EXTRACTION , ISOLATION AND CHARACTERISATION OF AQUOUS ETHANOLIC EXTRACT OF *ALLIUM HOOKERI* Thaw. Enum LEAVES AND STUDY ITS INVITRO ANTIHYPERTENSIVE POTENTIAL

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***Abstract*— Allium Hookeri is a grassy, perennial herb belongs to Alliaceae family, Its leaves and roots are used by Manipuris as a spice and condiments daily. . Its leaves are used by local traditional healers in lowering Blood pressure and the cholesterol level . The present study deals with isolation and characterisation of the leaf extract of *A. hookeri* . Ethanol fractions was subjected to column chromatography on silica gel and eluted stepwise using suitable solvent system .Two compounds were isolated ie compound A and compound B. Compound A isolated by hexane and ethylacetate showed positive test for flavanoid and compound B isolated by chloroform and methanol showed positive for flavanoid and glycoside and characterization of isolated compounds were carried out by FTIR, ^{1}HNMR , and Mass spectroscopy.**

Thus we can rationalise many folk lore medicines with modern techniques and in a scientific way.

I. INTRODUCTION

Now, herbal therapy is becoming more focused as up-to-date analysis and research show their value in the treatment and prevention of diseases with lesser side effect, better acceptability and economical.Manipur ,one of the north eastern states of India, is one of the hot- spot area of biodiversity. Manipuris are having good knowledge of herbs and its uses. And the local healers (Maiba and Maibis) are practicing folk lore medicines since time immemorial. *Allium hookeri Thaw Enum* is a perennial plant widely grown in dampy soil and in every kitchen garden in Manipur. Its leaves and roots are used as condiment and spice in regular house hold diet. Its taste is preferred over the use of onion by Manipuris.

Hypertension is a worldwide epidemic accounting about 6% of deaths globally (Gudina *et al.*, 2013). Overall, the prevalence of hypertension appears to be around 30–45% of the general population, with a steep increase with ageing (Mancia *et al*., 2013). Although there has been recent progress in the prevention, detection and treatment of hypertension, It persists as a major public health challenge affecting more than one quarter of the population worldwide (Okumura *et al*., 2014).

The general aim of pharmacological therapy is the reduction of BP *per se* by whatever means. As arterial pressure is product of CO and PVR, it can be lowered by the action of drug on either the peripheral resistance or CO, or both (Kolck *et al.*, 2004; Siyad, 2011). The ultimate goal of antihypertensive therapy is adequate control of BP to reduce the cardiovascular and renal morbidity and mortality (Taylor and Rahman, 2009). This can be achieved using a single therapy but most commonly concurrent use of drug from different classes is a strategy for achieving effective control of BP while minimizing the dose related adverse effects (Tran and Giang, 2014).

The number of synthetic antihypertensive agents available in clinical practice is also not effective in all cases This would help to impart adequate and scientific justification for the traditional use of *Allium hookeri* as well as it can be the source of lead compounds in the treatment of hypertension in addition to the worldwide severity of the diseases itself. Moreover, these agents are characterized by high cost, lack of definitive curative regimen, combinational therapy, which discourages drug adherence, many side effects and drug-drug interaction (Kagathara *et al*., 2009). That is why most of the patients and even medical professionals prefer herbal medication and preventive strategies in hypertension management (Kagathara *et al*., 2009). In the last three decades, many concerted efforts have been channeled into researching local plants with antihypertensive effect, of which some of these medicinal plants have been validated and others disproved (Tabassum and Ahmad, 2011). Therefore, those herbs, including the current experimental plant, can be source of drugs with fewer side effects and better bioavailability in the treatment of hypertension (Soncinia *et al)* The plant extracts usually occur as combination of various type of bioactive compounds or phytochemical with different polarities, Their seperation still remains a big challenge for the process of identification and characterization of bioactive compounds. The pure compounds are then used for the determination of structure and biological activity.., *Allium hookeri* (Alliaceae family) is an important ethnomedicinal plant native to the Himalayan region of Asia (Ayam, 2011; Pandey *et al.*,

2008).Manipuris folk lore practioner are using it for lowering blood pressure and cholesterol level. Allium hookeri has gained significant attention recently because it has many useful compounds such as phenolic antioxidants flavanoids fiber, ascorbic acid,allicin and unknown sulphur compounds distinct from A. cepa (Kala,2005;Rhyu and Park 2013)All these compounds have potent pharmacological properties like anti;inflammatory, antimicrobial activity, ability to lower blood pressure and cholesterol. According Medical Dictionary of Mayanmar ,A. hookeri is used to treat cancer or inflammation because of methyl sulfonyl methane (Bae- Bae,2012). A. hookeri contains proteins, glucose,fibre,ascorbic acid, phytosterol, total phenol than onion (Ayam.2011). However the chemical structure of many novel compounds in A.hookeri and their biological effects on health remain to be elucidated. Considering the above facts this study is an attempt to isolate the compounds in A. hookeri and to characterise the compounds by FTIR ,NMR, Mass spetroscopy . This would help to impart adequate and scientific justification for the traditional use of *Allium hookeri* as well as it can be the source of lead compounds in the treatment of hypertension.

II. MATERIALS AND METHODS

A. Collection and Authentification of plant materials:-

The plant material of Allium hookeri was collected in the month of june 2014 from Khongman ,Imphal East District of Manipur , India.. The fresh harvested leaves were washed with tap water ,dried under shade at room temperature . The plants were wrapped with plastic sheets during transportation to avoid any possible damage. Identification and authentication of the plant was done by chief scientist Dr Sunita Garg of CSIR-NISCAIR New Delhi and a voucher specimen was deposited at CSIR-NISCAIR Ref No NISCAIR/ RHMD CONSULT/ 2014/2479-58 Dated 8-7-2014 for future reference

B. Extraction procedure

The air dried leaf material was cut into small pieces and dried in shadow for at least two weeks for complete drying. Then it was grinded into a mixer and converted into fine powder. The 1000 g of powdered leaf materials were packed in soxhlet apparatus and successive extraction was performed using Hexane, Chloroform, Ethyl acetate and Methanol . The solution of the extract was filtered through Whatman No.1 filter paper and concentrated using rotary flash evaporator and dried under vacuum.

Preliminary Photochemical Analysis for alkaloids, carbohydrates , flavanoids, tannins polysterols ,saponins and glycosides were performed.

Total phenolic content and Total flavanoid content in A. hooker leaf extract were determined

Estimation of total phenolic content:- the total phenolic content was determined by the spectrophotometric method using Park *et al* (2008) with slight modifications.

In brief , 0.5 mL of the leaf extract was mixed with 2.5 ml of 0.2 (N) Folin- Ciocalteu"s phenol reagent. After 5 mins ,2ml of 7.5 % Na Co_3 solution was added to the mixture followed by addition of 13ml of deionized distilled water and mixed throughly. The mixture was kept in dark for 90 mins at 23 ^{o} C, after which the the absorbance was read at 750 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The estimation of phenolic compound was carried out in triplicate and expressed in as milligrams of gallic acid equivalents (GAE) per gram dry material.

C. Estimation of Total Flavanoid Content

Total flavanoid content was determined by Dowd method described by Meda *et al* (2005) with some modifications 1.5 ml of extract was reacted with 2% (w/v) aluminium chloride for 10 mins at room temperature and absorbance was read at 415nm. Total flavanoid content was determined by standard curve of quercetin (0-50mg/L) and expressed as mg of quecertin equivalents (QE) per gram dry material.

DPPH radical scavennging activity :- The free radical scavenging activity of the leaf extracts were measured with DPPH ,using the slightly modified method described by Yadav *et al* (2014) the leaf extracts (0.5Ml) various concentrations namely 5,10,20,50,100 **µg** were mixed with 2.5 ml of DPPH solution respectively. The reaction mixture was vortexed thoroughly and left for 30 mins. The absorbance of the mixture was measured at 517 nm. The ascorbic acid was used as the reference drug. The ability of plant extract to scavenge DPPH radical was calculated from the following formula:-

DPPH scavenging effect (%) = (OD of control – OD Test / OD control) x 100

Where OD control is the absorbance of the control reaction and OD test is the absorbance of the extracts. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph and inhibition percentage was plotted against extract concentration

Invitro ACE inhibition activity:- ACE from rabbit lung (purified ACE) ,hippuric acid (HA) ,hyppuryl-L- histidyl-L-leucine(HHL), boric acid , Nacl were obtained from Sigma – Aldrich, India.

The ACE inhibition activity measured as reported by Nakamura *et al* with minor modification . A sample solution (200**(µ**l), of different concentrations (10,20,30,50,100,200 **(µ** g/ml) of *A. hookeri* extracts, was added to 200**(µl)** containing 5Mm hippuryl –L hystidyl- L- leucine (HHL) and the pre -incubated for 3 mins at 37 ^{0} C .Allium hoookeri extracts and HHL were prepared in 100mM borate buffer ,having PH 8.3 containing 300Mm NACl . The reactions were then initiated by adding 20(µl) OF 0.1 U/ml ACE prepared in the same buffer and incubated for 30 mins at 37*c The enzymatic reaction was terminated by addition of 250(µLOF 1.0 M HCl. The hippuric **acid (**HA) liberated was extracted with 1 ml of ethylacetate and 0.8 ml of the

extract was evaporated and the residue was then dissolved in 1ml of sodium borate buffer . Absorbance at 228 nm was measured to estimate the ACE inhibitory activity. The concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined as IC_{50} ACE inhibitory was calculated using the following formula

$$ACE\ inhibition\ (\%) = \frac{A_1 - A_2}{A_1 - A_3} \times 100$$

Where A1 is the absorbance of HA generated without Extract/inhibitors, A2 is the absorbance of HA generated in the presence of Extract/ inhibitors and A3 is the absorbance of HHL + buffer solution.

D. Thin Layer Chromatography

Of the various methods of separating and isolating plant constituents, thin layer chromatography (TLC) is one of the most powerful techniques used for the separation, identification and estimation of single or mixture of components present in various extracts. Mechanism employed in this reliable technique is adsorption in which solute adsorbs on the stationary phase according to its affinity. Substances are separated by differential migration that occurs when a solvent flows along the thin layer of stationary phase. The substance which is having more affinity towards mobile phase moves faster when compared to the substance which has less affinity leading to the separation of the compounds.

E. TLC Plates

Precoated silica gel were used as a stationary phase.

F. Sample application

The extracts to be analysed were diluted with respective solvents and then spotted with help of capillary tube just 2 cm above its bottom.

G. Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Solvents were analysed as in its order of increasing polarity. Several mobile phases were tried for the separation of maximum components. After several trials, the best solvent system was selected which showed good separation with maximum number of components.

H. Solvent system

Hexane extract - Methanol: Chloroform (9:1)
Chloroform extract - Methanol: Chloroform (9:1) Ethyl acetate extract - Methanol: Ethyl acetate: Hexane: Acetic acid (2:7:1: 0.5) Methanol extract - Ethyl acetate: Water (6:3:1)l
Ethanol fraction was subjected to column chromatography on silica gel and eluted with stepwise gradient polarity using hexane: Ethylacetate (100:0, 70:30.60:40,50:50,30:70, 20:80,0:100. ;Each 250 ml) . The system was further eluted with ethylacetate chloroform (100:0,90:10,80:20,60:40,50:50,40:60,20:80;0:100; each 250 ml. Again it is further eluted with high polar solvent Chloroform- Methanol ((98:2,96;4,92:8,90:10;88:12;86:14;84:16;82:18,80:20,; each 250ml). fractions were subjected to TLC studies . These fraction having prominent spot and having more or less similar Rf value were combined together and the solvent was evaporated to get pale green solid mass. This solid was dissolved in hot ethanol and the colour was removed by activated charcoal and recrystallized using ethanol. The solid is again subjected to TLC using different solvent systems ie Hexane;Ethylacetae (90:10); Hexane : ethylacetate (50:50) and Chloroform : ethanol (95:5) to check the homogeneity of the compound. It is observed from the Rf value of the TLC spot.. Then, the isolated compounds were submitted for Mass, FTIR and ^{1}H-NMR for spectral analysis for structural elucidation.

III. RESULTS AND DISCUSSION

Successive extraction by Soxhlet apparatus shows the presence of alkaloids, flavanoids ,terpenoids, tanins, polyphenols nd phytosterols.

Table 1 shows the phytochemical screening of A. hookeri leaf extracts

extract	ster	alk	fla	sap	terp	tan	polyp
hexane	-	-	-	-	-	+	_
chloro	+	-	+	-	+	+	+
Eth.acet	+	-	+	-	-	-	+
ethanol	+	+	+	-	-	+	+
aqueous	+	-	+	-	-	+	+

(Ster- steroids, alk- alkaloids, fla- flavanoids ,sap- saponins, ter- terpenoids, tan- tanins, polyp- polyphenols.).

It was noted that tanins were only present in hexane extract whereas saponins and alkaloids were absent in chloroform extract . Steroids ,flavanoids and polyphenols were present in ethyl acetate extract. Steroids, alkaloids, flavanoids tanins and poly phenols were present in ethanol extract.

Table 2 .physicochemical property of A .hookeri
Different Physicochemical Parameter

S. No.	Parameters	Result
1.	Total Ash value (%w/w)	**14.91**
2.	Acid insoluble ash (%w/w)	**0.56**
3.	Water soluble ash (%w/w)	**6.15**
4.	Sulfated ash	**19.98**
9.	Soluble ash	**5.22**
10.	PH of the powdered drug	**5.92**

All the parameters were performed according to WHO and Ayurvedic guidelines. Heavy metals like lead, mercury, arsenic and cadmium were not detected .

Table 3 shows the TLC profile of different extract

EXTRACT	SOLVENT SYSTEM	NUMBER OF SPOTS	Rf value
Ethyl acetate	Hexane : Ethyl acetate (50:50)	6	0.29; 0.42; 0.64; 0.73; 0.82; 0.96
Ethanol	Dichloromethane : methanol (95:5)	4	0.19; 0.28; 0.83; 0.92
methanol	Chloroform : Methanol (90:10)	9	0.12; 0.43; 0.52; 0.59; 0.62; 0.73; 0.79; 0.84; 0.87
Aqueous	Butanol : Acetic Acid: water (4 :1:2)	3	0.24; 0.48; 0.76

Table 4 shows the Total phenolic content in A.hookeri leaf extract

S.NO.	SAMPLE	TOTAL PHENOLIC CONTENT (µg/ml)
1.	Aquous extract	**267.58 ± 2.07**
2.	Ethanol extract	**118.42 ± 1.88**
3.	Ethyl acetate extract	**214.71 ± 2.46**
4.	Methanol extract	**303.57 ± 2.07**

Phenolics hinders oxidative degradation of lipids and thereby enhance the excellence and nutritional value of food. Phenolics possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic as well as modifying gene expression(Srivastava *et al* ,2013) the ethanolic extract recorded highest phenol content (303.57**±2.07)** compared to aqueous extract (267.58 ±2.07)

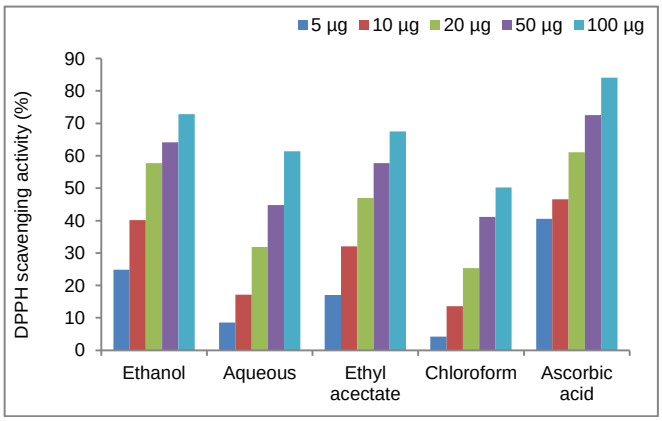
ethyl acetate± (214.71 **± 2.46) and chloroform (118.42±1.88)**

Table 5 shows the total flavanoid content

S.NO.	SAMPLE	TOTAL FLAVANOID CONTENT (µg/ml)
1.	Aquous extract	**7.16 ± 1.25**
2.	Ethanol extract	**149.78 ± 4.58**
3.	Ethyl acetate extract	**134.4 ± 12.09**
4.	Methanol extract	**40.4 ± 1.85**

Flavanoids are group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and and antiinflammatorybaction (Frankel ,1995) it was noted that ethanol extract recorded highest flavanoid content (149.78 ± 4.58) compared to ethyl acetate (134.4 ±12.09) ,chloroform (40.4 ±1.85) and aqueous (117.16 ±1.25) extracts .Srivastava *et al* (2013) in his study recorded total flavanoid content of 18.50 mg catechin equivalents /100 gms of fresh mass in stem of Allium. Cepa.

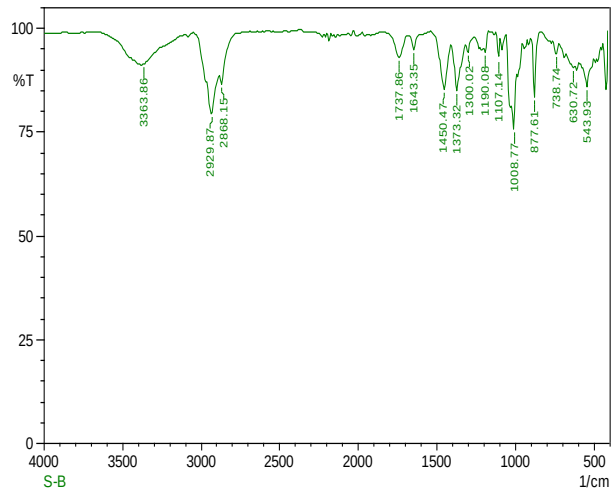
Fig 1showing DPPH scavenging activity



In vitro antioxidant activities of allium hookeri leaf extract were evaluated at various concentrations using DPPH assay.

It was noted that inhibition percentage increased by increasing the concentration providing(50%) inhibition (IC 50) and it was noted that ethanol leaf extract recorded the highest inhibition percentage of 72.86% followed by ethyl acetate (67.5%) aqueous (61.3%) and chloroform (50.2%)

Fig. 2 FT-IR spectra of isolated column A.



Compound–A:
Mass: m/z = 443 (M+1) , m.p. : 249-252 ^{o}C

FT-IR (KBr cm-1): 3363 cm^{-1} (-OH stretching); 2929, 2868 cm^{-1} (-CH stretching); 1643 cm^{-1} (-C=C stretching); 1450 cm^{-1} (-CH bending); 1373 cm^{-1} (-CH bending).

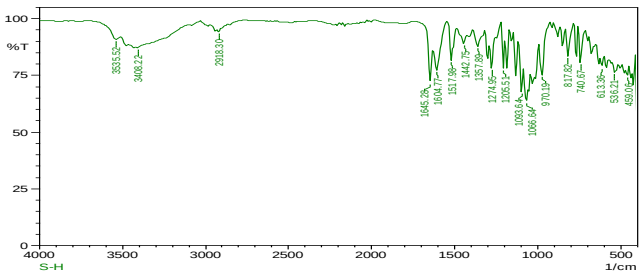
^{1}H-NMR ($CDCl_3$): δ ppm: 0.75 (3H, s, H-24); 0.82 (3H, s, H-25); 0.96-0.97 (6H, d, - H-27,H-23); 1.02-1.09 (6H, s, H-26); 1.23-1.25 (7H, m, H-13, H-29); 1.36-1.39(5H, m, H-16); 1.68(4H,m, H-30); 1.84 - 2.03 (10H, m, H-1, H-5, H-6, H-7, H-9, H-11, H-13, H-18, H-21, H-22); 2.58 -2.62 (1H,d, H-15); 3.16-3.19 (1H,m,H-3); 3.30-3.33 (1H,d, 28a); 3.77-3.79 (1H,d, 28b); 4.57 (1H,s, 29a); 4.68 (1H, s, 29b).
Compound is identical to BETULIN
The chemical formula of compound A is $C_{30}H_{50}O_2$

And molecular weight is 442.73 and Rf value is 0.64

Compound B:
Mass: m/z = 611.31 (M+1) , m.p. : 246-248 ^{o}C

FT-IR: 3535 cm^{-1}, 3408, 2918, 1645, 1604, 1517, 1442, 1274, 1205. The IR spectrum as KBr disk showed a strong band of -OH group at 3535 cm^{-1}, aliphatic -CH at 2918, aromatic -C=C at 1604, 1517, 1442 cm^{-1} and of -C=O at 1645cm-1, -C-O at 1274, 1205 cm^{-1}.

Fig. 3. FT-IR spectra of isolated compound B.



^{1}H-NMR (DMSO-*d6*): δ ppm: 1.09-1.10 (1H,m, H-6’’’); 2.73-2.79 (1H, m, H-3a); 3.09-3.18 (2H, m, H-3b); 3.24-3.29 (3H,m, H -2, H-3); 3.35-3.43 (3H, m, H-4); 3.51 -3.53 (1H, d, H-5); 3.64 (1H, m, H-6); 3.78 (3H, S, -OCH_3); 4.45-4.47 (1H, d, H-1); 4.53(2H, m, H-1’’’); 4.59-4.62 (1H, m, -OH glucose); 4.67-4.70 (1H, m, -OH glucose); 4.97 -5.00 (1H, t, -OH glucose); 5.16 -5.19 (2H, m, -OH glucose);

5.39 -5.40 (1H, d, -OH glucose); 5.49-5.52 (1H, m, H-2); 6.13-6.15 (2H, m, H-6,

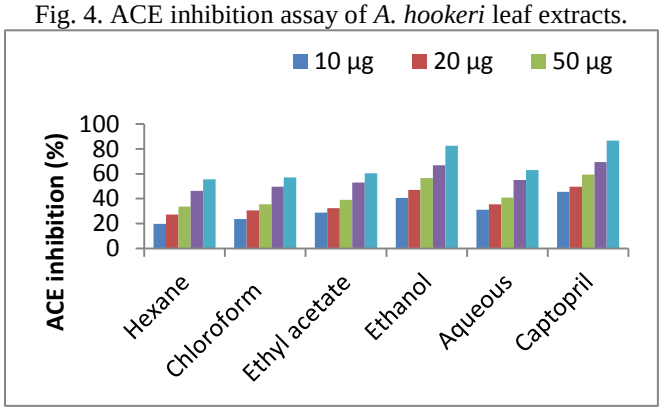
The chemical formula of Compound B is identical to Hesperidine : $C_{28}H_{34}O_{15}$ and molecular weight is 610.57 and Rf value is 0.32

***In vitro* ACE inhibition assay**
The ACE inhibition activity of different concentrations (10, 20, 50, 100, 200 µg/mL) of Allium *hookeri* leaf extracts were evaluated as reported by Nakamura et al. (year) with minor modifications. It was noted that inhibition percentage increased in increasing concentrations of the extracts. The extract concentration providing 50% inhibition (IC 50) was calculated and inhibition percentage was plotted against extract concentration. It was noted that ethanol leaf extract recorded the highest inhibition percentage of 82.67% followed by aqueous (63.02%), ethyl acetate (60.32%) and chloroform (57.01%) leaf extracts (Fig. 2). Standard drug captopril recorded 86.72%.

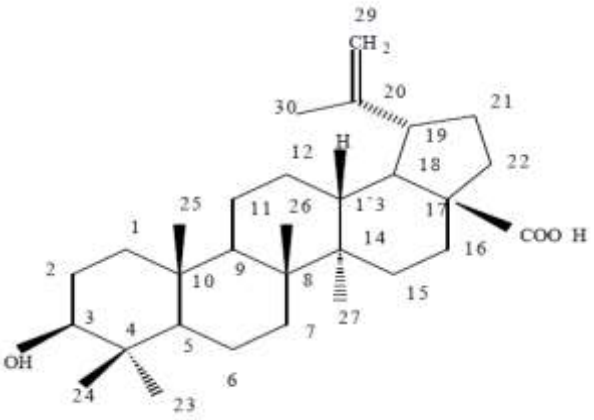
Table 6. ACE inhibition assay of *A. hookeri* leaf extracts.

Extracts	ACE inhibition (%)					
	Hexane	Chloroform	Ethyl acetate	Ethanol	Aqueous	Captopril
10 µg	19.74	23.52	28.82	40.57	31.05	45.42
20 µg	27.19	30.61	32.49	47.03	35.36	49.73
50 µg	33.57	35.54	38.95	56.64	40.93	59.34
100 µg	46.31	49.73	52.87	66.87	55.02	69.56
200 µg	55.56	57.01	60.32	82.67	63.02	86.72

Fig. 4. ACE inhibition assay of *A. hookeri* leaf extracts.

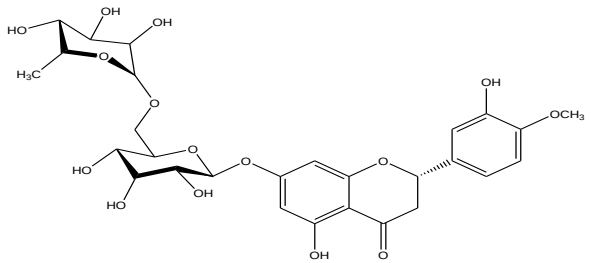


Compound A



This pattern of spectrum was similar to that of BETULIN.

Compound B



Hesperidin

This pattern of spectrum is identical to HESPIRIDINE

IV. CONCLUSION

Plants used by the traditional healers are unexplored source for the develpoment of potential new lead compound.(Lindequist *et al* ,2005). It is always needed to investigate scientifically for its potential and toxicities hence there is need to isolate and characterize the compound from the extract. Further Studies for its mechanism of action, formulation and toxicity studies can be extended. This study have isolated two compounds which are having similar spectra that of Betulin and Hesperidine.

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