentactivity. So the ethanol extract was subjected to column chromatography,by means of gradient elution technique.The fractions 260 – 300 and 301 – 344

,gave two compounds A 1 and A 2.Later by spectral analysis they were found to be

Quercetin and **Luteolin 7 – glucoside., Interpretation and observation of A 1 sample[14-15]:** The isolate A 1 was a Yellow powder , of M.P316^{0}c,give a single spot on TLC with BAW of Rf value 0.96 and of λ max **:** 370nm Figure: 1.This shows the isolate A1 is **Quercetin** Figure : 16**.**The compound in its IR spectra exhibited absorption bands at 1000 – 750 cm^{-1} for aromaticity,1500 – 1250 cm^{-1} for alkyl, 1662 cm^{-1}a broadband for ketone and bands at 3406 cm^{-1} and 3315 cm^{-1} for phenolic OH group.Figure: 2.The LC-MS data shows a peak at retention time 11.4 and molecular weight of 303 in positive mode. Figure: 3.In its ^{1}H-NMR spectra shows, bands between δ 6 – 6.7 shows aromaticity, δ 7 – 7.6 shows presence of phenolic OH group, δ 3.2 - 3.5 shows presence of –CH2- , - CH and an instance peak at δ 4.79 shows the presence of OH group.Figure : 4.^{13}C-NMR spectrum exhibits a signal at the range δ 116 -177 shows the presence of aromaticity with 12 carbon atoms. So two aromatic rings may be present. An instance band at δ 49 shows the presence of the alkyl group.Figure : 5.

Interpretation and observation of A 2 sample[16]: The isolate A 1 was a dull ochre powder , of M.P 256^{0}c,give a single spot on TLC with BAW of Rf value 0.69 , of λ max **:** 348nm and bright yellow coloration with ammonia**.** Figure: 6.This shows the isolate A2 is **Luteolin 7 - glucoside** Figure : 17**.**The compound in its IR spectra exhibited absorption bands at 1000 – 750 cm^{-1} for aromaticity,1500 – 1250 cm^{-1} for alkyl, 1662 cm^{-1}a broadband for ketone and bands at 3406 cm^{-1} and 3315 cm^{-1} for phenolic OH group. Figure : 7.The LC-MS data shows a peak at retention time 8.1 and molecular weight of 609 in negative mode. Figure :8.In its ^{1}H-NMR spectra shows, bands between δ 6 shows aromaticity, δ 7 shows the presence of phenolic OH group, δ 3 shows presence of –CH2- , - CH and an instance peak at δ 5 shows the presence of OH group. Figure: 9.^{13}C-NMR spectrum exhibits presence of 25 carbon atoms, a signal at the range δ 116 – 179 shows the presence of aromaticity with 12 carbon atoms. So two aromatic rings may be present. An instance band at δ 49 shows the presence of the alkyl group. Figure: 10

CONCLUSION

The plant ***Mathuka Neriifolia*** **(Moon) H.J. Lam,** ethanol extract was having good antioxidant activity when compared to the other extracts, showed minimum IC_{50} value. So further studies were conducted for isolation and identification of biologically active substances by column chromatograph.It confirmed the presence of two phenolic compounds, **Quercetin** and **Luteolin 7 – glucoside.**As phenolic compounds are having antioxidant activity,we concluded that the antioxidant activity of the ethanolic extract was due to the presence of these compounds.

Fig:1 UV spectra data of A 1

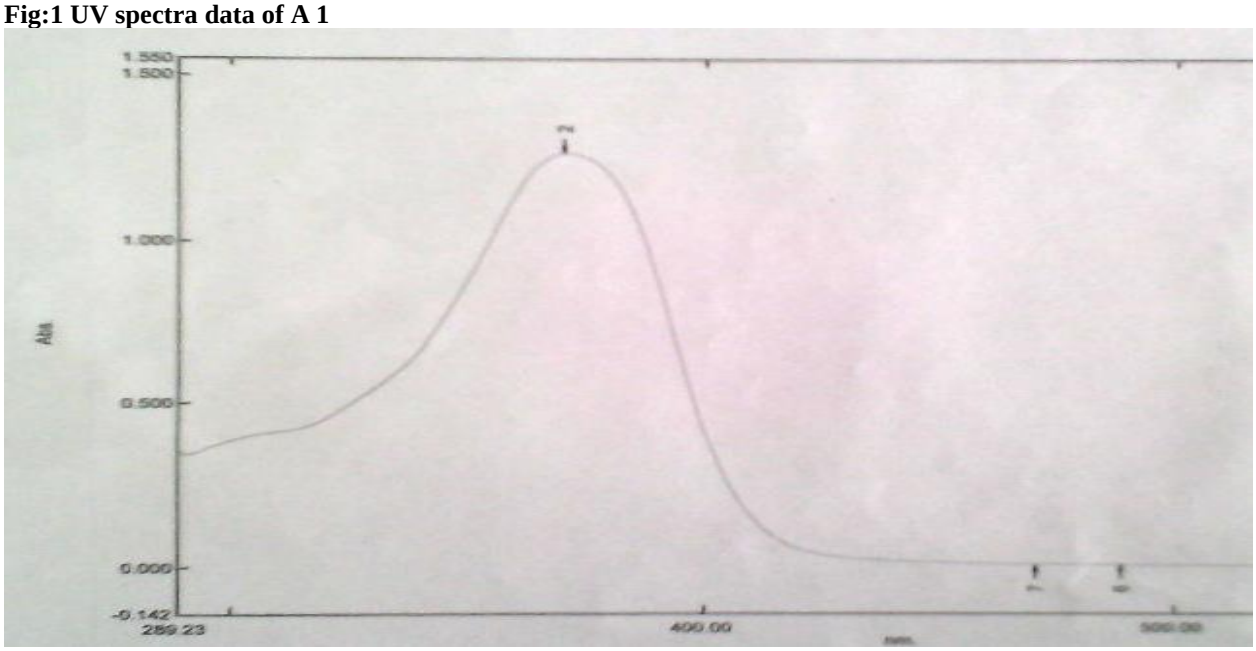

Fig 2: FTIR data of A 1

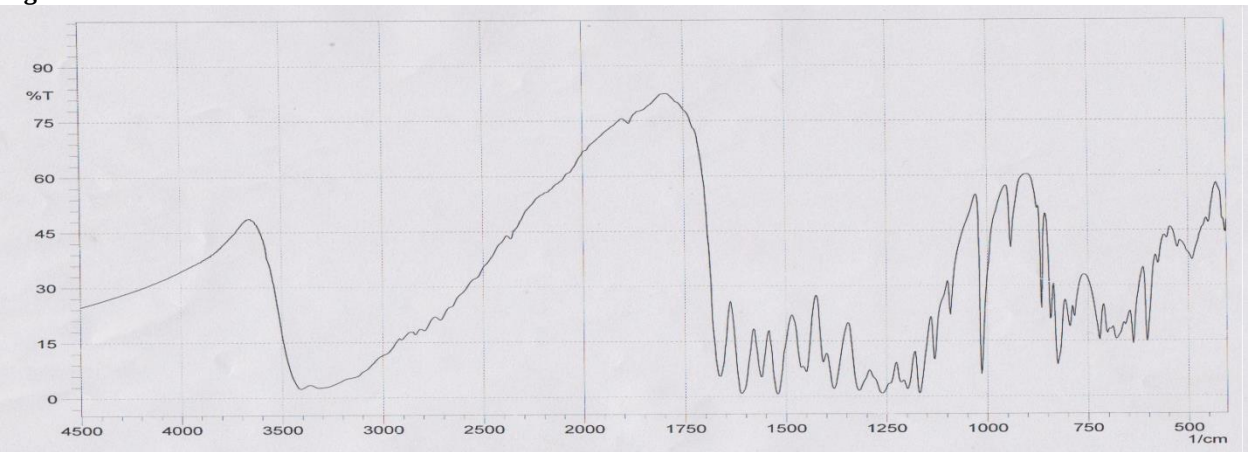

Fig 3: LC-MS data of A1

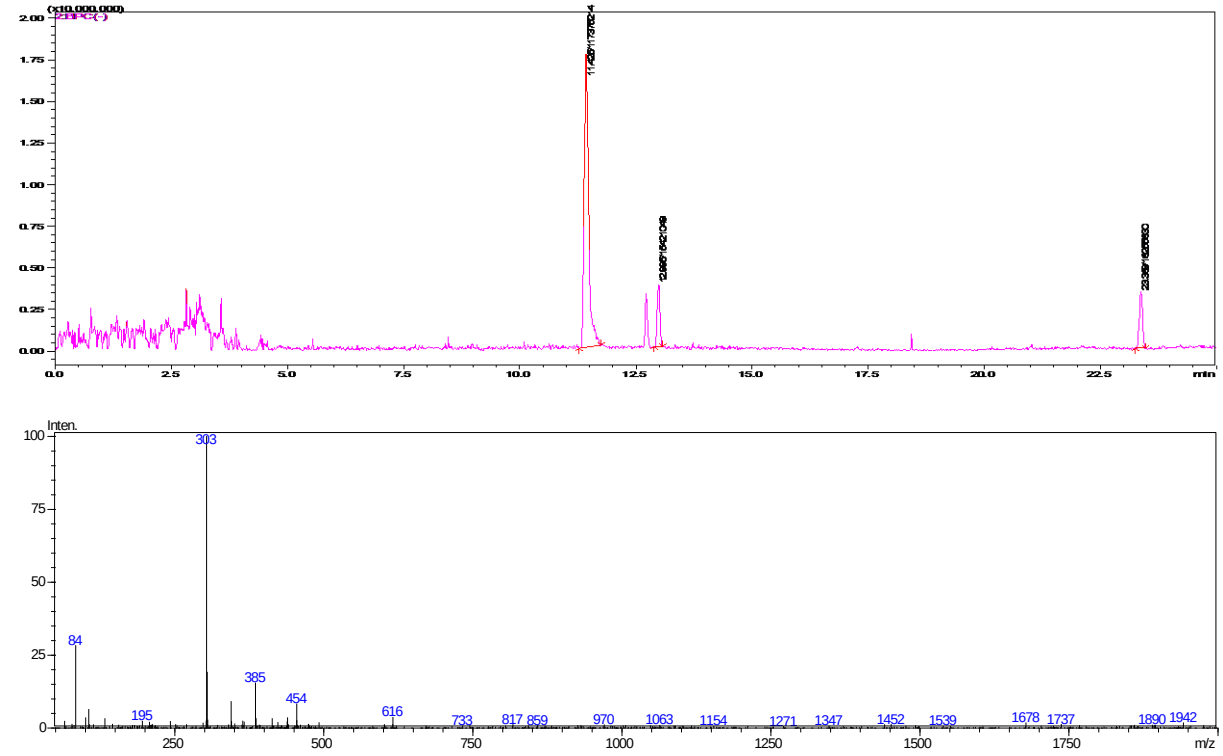

Fig 4:[1]H-NMR data of A 1

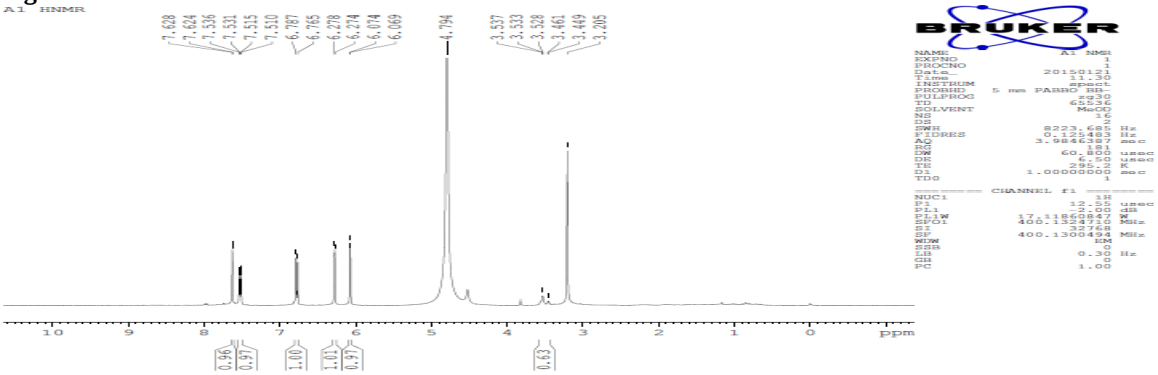

Fig 5: ^{13}C-NMR data of A 1

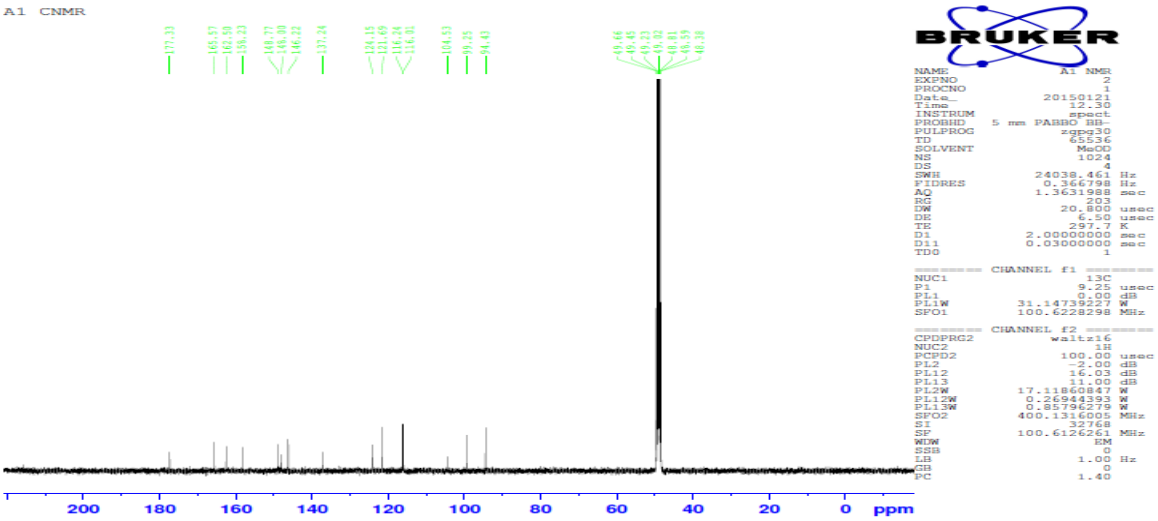

Fig 6: UV spectral data of A 2

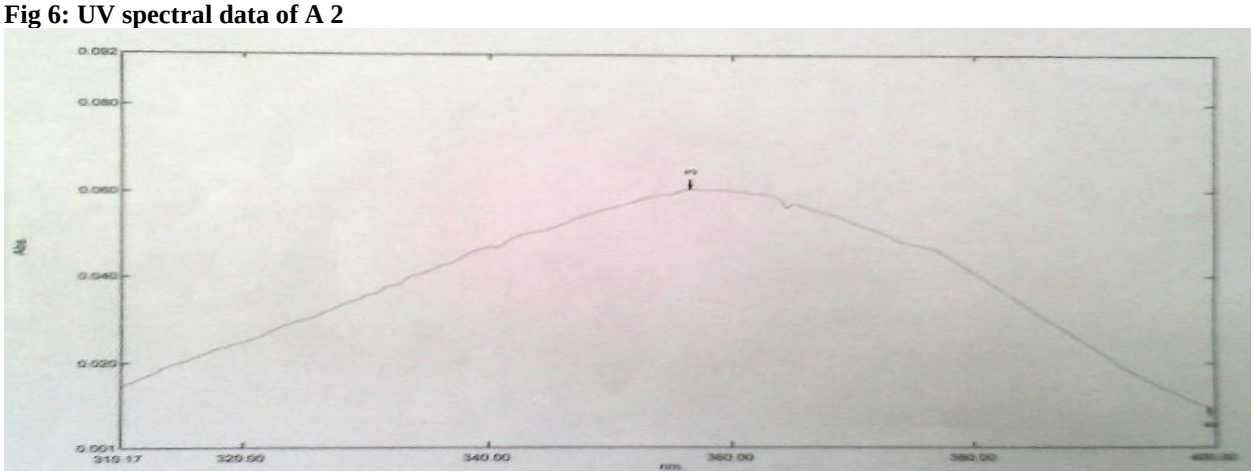

Fig 7: FTIR spectral data of A 2

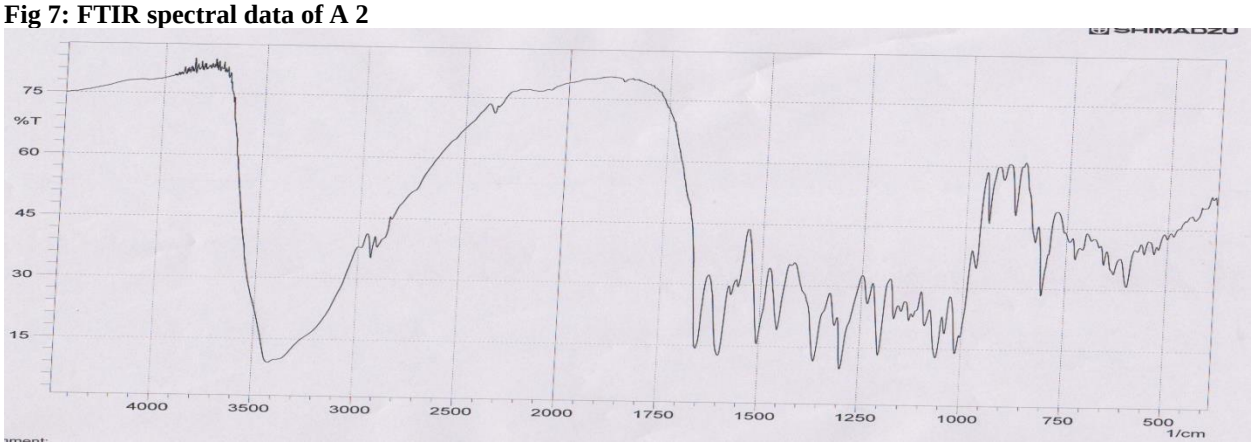

Fig 8: LC-MS data of A 2

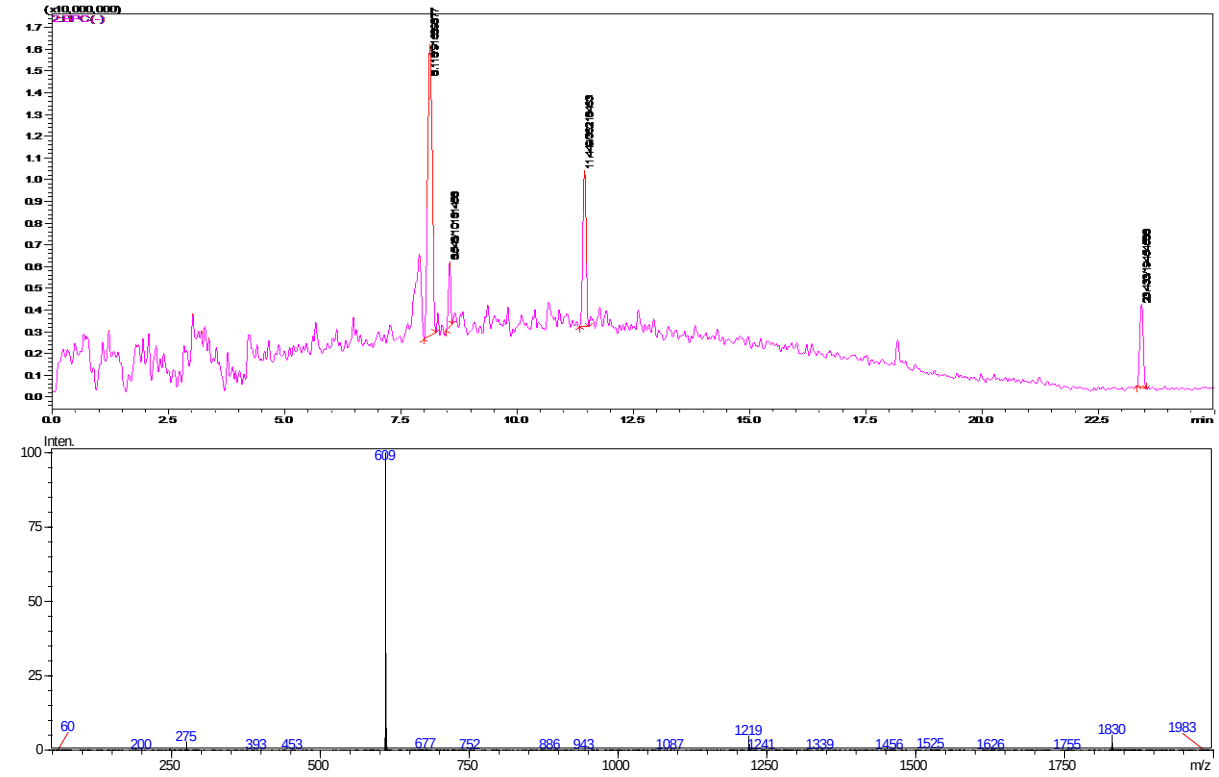

Fig 9: ^{1}H-NMR data

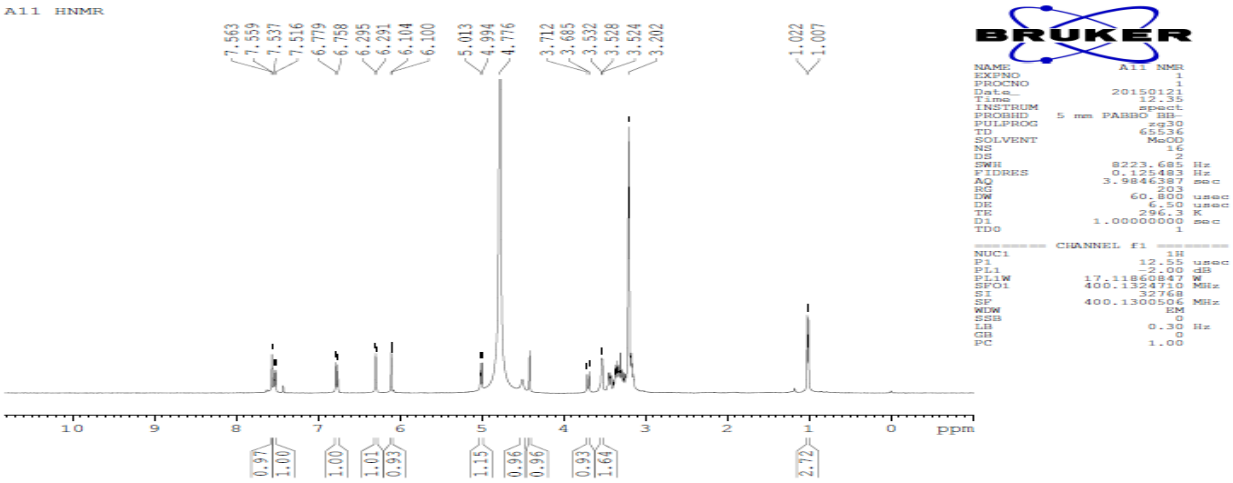

Fig 10 : ^{13}C-NMR data

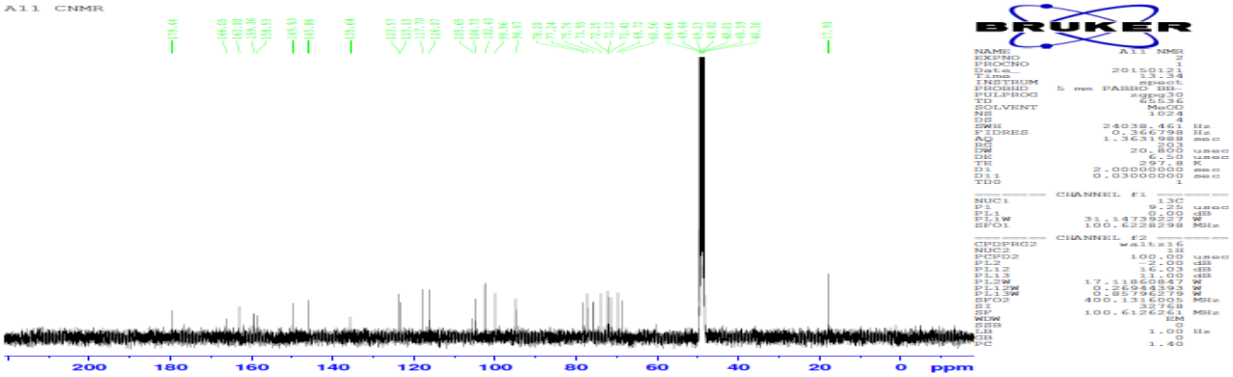

Fig 11:Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. By DPPH

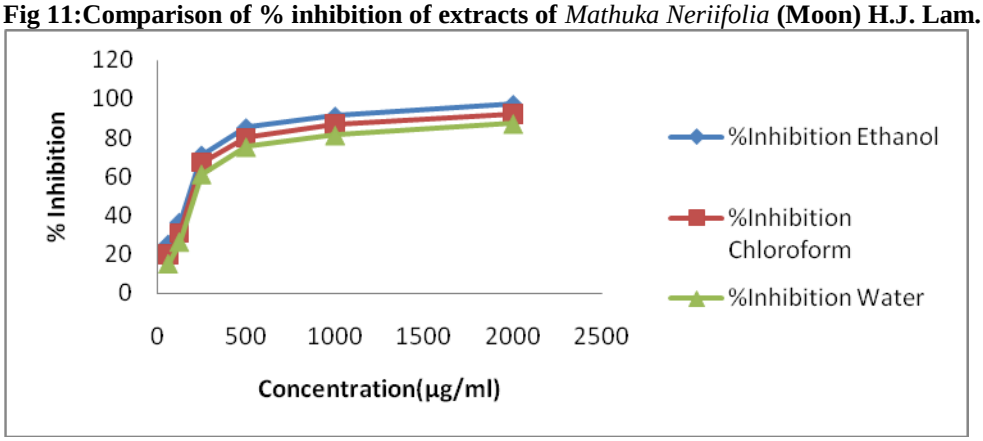

Fig 12: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by hydroxyl radical scavenging activity

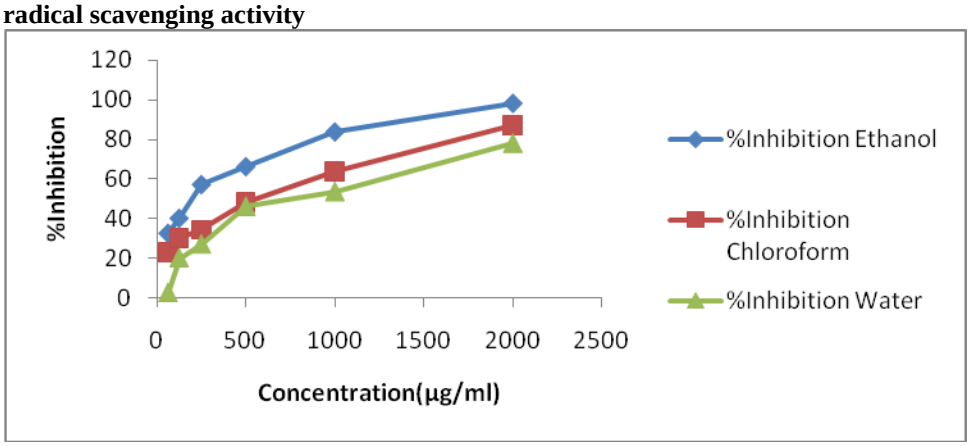

Fig 13: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by nitric oxide radical scavenging activity

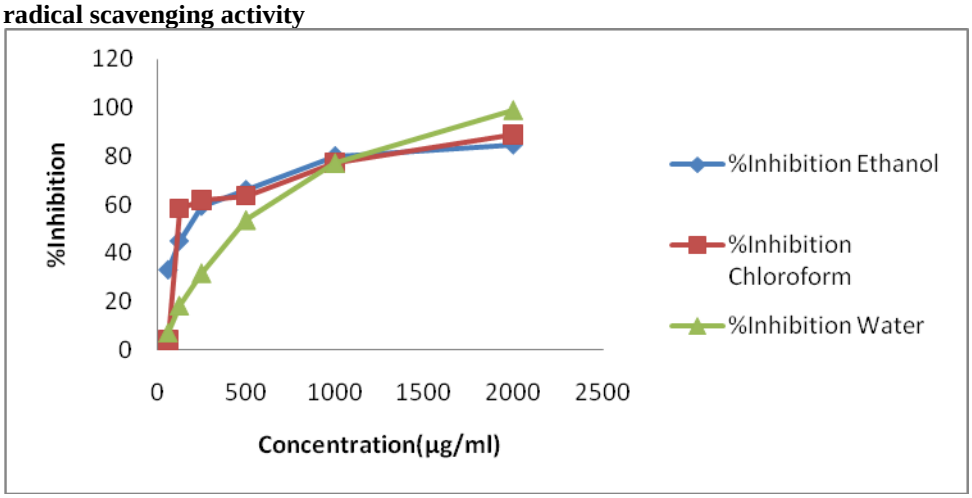

Fig 14: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by super oxide free radical scavenging activity

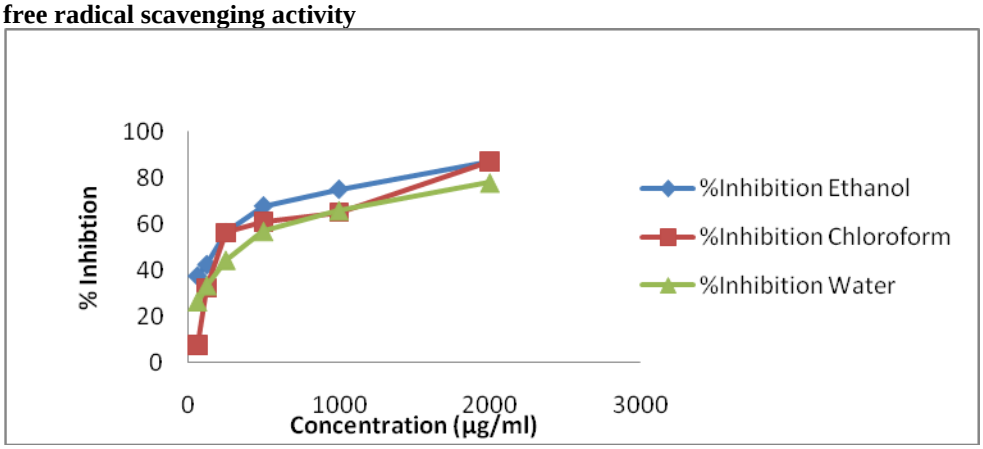

Fig 15: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by total antioxidant activity

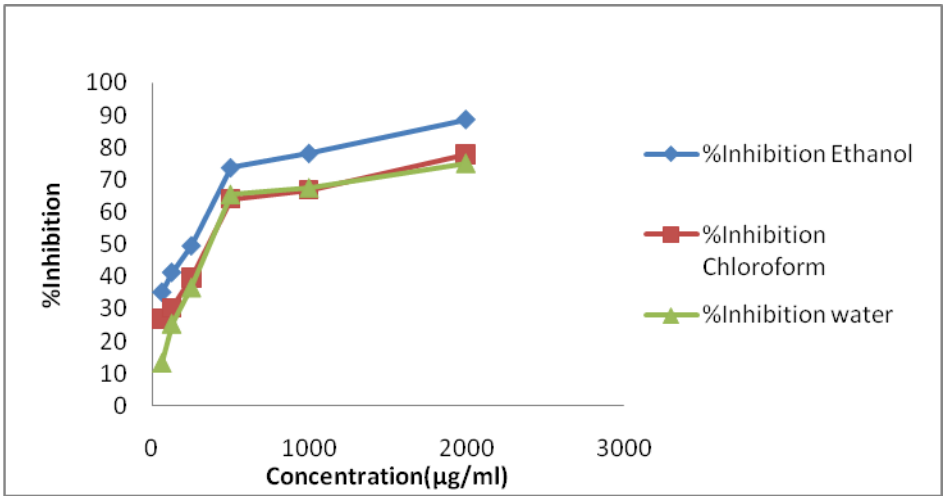

Fig 16: Quercetin

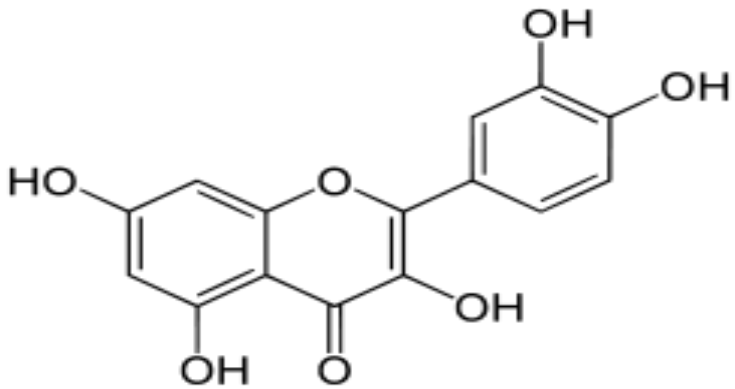

Fig 17 : **Luteolin 7 – glucoside**

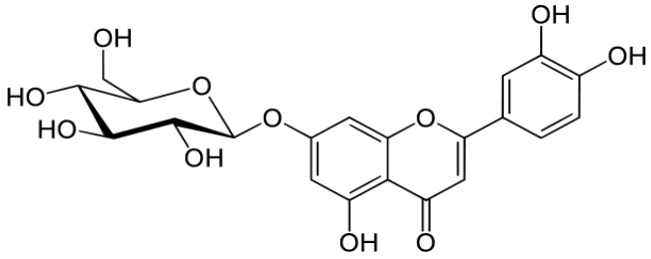

Table 1: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by DPPH

Con(µg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**Water**
62.5	25	20	15
125	36.29	31.09	26.19
250	71.05	67.45	61.23
500	85.19	80.24	75.34
1000	91.20	87.13	81.42
2000	97.25	92.55	87.35

Table 2: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by hydroxyl radical scavenging activity

Con(µg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**Water**
62.5	32.68	22.88	2.68
125	40.24	30.22	20.24
250	57.16	34.38	27.16
500	66.17	48.25	46.17
1000	83.61	63.54	53.61
2000	97.99	87.12	77.99

Table 3: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by nitric oxide radical scavenging activity

Con(µg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**Water**
62.5	33.32	3.82	7.12
125	45.15	58.29	18.29
250	59.47	61.68	31.68
500	66.24	63.53	53.53
1000	80.15	77.07	77.07
2000	84.76	88.86	98.86

Table 4: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by super oxide free radical scavenging activity

Con(µg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**Water**
62.5	37.36	7.36	26.13
125	42.28	32.28	33.19
250	56.14	56.14	44.25
500	67.7	60.70	56.8
1000	74.91	64.91	65.82
2000	87.01	87.01	77.91

Table 5: Comparison of % inhibition of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by total antioxidant activity

Con(µg/ml)	%Inhibition		
	Ethanol	Chloroform	water
62.5	35.22	26.79	13.42
125	41.34	30.21	25.54
250	49.55	39.71	36.75
500	73.67	64.20	65.54
1000	78.11	66.97	67.6
2000	88.61	78.03	75.21

Table 6: Comparison of IC 50 of extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. by antioxidant tests

Antioxidant test	DPPH	Hydroxyl radical scavenging activity	Nitric oxide radical scavenging activity	Super oxide free radical scavenging activity	Total antioxidant activity
Chloroform	240.645µg/ml	749.345µg/ml	345.71µg/ml	612.91µg/ml	627.2µg/ml
Ethanol	90.645µg/ml	244.61µg/ml	155.22µg/ml	197.39µg/ml	270.43µg/ml
Water	411.6129µg/ml	1034.848µg/ml	708.8µg/ml	641.66µg/ml	756.79µg/ml

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