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RESEARCH ARTICLE

GC-MS Analysis and Biological Activity of Different Fractions of Sudanese *Albiza amara*(Vohl)Benth. Roots.

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Abstract

Abiza amara (Fabaceae) is an important plant in ethno medicine . Most of the previous studies focused on the leaves, barks and seeds. Phytochemical screening of the ethanolic extract of the roots revealed the presence of alkaloids ,tannins, saponins, carbohydrates, and flavonoids. Different fractions obtained from roots were studied by (GC-MS) . The volatiles present varied according to the solvent used for extraction. The ethyl acetate fraction yielded highest amount of volatile compounds (17components) ,petroleum ether (2 components) ,chloroform (4 components) ,ethanol (9 components) and n-butanol(7components) .2,2'-Methylene-bis[6-(1,1dimethylethyl)-4-ethyl] phenol was the most frequently occurring compound being detected in chloroform, ethyl acetate and n-butanol extracts(26.56,21.04 and 7.73% respectively) . Also hexadecanoic acid methyl ester is another major constituent being detected in ethyl acetate , n-butanol and ethanolic extracts (12.58,15.07,22.82% respectively).Thirty one components were identified in *A. Amara* roots by GC-MS analysis.

Introduction:-

Since the ancient time, medicinal plants are important part of healthcare system.The heavy reliance on plant medicine is attributed to their relative accessibility, low prices, local availability and acceptance by local communities. Due to shortage of dispensaries and professional standard doctors for healthcare needs in developing countries, especially in rural areas, medicinal plants have found extensive applications in traditional medicine. The use of medicinal herbs for curing diseases has been documented in the history of all civilizations(Kumar and Pandey ,2013) . Medicinal plants and derived products are used for the treatment of major diseases such as typhoid fever, diabetes, oedema, obesity and high blood pressure(Leopoldini *et.al.*,2006) . Many herbal drugs are commonly used today by local healers in different communities. According to recent studies conducted by the World Health Organization (WHO), about 80% of the world's population relies on traditional medicine(WHO News,2002) .About 121 drugs prescribed in USA today come from natural sources, 90 of which come either directly or indirectly from plant sources(Benowitz ,1996) . Forty-seven percent of the anticancer drugs in the market come from natural products(Newman, Cragg,2007) .

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Albiza amara **(Vohl) Benth** is one of the 150 species available from the genus *Albizia*. The genus is pan tropical .It has a wide distribution in Africa , occurring in Sudan ,growing as a savanna tree .it is also found in many other parts of Africa including Kenya ,Zambia and Chad. The plant has been used for various ailments in traditional medicine(Orwa ,2009) .

A.amara is a tree of moderate size, much branched with smooth, dark green, scaly bark. Leaves; pinnately compound, with 15-24 pairs of small, linear leaflets, on 6-15 pairs of pinnate. Fruits are oblong pods, about 10-28 X 2-5 cm, light brown, thin, and 6-8 seeded(Orwa,2009) . The seeds are used as astringent. They are also used in treating piles, diarrhea, gonorrhea, leprosy and abscesses(Rajkumar *et.al.* ,2010) .The leaves are applied for boils, eruptions, swellings, coughs, ulcer, dandruff and malaria(Woongchon *et.al.*,1991; Cook and Samman,1996). The tree yields a gum which is used against ulcers while fruits are said to cure malaria and cough. It is reported that soap for hair can be made from the roots and leaves(Narayana *et.al*.,2001). Phytochemical investigation revealed the presence of triterpenes, flavonoids, rare amino acids, lipids, steroids and alkaloids(Narayana *et.al*.,2001) . Previous studies indicated that some compounds isolated from bark of *A.amara* showed anti-oxidant activitiy(Murugan *et.al*.,2009), whereby compounds isolated from leaves showed antifungal, antioxidant ,antibacterial and anticancer properties. In addition, the root of *A. Amara* showed anti-inflammatory and anti-analgesic activities. *Albizia amara* belongs to a family rich in alkaloids, and its extracts have been reported to possess various bioactivities(Prashanth *et.al.* ,2011) ..

In continuation of our interest in species of Fabaceae used in Sudanese ethno medicine it was aimed to identify and quantify the volatile components present in the ethanolic , petroleum ether ,chloroform, ethyl acetate and n-butanol fractions. The study was also designed to evaluate the antimicrobial and antioxidant potential of the Sudanese material of *A.amara* roots.

Materials and Methods:-

Materials:-

Plant material:-

The roots of Sudanese *Albizia Amara* were collected from the "Fulla Forest" - western Sudan in January,2015. The plant was kindly authenticated by Dr. Abdel Halem, Department of Forests, College of Agriculture, University of Bahri, Sudan .The freshly collected plant material was dried under shade at room temperature, cut into small pieces and powdered . The powdered plant material was stored in sterile containers for further use.

Methods:-

Soxhlet extraction:-

(3.5 Kg) of powdered *A.Amara* roots were extracted with 95% ethanol for 10 h. at 60 ^{o}C in a Soxhlet apparatus.The extract was filtered and the solvent was removed under reduced pressure. The dried extract was stored at 5^{o}C in an airtight container for further manipulation.

Solvent-solvent extraction:-

The ethanolic extract(24g) was suspended in water(100ml) and successively extracted with petroleumm ether, chloroform, ethyl acetate and n-butanol.In this way fractions of different polarities were obtained.

Phytochemical screening:-

All fractions were screening for the presence of phenolic compounds, flavonoids, tannins, terpenoids, saponins, alkaloids and carbohydrates using standard methods(Kokate ,2000).

Determination of volatile components:-

Volatiles of all extracts were determined via Agilent Technologies 7890A Gas Chromatography (GC) system coupled with Mass Spectrometry (MS) detector. Each sample was prepared at 1000 ppm via dilution in respective solvents and was injected into the system. Blank analysis was also performed. The chromatography settings are; injection source: GC auto sampler and Thermal separation probe (TSP); injection volume: 1 µL (organic sample); injection mode: split less and split ratio 1:5 and oven temperature: initial 35 °C, increased to 180 °C (6 °C/min), held 5 min, increased to 230 °C (1 °C/min) and held 20 min; and initial 35 °C (2 min), increased to 180 °C (2 °C/min), held 5 min, increase to 230 °C (6 °C/min) and held 30 min; for organic and aqueous samples, respectively. Other settings; column: non-polar capillary DB-1 of 100% dimethyl-polysiloxane (30 m x 0.53 mm id, film thickness 0.25 µm); carrier gas: helium (1 mL/min); ionization energy: 70 eV; front inlet pressure: 6.78 psi, oven equilibrium time: 3 min; maximum oven, post run, front inlet, MS source and MS quad temperature: 350, 290, 250, 230 and

150°C,respectively, for both organic and aqueous samples. The compounds were characterized with the National Institute of Standards and Technology (NIST) Library ChemStation software.

Antioxidant techniques:-

Antioxidant activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH):-

The determination of antioxidant through DPPH scavenging was carried out according to the standard method[8] . Stock solution was prepared by dissolving 40 mg DPPH in 100 ml methanol and kept at -20^{o} C until used . About 350 ml stock solution was mixed with 350 ml methanol to obtain the absorbance of 0. 7 ± 0.01 unit at wavelength 516nm using spectrophotometer (Epoch .Biotek.USA) About 100 µl of sample (extract) were added to 1 ml methanolic DPPH and kept overnight for scavenging reaction in the dark.Percentage of DPPH solution activity was determined as follows :

DPPH scavenging activity (%) = [(A_{blank} - A_{sample}) /A_{blank}] × 100 ; where A is absorbance .

Ferric reducing antioxidant power (FRAP):-

The determination of antioxidant through FRAP was carried out according to the standard method[14].FRAP reagent was prepared freshly using 300 mM acetate buffer , p^{H} =3.6(3.1g sodium acetate trihydrate,plus16 ml glacial acid made up to 1:1 with distilled water ; 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), in 40 mM HCL ; and 20 mM $FeCl_3$.6H2O in the ratio of 10:1:1 to give working reagent . About 1ml of FRAP reagent was added to 100 µl sample and absorbance was measured at 595 nm wavelength. Calibration curve of Trolox was set up to estimate the activity capacity of sample .The results were expressed as mg of Trolox equivalent per 100 g of fresh sample (mg TE/100g of FW).

Determination of total phenolic content(TPC):-

The determination of antioxidant through TPC was carried out according to the standard method[14]. About 100 µl of extract was added to 0,5 ml dilute Folin –Ciocateu reagent. The mixture was left for 5 minutes before 1 ml 7.5% sodium carbonate(w/v)was added. The absorbance was measured at 765 nm wavelength with spectrophotometer after 2 hours. Gallic acid was used as the standard phenolic acid to set up the calibration curve. The results were expressed as mg of gallic acid equivalent per 100 g of fresh sample (mg GA/100g of FW) using the equation obtained from the calibration curve:

y= 2.683x

where: y = absorbance ; x = gallic acid equivalent (µg/g).

Cupric reducing antioxidant capacity (CUPRAC):-

Cupric reducing antioxidant capacity assay was carried out according to a standard procedure[14] ; where 1 m: of 10 mM Cu(II),7.5 mM neocuprine, 1 M ammonium acetate buffer (p^{H}7) solutions and 0.6 mL water were mixed in a test tube.(0.5)mL of dilute sample (or Trolox standard solution) were added to the initial mixture.The absorbance was measured at 450 nm after 30 minutes. Trolox standard solutions were prepared at a concentration ranging from: 40 to 400 µM.

Well diffusion method:-

An inoculum suspension was swabbed uniformly to solidify 20 mL Muller-Hinton Agar (MHA) for bacteria, and the inoculum was allowed to dry for 5 min. Holes of 6 mm in diameter were made in the seeded agar . Aliquot of 20 µl from each plant crude extract (200 mg/ml) was added into each well on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37^{o}C for 24 h. The resulting inhibition zones were measured in millimeters (mm).The assays were repeated in triplicate and the concurrent values were taken.The activity is expressed as less active, if the zone of inhibition is 9-12 mm, moderate 13-16 mm and high : greater than 17 mm.

Results and discussion:-

Plant extracts:-

Soxhlet extraction of *Albiza amara* roots gave 174 g of crude ethanolic extract which was successively partitioned with petroleum ether, chloroform ,ethyl acetate and n-butanol . Yields for these fractions are depicted in Table 1(see also Fig.1).

Table 1:- Yield of different crude extracts of *A.Amara* root

Fraction	Extract in gram	Yield (%)
Petroleum ether	5	6.7
Chloroform	6.3	8.5
Ethyl acetate	9.7	12.8
n-Butanol	14.2	19.1

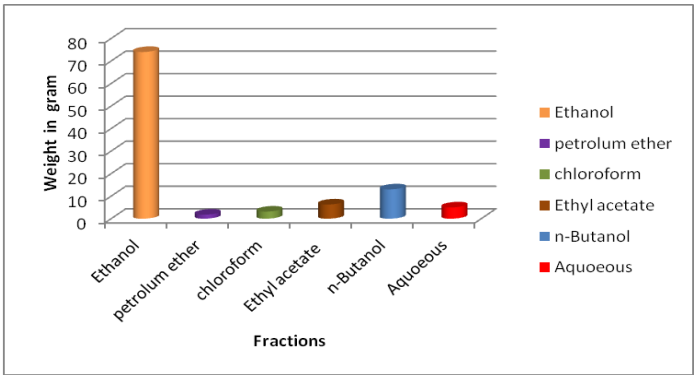

Fig.1:- Weights of different fractions

Qualitative phytochemical analysis:-

The results of qualitative phytochemical analysis of chloroform ,ethyl acetate, n-butanol and ethanolic extracts of *A .Amara* roots are given in Table 2. Results indicated the presence of many phyto-components in ethanolic and other fractions.

Table 2 :- Phytochemical screening of different fractions

No	Constituent	Test	Results				
			ET	PE	CH	EA	BU
1	Carbohydrates	Molish's	+++	-	-	-	+++
2	Flavanoids	Alkaline reagent	++++	-	++++	++++	-
3	Tannins,Phenolic	Ferric chloride Lead acetate	+++++	+++	++	+++	++
4	Saponins	Forth	+++	-	-	-	-
5	Alkaloids	Mayer's,Wagner's reagent	++++	-	++	++	++++

(+++) – Heavy ; (++) – Medium ; (+) - Low ; (-) indicates absent
ET= ethyl acetate ; PE= petroleum ether ; CH= chloroform; Bu= butanol

GC-MS analysis:-

Different extracts were subjected to GC-MS analysis. Volatile substances varied according to solvent. The ethyl acetate fraction yielded highest amount of volatile compounds (17compounds),petroleum ether (4 compounds), chloroform(2 compounds) , ethanol (9 compounds) and n-butanol(7compounds). 2,2'-Methylene bis[6-(1,1dimethylethyl)- 4-ethyl-phenol was the most frequently occurring component being detected in ethyl acetate , n-butanol and ethanolic extracts (26.56,21.04 and 7.73% respectively) . Also Hexadecanoic acid methyl ester was detected in ethyl acetate , n-butanol and ethanolic extracts(12.58,15.07,22.82% respectively) . Thirty two compounds were identified in *A . Amara* roots extract by GC-MS analysis.The percentage of volatile components in different fractions is shown in Fig.2. The active principles in ethyl acetate fraction with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are shown in Table 3.The corresponding chromatograms are shown in Fig.3.

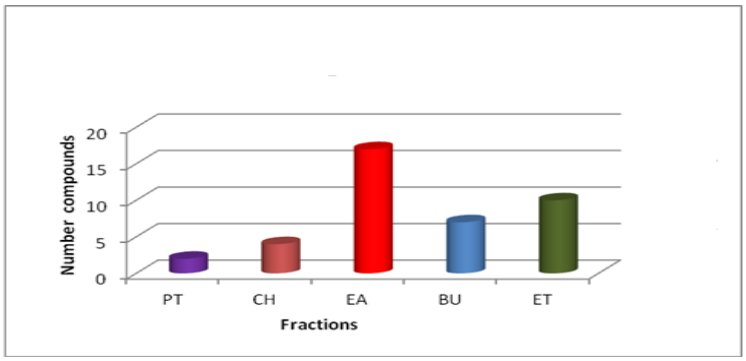

Fig.2:- Volatile components in different fractions

Table 3:- Volatile compounds in ethyl acetate fraction

Compound	Percentage of compound in fractions (%)					RT	MF	MW
	PE	**CH**	**EA**	**BU**	**ET**			
Hexadecanoic acid,14-methyl-,methylester	54.0	-	-	14.04	-	8.393	$C_{18}H_{36}O_2$	284.47
Tetrasiloxane, decamethyl-	46.0	-	-	-	-	19.385	$C_{10}H_{30}O_3Si_4$	310.69
Phenol,2,2'-methylene bis[6-(1,1-dimethylethyl)-4-ethyl-	-	26.56	21.4	7.73	-	12.244	$C_{25}H_{36}O_2$	368
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	-	15.71	-	-	-	12.558	$C_{16}H_{22}O_4$	278
1,2-Bis(trimethylsilyl)benzene	-	11.84	-	-	-	14.407	$C_6H_4[Si(CH_3)_3]_2$	222.47
Trimethyl[4-(2-methyl-4-oxo-2 pentyl)phenoxy]silane	-	45.89	-	-	-	19.837	$C_{15}H_{29}O_2Si$	264.43
Hexadecanoic acid, methyl ester	-	-	12.58	15.07	22.2	7.466	$C_{17}H_{34}O_2$	270.45
Heptadecanoic acid, methyl ester	-	-	16.79	-	-	8.393	$C_{18}H_{36}O_2$	284.47
9,15-Octadecadienoic acid, methylester, (Z,Z)-	-	-	3.09	-	-	8.959	$C_{19}H_{34}O_2$	294.47
9-Octadecenoic acid, methyl ester,(E)-	-	-	12.83	16.06	-	9.039	$C_{19}H_{36}O_2$	296.48
Heptadecanoic acid, 14-methyl-,methyl ester, (.+/-.	-	-	1.19	-	-	9.291	$C_{18}H_{36}O_2$	284.47
Eicosane	-	-	2.05	-	-	10.069	$C_{20}H_{42}$	282.54
8-Methyl-6-nonenamide	-	-	2.46	-	-	11.088	$C_{18}H_{27}NO_3$	305.42
1-Bromoeicosane	-	-	2.75	-	-	14.653	$C_{20}H_{41}Br$	364.46
Cyclotrisiloxane, hexamethyl-	-	-	1.3	0.19	-	15.339	$C_6H_{18}O_3Si$	222.47
1,4-Phthalazinedione, 2,3-dihydro-6-nitro	-	-	4.5	-	-	19.110	$C_8H_5N_3O_4$	207.14
2-p-Nitrophenyl-oxadiazol-1,3,4-one-5	-	-	2.51	-	--	19.838	$C_8H_5N_3O_4$	207.02
9-Octadecenamide, (Z)-	-	-	-	42.12	-	11.071	$C_{18}H_{35}NO$	281.47
2,4,6-Cycloheptatrien-1-one,3,5-bis trimethylsilyl-	-	-	-	4.79	-	15.048	$C_{13}H_{22}OSi_2$	250

n-Hexadecanoic acid	-	-	-	-	1.58	7.758	$C_{16}H_{22}O$	256.4
Heptadecanoic acid, methyl ester	-	-	-	-	38.2	8.399	$C_{18}H_{36}O_2$	284.47
Octadecanoic acid, methyl ester	-	-	-	-	2.52	9.297	$C_{19}H_{34}O_2$	294.47
5-Methyl-2-trimethylsilyloxy-acetophenone	-	-	-	-	1.44	15.402	$C_{15}H_{18}O_2Si$	222
Cyclodecasiloxane, eicosamethyl-	-	-	-	-	1.19	9.818	$C_{20}H_{60}O_{10}Si_{10}$	741.53.
1,3-Diphenyl-2-azafluorene	-	-	-	-	2.89	9.715	$C_{24}H_{17}N$	319.39

9,12-Octadecadienoic acid (Z,Z)-,methyl ester	-	-	-	-	5.23	8.971	$C_{19}H_{34}O_2$	294.25
Hexadecane	-	-	3.28	-	-	10.905	$C_{17}H_{34}$	226
Tetracosane	-	-	4.05	-	-	11.712	$C_{24}H_{50}$	338
Heptadecane	-	-	3.35	-	-	12.484	$C_{17}H_{36}$	240.4
Hexacosane	-	-	3.43	-	-	13.234	$C_{26}H_{54}$	366.7
Heptacosane	-	-	2.69	--	-	13.960	$C_{27}H_{54}$	380

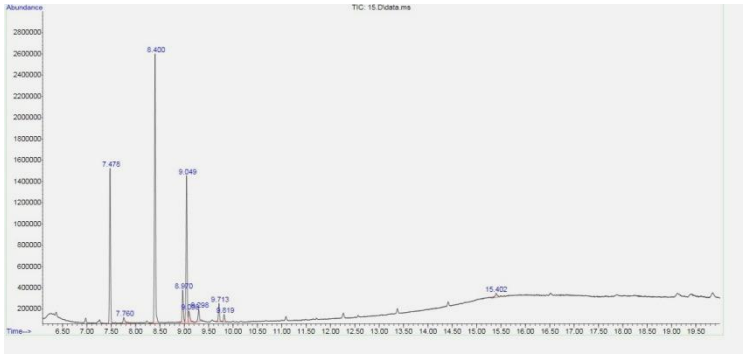

Fig.3:- GC chromatograms for ethyl acetate fraction

GC chromatograms for petroleum ether,chloroform and n-butanol fractions are depicted in Figures 4-6.

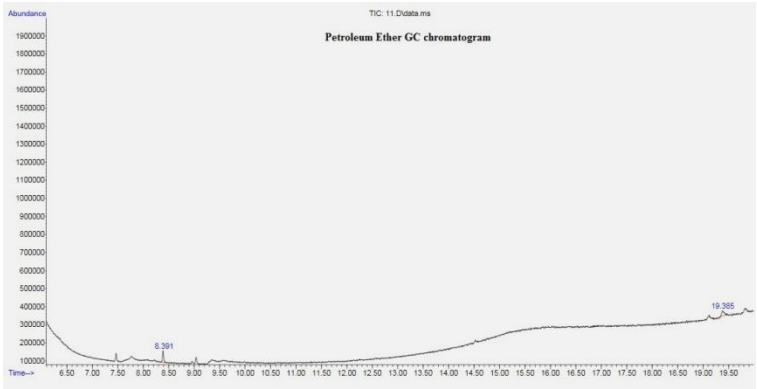

Fig.4:- GC chromatograms for petroleum ether fraction

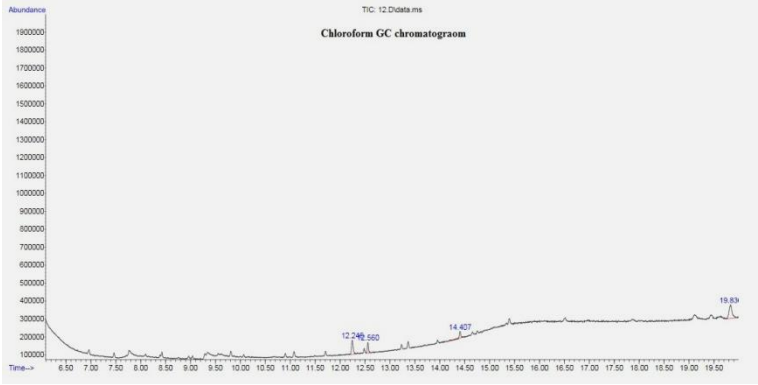

Fig.5:- GC chromatograms for chloroform fraction

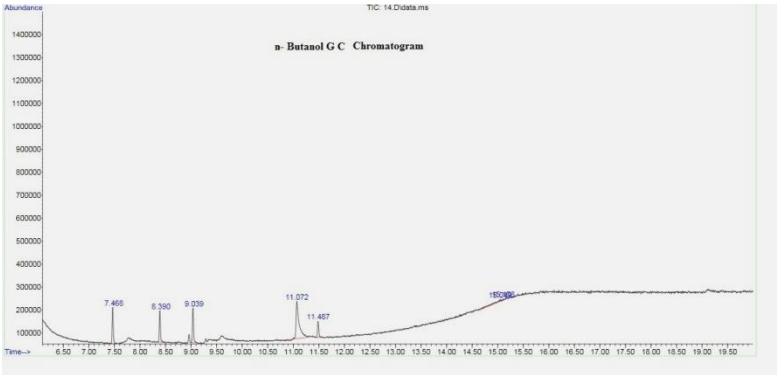

Fig.6:- GC chromatograms for n-butanol fraction

Total antioxidant activity and total phenolic content of fractions:-

The total antioxidant capacity (TAC) and total phenolic content (TPC) of different fractions are presented in table 4 **.**The antioxidant activity of the *A.Amara* extracts were determined by different assays: DPPH, FRAP and CUPRAC using µg Trolox equivalents per g of sample (µg TE/g dry weight ,DW) . Total phenolic content were expressed as mg of gallic acid equivalents per liter of wine (mg GAE/g DW). Table 4 shows the antioxidant activity of different fractions obtained by FRAB,TPC, DPPH, and CUPRAC methods.The results of DPPH and CUPRAC assays ranged from (771.83 to 24.28 mg/g) and (1902.69 to 75.79µg/g) respectively. The reducing power (FRAP) of ethanolic extract and its fractions ranged from 132.35 to 538.09 µg of TE/g extract .The total phenolic content varied from 719.48 to 3825.47 µg/g of dry weight of extract, expressed as gallic **.** Overall, the results showed that the ethyl acetate fraction has highest antioxidant activity at a mean of : (DPPH)**,** 771.83 µg/g ; (TPC), 3825.47 µg/g;(CUPRAC) 1902.686µg/g. (FRAP), 538.09 µg/g . The chloroform fraction gave the lowest antioxidant activity at a mean of: (DPPH) µg/g 24.28 ; (TPC) 719.48 µg/g ; (CUPRA)C75.79 µg/g ; (FRAP) 132.35 µg/g**(**see Figures 7-10) .

Table 4:- Total antioxidant capacity and total phenolic content of different extracts

µg g^{-1}	**PT**	**CH**	**EA**	**BU**	**ET**	***r2***	**equation**
TPC	2085.29	719.48	3825.47	1738.29	2198.12	0.996	*y = 0.0039 x + 0.0577*
DPPH	311.59	24.28	771.83	227.16	339.27	0.998	*Y* = 0.7344 x +1.1155
FRAP	424.97	132.35	538.09	343.82	440.79	0.999	*Y = 1.243 x + 0.1558*
CUPRAC	433.94	75.79	1902.68	663.28	624.01	0.9988	*y* = 0.4244 x + 0.0525

PT = petroleum ether , CH = chloroform , EA = ethyl acetate , BU = n- butanol , ET = ethanol

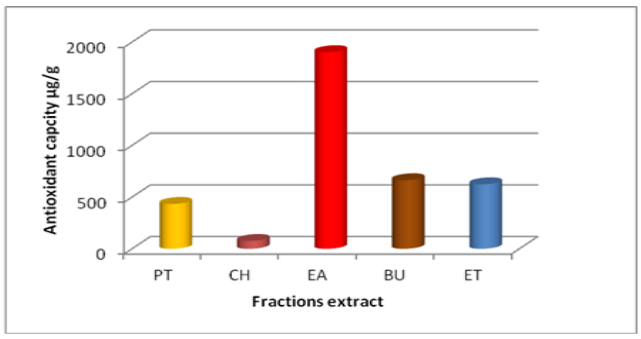

Fig.7:- Antioxidant activity of different extracts using(CUPRAC) assay

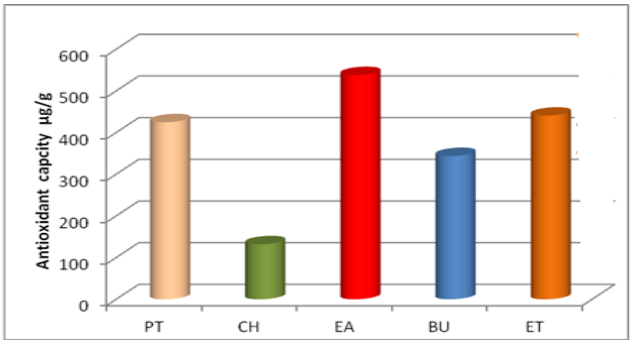

Fig.8:- Antioxidant activity of different extracts using(FRAP) assay

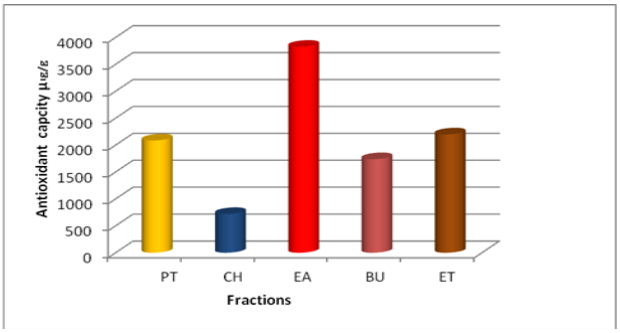

Fig.9:- Antioxidant activity of different extracts using(TPC) assay

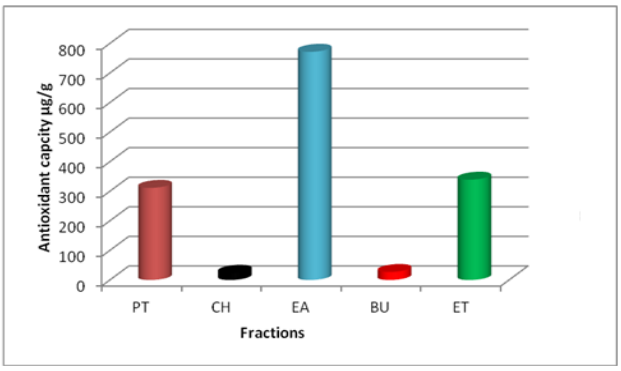

Fig.10:- Antioxidant activity of different extracts using (DPPH) assay

Antimicrobial activity of different fractions:-

In well diffusion method, all fractions from *Albizia.Amara* roots showed inhibitory activity against *Streptococcus mutans* (Sm) and *Lacto bacillus* (Lb).The activity is expressed as partial, if the zone of inhibition is 9-12 mm; active: 13-18 mm and high if greater than 18 mm. The ethyl acetate extract showed high activity on *Streptococcus mutans*

(Sm) , while the n-butanol extract showed high activity on *Lacto bacillus* (Lb). The results of antimicrobial activity are depicted in Table 5.

DMSO was used as solvent since it has no effect on the growth of any of the test microorganisms. Standard discs inhibited the growth of all test microorganisms. . There has been an increasing effect on microbial growth inhibition with increasing concentration of the extracts However, the effects observed were less than those produced by standard chemotherapeutics. Further research is in progress to isolate active metabolites in the extracts of *A.amara* root.

Table 5:- Antimicrobial activity of different fractions

Microrganism	Fractions	300µl	200 µl	100 µl
Lacto bacillus (LB)	PT	20	19	18
	CH	12	11	12
	EA	14	12	11
	BU	22	22	20
	ET	21	19	18
	AMP	25	25	25
	DMSO	-	-	-
Streptococcus mutans (SM)	PT	9	10	11
	CH	15	14	12
	EA	20	23	22
	BU	18	14	15
	ET	20	20	20
	AMP	25	25	25
	DMSO	-	-	-

(ET = 0.09 , CH = 0.08, EA = 0.1 , P =0.03 , BU = 0.03 mg/ml) ; 100 µl of sample + ml of DMSO

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