CHAPTER IV

RESULTS

The investigation was carried out with a view to explore new medicinal compounds from two known traditional medicinal plants to determine their efficiency in addressing hair fall related problems. The selected plant species were assessed with respect to isolation and purification of compounds, antimicrobial potentials of the isolated compounds, acute dermal toxicity against the mammalian skin and cytotoxic effect in case the of RAW 264.7. *In-vivo* study was done for the purified compounds in hair fall induced animal models at dermal and follicular level. The investigations generated interesting results with respect to the above mentioned parameters, which are presented in an orderly fashion.

4.1. Morpho-phenological characters of the plants

Morpho-phenological characters like plant height, plant girth, leaf length, leaf breadth, petiole length, flowering time, flowers per inflorescence, length of inflorescence, diameter of inflorescence, flower length, flower diameter, number of sepals in each calyx, number of petals in each corolla, period of fruiting and seed descriptions were recorded on five randomly selected plants.

4.1.1. *Eclipta alba*

E. alba belongs to the family *asteraceae*, is a small herb with white flower heads. The species grows in moist and water-logged locality and is not usually found in dried areas and grows just after the first showers of rainy season. It is an annual, erect or prostate, much-branched herb, rooting at nodes. The mature plant attains an average height of 40.4 ± 3.36 cm and girth 4.06 ± 0.69 cm, covered with white hairs rising from the base. The root system consists of finely branched thin roots penetrating up to a depth of about 15-20 cm. Leaves of the plant are sessile, lanceolate or elliptic and oblong-lanceolate, distantly toothed, sharp, narrowed and pointed at both ends. Lengths of the leaves ranges from 2.56±0.67 to 3.53±0.85 cm and breadth varied in between 1.29±0.24 to1.69±0.25 cm. The data on morphological characters of five randomly selected plants are presented in Table 4.1a.

E. alba plants flower for 15 days and flowers are solitary. The phenological data of the plant is presented in Table 4.1b. Flowers are axilliary or terminal, tubular, 6 sepals present in each calyx, occasionally very minor tooth on the top of the achene. Flower heads are sub-globose and small. The length of the flowers ranged from 1.0 ± 0.09 to 1.3 ± 0.14 cm and the diameter 0.63 ± 0.16 - 1.02 ± 0.34 cm. On the other hand, the length of the inflorescence ranges from 4.17 ± 1.23 - 6.89 ± 2.41 cm and the diameter varies from 0.46 ± 0.32 - 0.74 ± 0.34 cm. Fruits are dark green, minute and length varies from 0.25 ± 0.11 - 0.51 ± 0.13 cm and width 1.5-2.0 cm. Seeds are minute, oval shaped, black in color and weight varies

between 1.1 ± 0.1 and 2.1 ± 0.3 mg. Different parts of the plant are shown in Fig.4.1.

Table 4.1. a) Morphological characteristics of *E. alba*

Plant no.	Plant height (cm)	Girth of the plant(cm)	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)
1	42	3.4	2.7±0.4	1.36±0.31	4.38±0.65
2	45	4.1	2.64±0.23	1.29±0.24	3.79±0.54
3	36	3.7	3.43±0.19	1.53±0.11	4.66±0.73
4	40	3.9	2.56±0.67	1.69±0.25	5.23±0.21
5	39	5.2	3.53±0.85	1.55±0.06	4.60±0.39

(N.B. The values presented here are average of three repetitions of study)

Table 4.1.b) Phenological characters of *E. alba*

Plant no.	Flower length (cm)	Flower diameter (cm)	Length of inflorescence (cm)	Dia. of the inflorescence (cm)	Fruit length (cm)	Seed weight (mg)
1	1.2±0.11	0.94±0.24	4.17±1.23	0.52±0.09	0.34±0.02	2.1±0.3
2	1.1±0.07	0.71±0.11	5.23±0.73	0.46±0.32	0.25±0.11	1.4±0.1
3	1.3±0.14	1.02±0.34	4.93±1.37	0.71±0.17	0.41±0.24	1.2±0.3
4	1.0±0.09	0.63±0.16	6.89±2.41	0.67±0.22	0.51±0.13	1.05±0.4
5	1.1±0.12	0.77±0.06	4.72±3.02	0.74±0.34	0.46±0.03	1.1±0.1

(N.B. The values presented here are average of three repetitions of study)

Fig.4.1. Different parts of the plant *E. alba*: flower (**A**), fruit (**B**), leaves (**C**) and stem (**D**)

4.1.2. *Aloe barbadensis*

A. barbadensis commonly referred to as *Aloe vera*, is one of the 420 species belonging to the family *liliaceae*. It is a stemless, perennial, succulent plant arising directly from the stem. The average height of the plant at maturity is 56.4 ± 6.35 cm and girth at the base 10.8 ± 2.77 cm and at the top 44.2 ± 5.17 cm. Leaves grow in a spiral rosette around the stem in the ground level but the stem can grow upto 12-15 cm in older plants while in younger plants, it is 5-8 cm and

the average width of the stem is 6-8 cm. There are 20-25 leaves per plant; older leaves are more erect as compared to the younger ones. In the young plants, leaves are bright green in color with whitish spots on both sides and on full maturity leaves become grey-green with the disappearance of the whitish spots. The average length of leaves varies from 41.76 ± 3.94 - 50.5 ± 6.08 cm and breadth ranges from 5.12 ± 1.11 - 9.7 ± 0.88cm, tapering in the middle with saw-like teeth along the margin of the leaves. The main root grows vertically inside the soil from the rhizosphere base, upto a length of 40-45 cm, from where root hairs arise laterally. Petiole is absent as leaves grow directly from the stem. The morphological data obtained from 5 randomly selected plants are presented in Table 4.2 and the different parts of the plant are shown in Fig.4.2. Plants of the species failed to flower during the study period.

Table 4.2. Morphological characters of *A. barbadensis*

Plant no.	Plant height (cm)	Girth of the plant (cm)	Leaf length (cm)	Leaf breadth (cm)
1	54	9	41.76±3.94	5.12±1.11
2	50	10	43.48±2.72	6.2±0.96
3	52	12	42.44±3.99	8.1±0.83
4	61	8	50.5±6.08	9.3±1.67
5	65	15	48.72±4.59	9.7±0.88

(N.B. The values presented here are average of three repetitions of study)

Fig.4.2. Different parts of the plant *A. barbadensis*

4.2. Genomic study of the plants

4.2.1. DNA isolation

Genomic DNA from *E. alba* and *A. barbadensis* was isolated using Doyle and Doyle[210] method with some modifications. The isolated DNA was electrophored alongside *Hind* III digested λ DNA marker. The gel is presented in Fig. 4.3. The purity of the isolated DNA from both the plants was calculated by taking OD at 260 and 280 nm in a UV/VIS spectrophotometer (Beckman DU® 530 Life Sciences). The purity of the isolated DNA samples was found to be 1.81 and 1.83,

respectively and the yield was calculated using optical density at 260 nm. Yields of the DNA isolated from the plants are presented in Table 4.3.

Table 4.3. Purity and yield of isolated DNA

Name of the plant	A260	A280	A260/A280	DNA yield (μg g^{-1})
E. alba	0.174	0.096	1.81	16.5
A. barbadensis	0.130	0.071	1.83	12.3

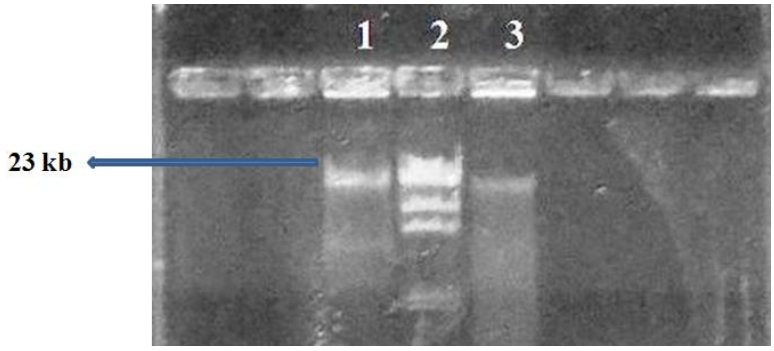

Fig.4.3. Isolation of genomic DNA from selected plants (lane 1: DNA of *E. alba*; lane 2: *Hind* III digested λ DNA marker; lane 3: DNA of *A. barbadensis*)

4.2.2. Genome size determination of the plants

Flow cytometric analysis of the isolated nuclei resulted in DNA content of both the standard and the tested plants. The gain in the instrument was set so that the fluorescence peak of the external reference standard *P. sativum* could be placed in channel 202 of the 1023-channel scale. The fluorescence peak of DNA nuclei of *E. alba* was recorded at channel 195 depicted in Fig.4.4 and that of *A.*

barbadensis at channel 201 depicted in Fig.4.5. The peak ratio of *E. alba* and *A. barbadensis* were 0.96 and 0.99, respectively. The absolute 2C nuclear DNA content of *E. alba* and *A. barbadensis* was calculated to be 8.73 and 8.85 pg (Table 4.4). The genome size of *E. alba* and *A. barbadensis* was estimated to be $4.27x10^9$ bp and $4.42x10^9$ bp.

Table 4.4. Genome size determination

Plant species	Fluorescence peaks	Peak ratio	2C DNA content (pg)	C-value (pg)	C-value (bp)
E. alba	195	0.99	8.73	4.36	4.27×10^9
A. barbadensis	201	0.96	9.045	4.52	$4.42x10^9$

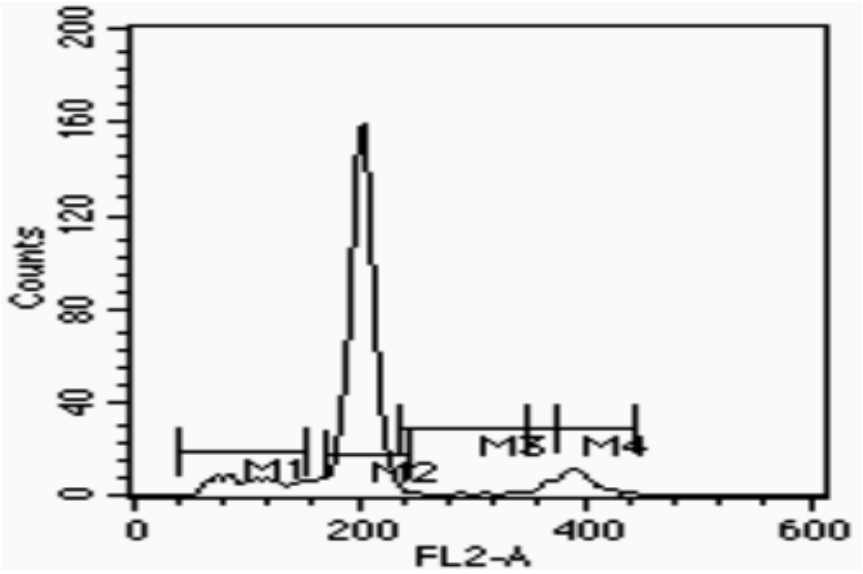

Fig.4.4. Genome size of *E. alba*

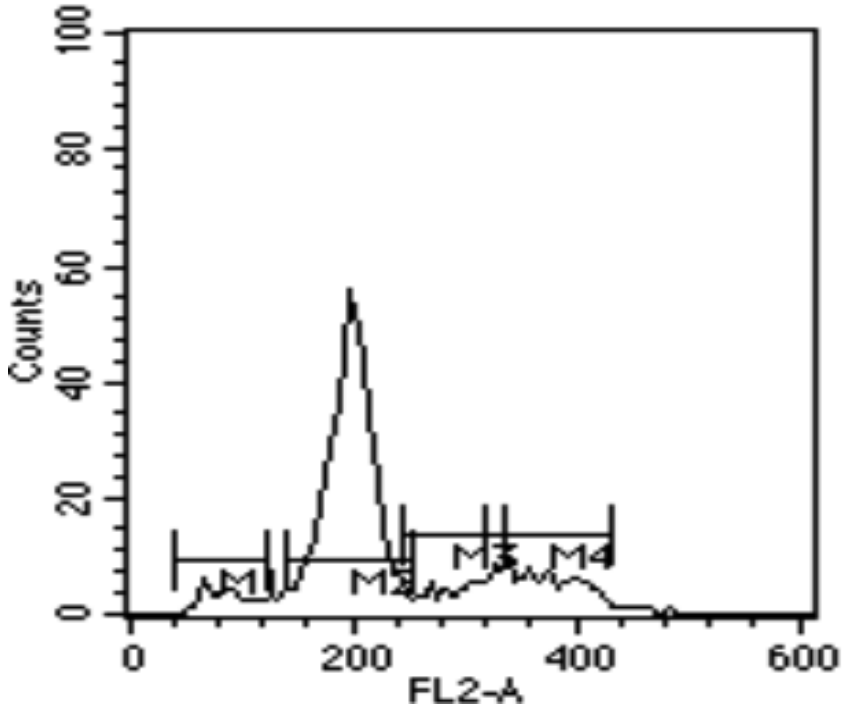

Fig.4.5. Genome size of *A. barbadensis*

4.3. Isolation and purification of medicinal compounds

4.3.1. *Eclipta alba*

Thin layer chromatography profile of the isolated compounds from *E. alba* is shown in Fig. 4.6. The isolated fraction Ea 1 (Fig.4.6a) present in the methanolic extract of *E. alba* was separated by TLC. Ea 1 was identified as saponin after spraying with iodine solution in thin layer chromatogram at R_f value 0.27. Preparative TLC was performed to collect the Ea 1 fraction in higher amount for further characterization. The fraction Ea 1 was then purified by HPLC with retention time (RT) 11.52 min as shown in Fig. 4.7a.

Another fraction Ea 2 isolated from *E. alba* ethylacetate extract, was also separated by TLC chromatogram (Fig.4.6b). After spraying with anisaldehyde reagent, the R_f value of the compounds Ea 2 was calculated as 0.33. For further investigation, preparative TLC was done in 20×20 cm plates. Purification of compound Ea 2 was done by HPLC with the standard gradient method. The RT of the compound Ea 2 was detected at 10.31 min as shown in Fig.4.7b.

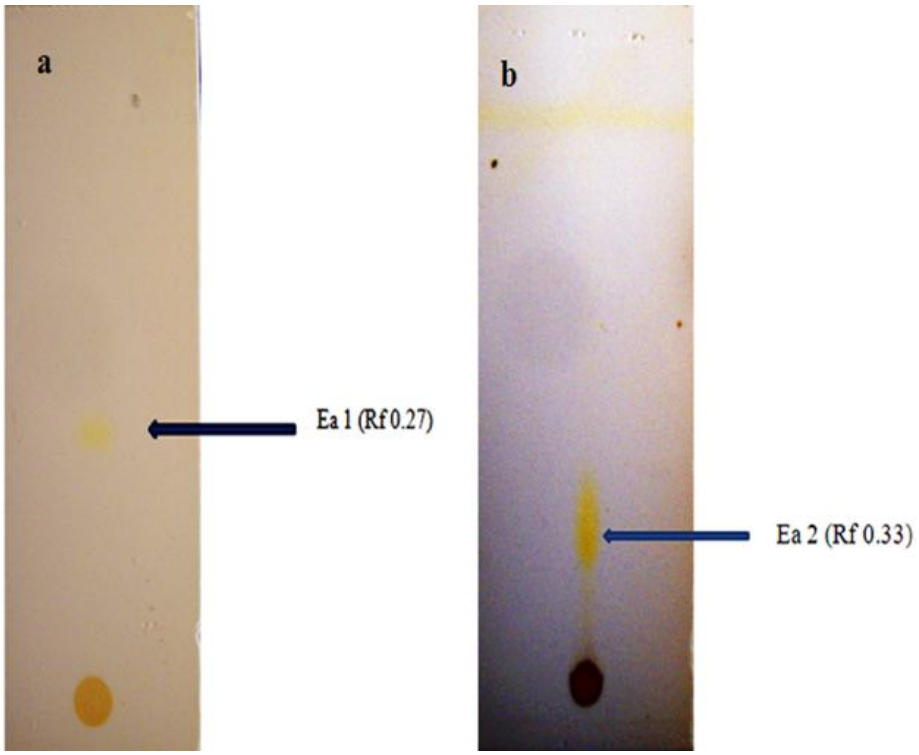

Fig.4.6. TLC profile of Ea 1 (a) and Ea 2 (b) isolated compounds from *E.alba*

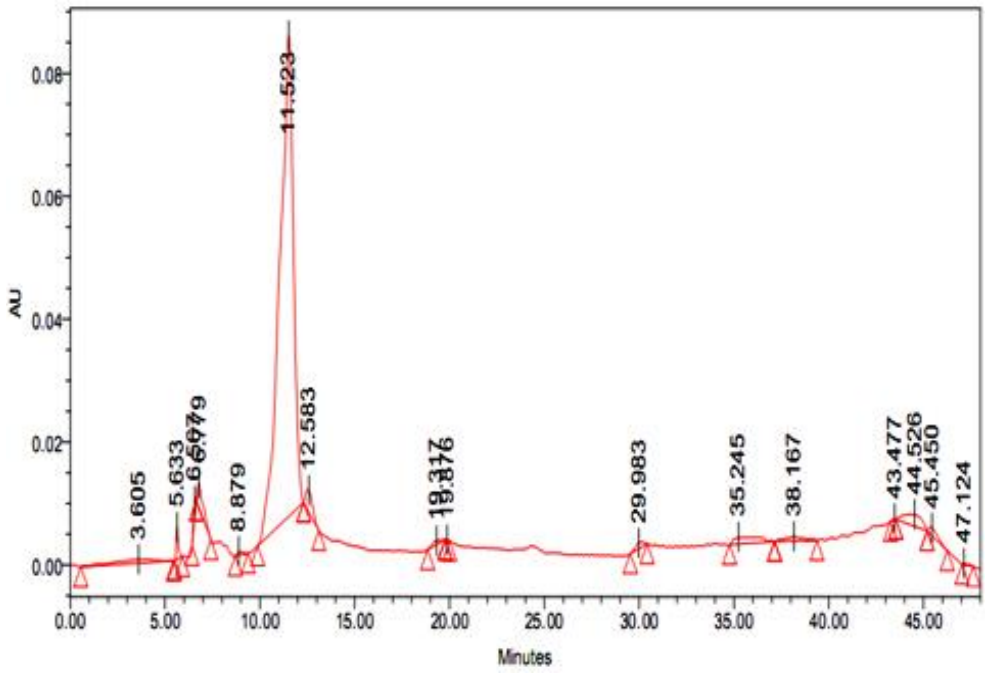

Fig.4.7a) HPLC profile of the compound Ea 1

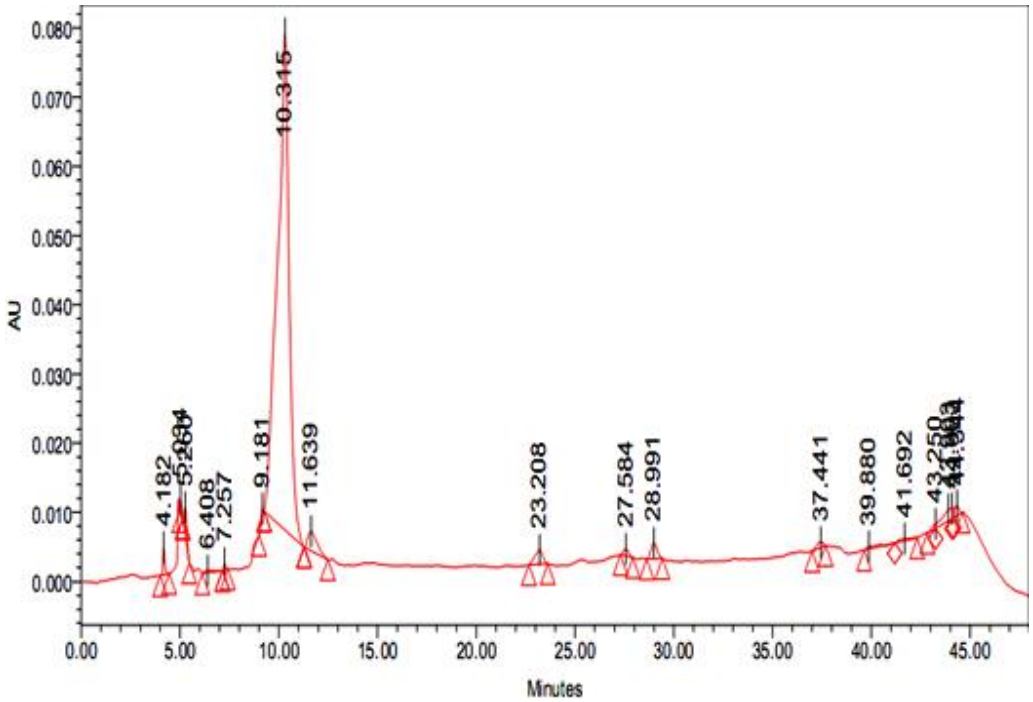

Fig.4.7b) HPLC profile of the compound Ea 2

4.3.2. *A. barbadensis*

The medicinal compounds isolated from *A. barbadensis* with ethylacetate extract, were separated using TLC. The chromatogram of the isolated fraction Av 3 and Av 4 were shown in Fig.4.8 (a and b). The R_f value of the fractions were calculated as 0.83 and 0.52 after spraying with anisaldehyde reagent followed by heating. Both phyto-compounds Av 3 and Av 4 were purified by HPLC at retention time 27.68 and 10.17 min as shown in Fig.4.9 (a and b).

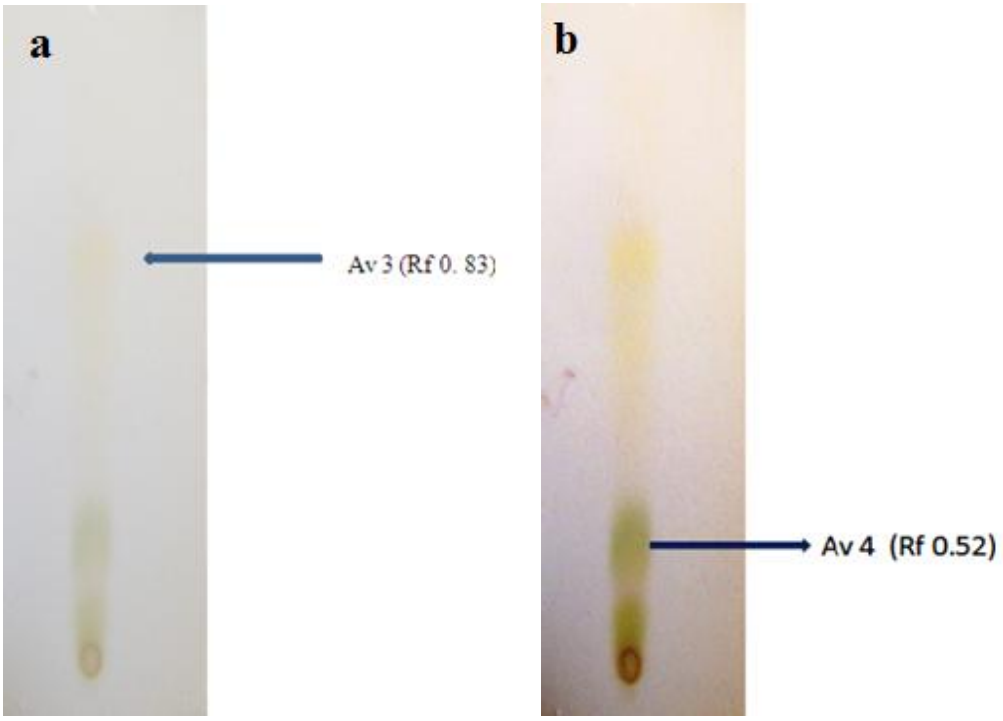

Fig.4.8. TLC profile of Av 3 (a) and Av 4 (b) isolated from *A. barbadensis*

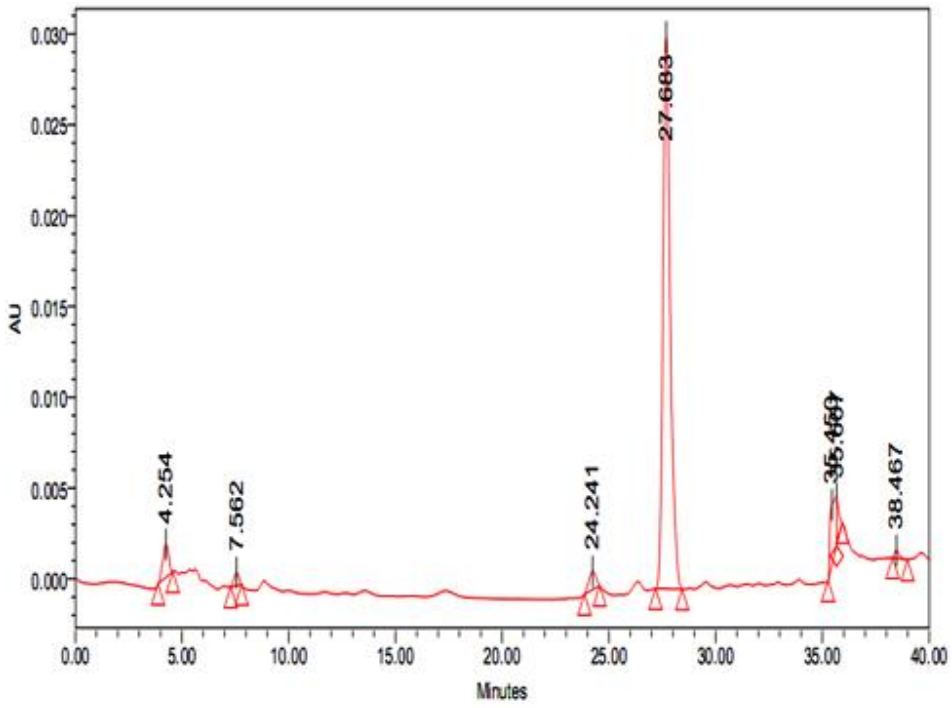

Fig.4.9a) HPLC profile of the compound Av 3

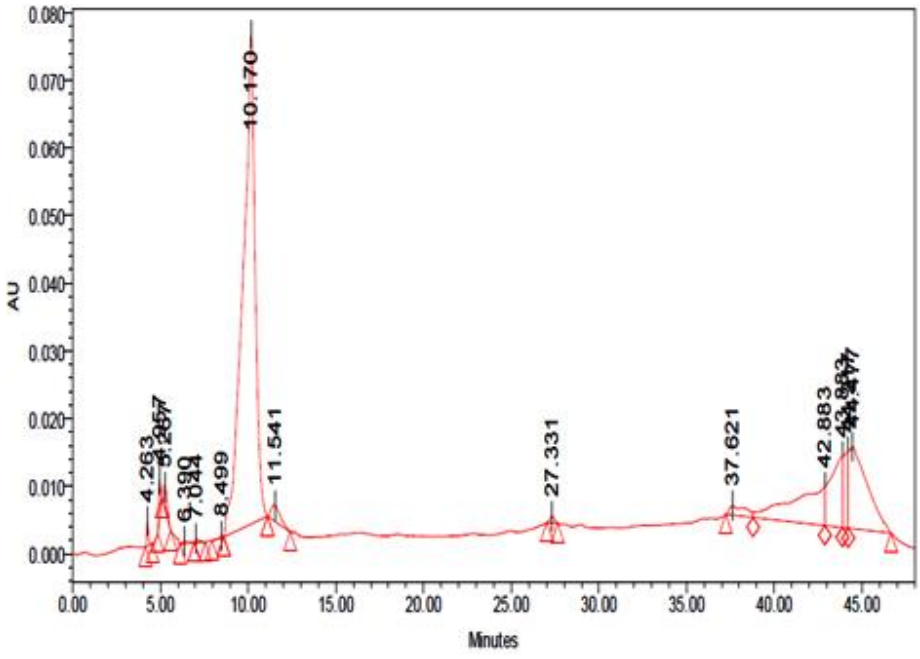

Fig.4.9b) HPLC profile of the compound Av 4

4.4. Chemical characterization and structure elucidation of the purified compounds

4.4.1. *E. alba*

The purified compounds were collected and subjected to characterization using IR, MS and NMR spectroscopy. Probable structures of the phyto-compounds were determined on the basis of the data generated.

For Ea 1 compound, the IR spectra exhibited absorption at 3410.97, 2925.35, 2857.04, 2194, 1564.52, 1414.63, 1258.49, 1166.26 and 1,097.72 as shown in Fig. 4.10. The purified fraction was analyzed by MS which gave a mass value of 620.3 in its negative ion mass (Fig.4.11). The molecular formula of Ea 1 compound was revealed as $C_{32}H_{62}O_8$ and the probable structure of the compound was derived from the mass and NMR spectra of the compound. The proton and carbon NMR spectra of Ea 1 (Fig.4.12a and b) are given below:

$^{1}H_1$ NMR: 0.79 ($-CH_3$), 0.95 ($-CH_3$), 1.05 ($CH-CH_3$), 1.22 (CH_2), 1.35 (CH_2-CH_3), 1.5 ($-CH$), 1.95 (CH_2-CH_3), 2.55 ($HC-C=O$), 3.17 ($-OH$), 3.34 (CH_3-O-), 3.6 ($HC-O$), 3.81 (CH_2-CH_2), 4.6 ($C=C-H$), 4.87 ($-OH$)

^{13}C NMR: 139.3 (aromatic ring, H), 114.08 (aromatic ring, H), 33.83 ($-CH-CH_2$), 31.95 (CH_3-CH_2), 30.06 ($-CH_3$), 29.37 ($-CH_2OH$), 28.98 ($-CH_2$), 22.7 ($-CH_2$), 14.1($-CH_3$).

The probable structure of the compound Ea 1 is shown in Fig.4.13.

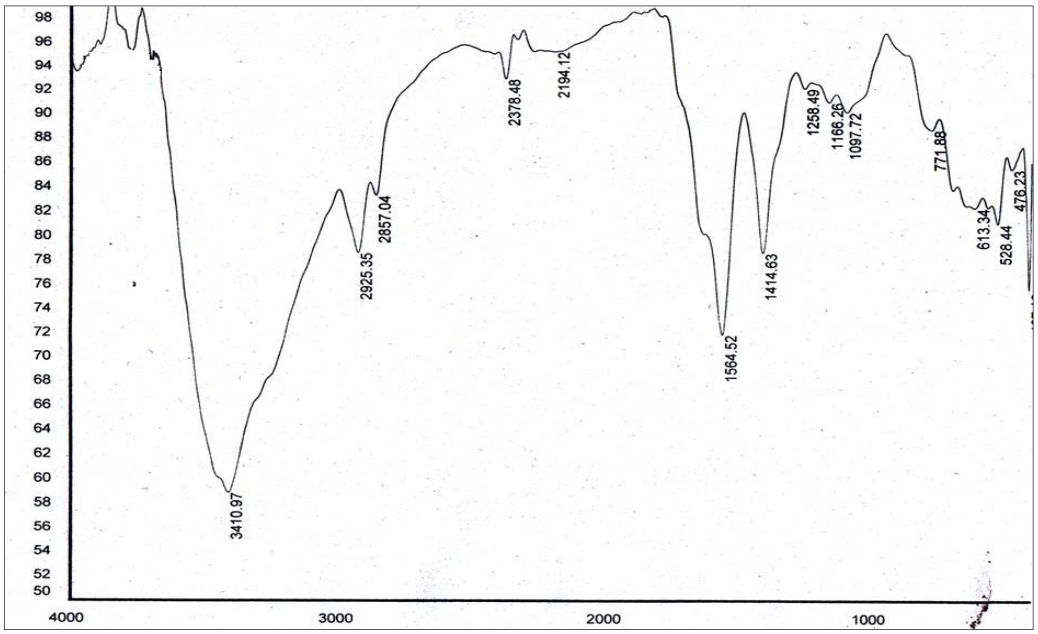

Fig.4.10. FTIR spectra of the compound Ea 1

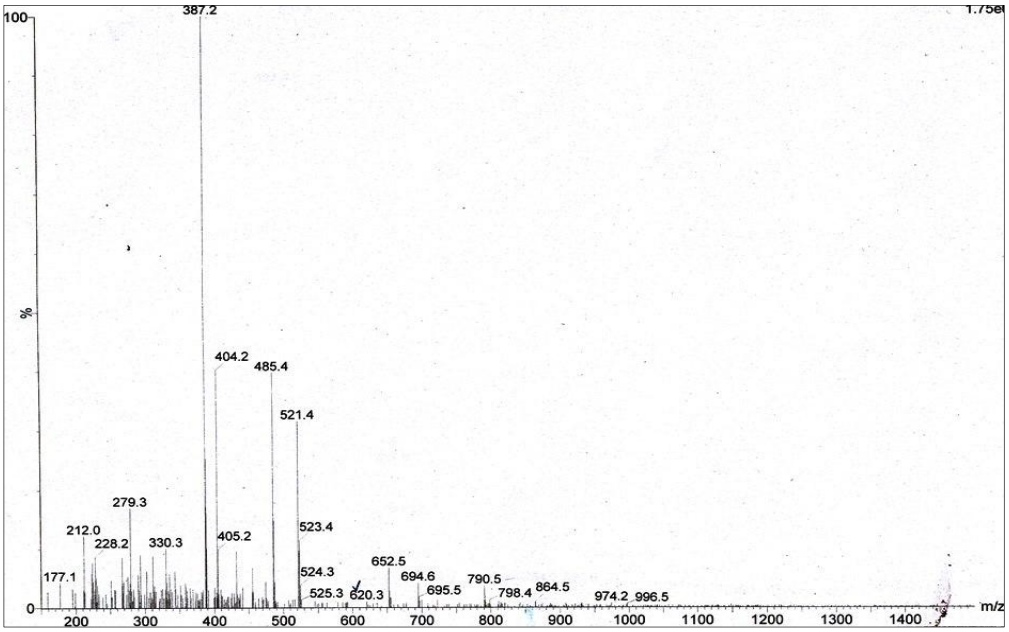

Fig.4.11. MS spectra of the compound Ea 1

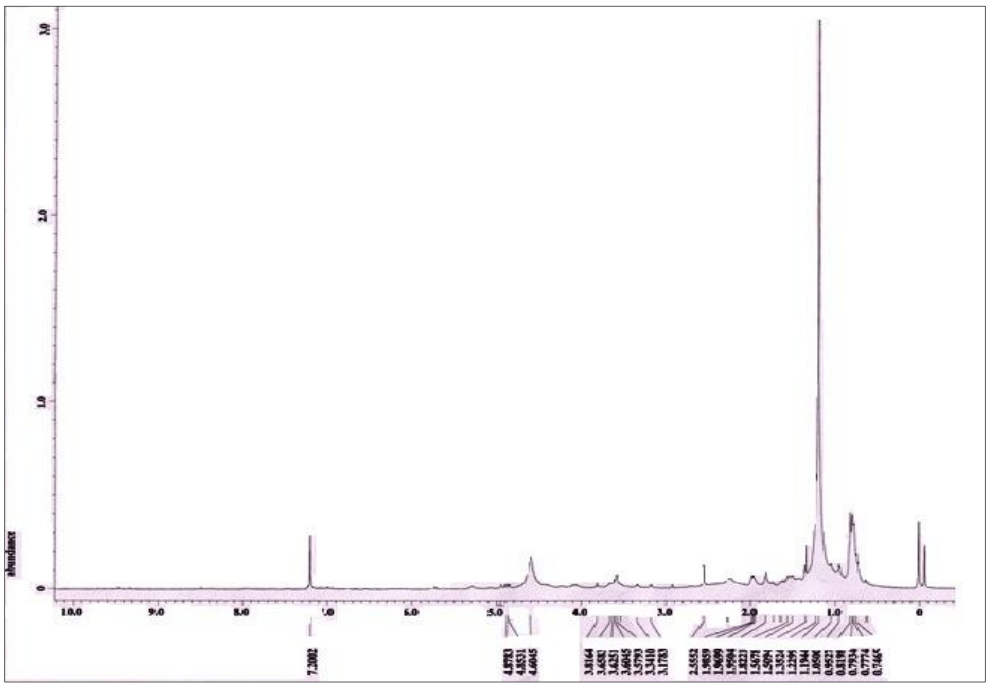

Fig.4.12.a) Proton NMR spectra of the compound Ea 1

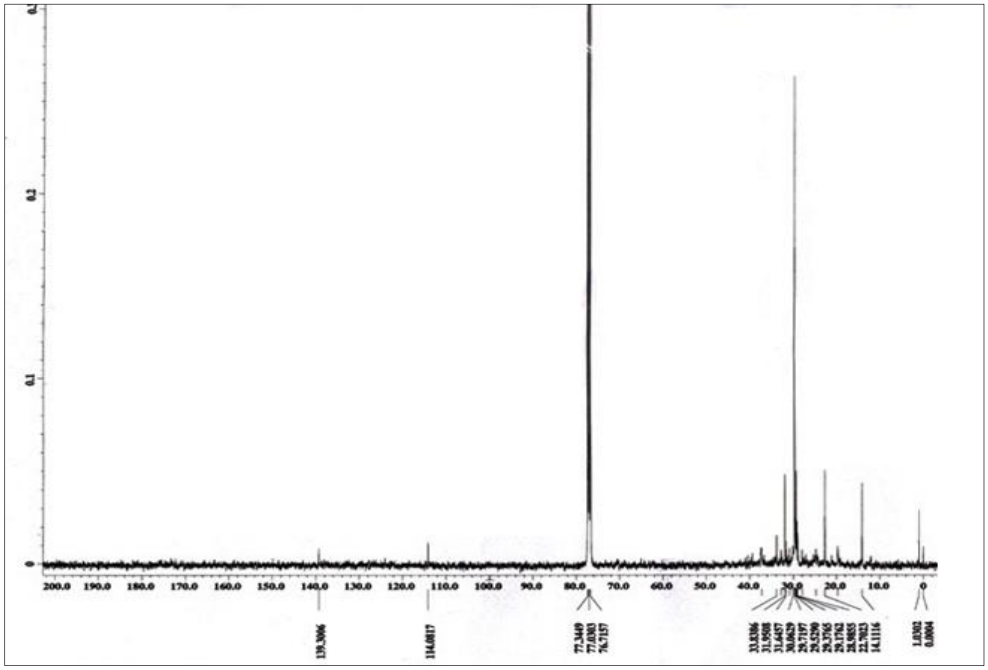

Fig.4.12.b) Carbon NMR spectra of the compound Ea 1

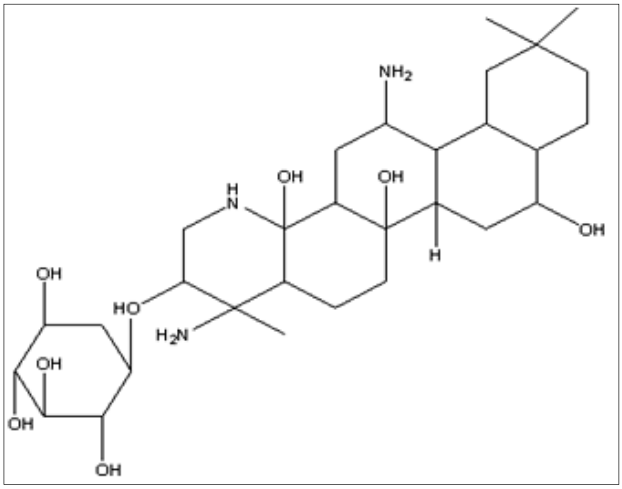

Fig.4.13. Probable structure of the compound Ea 1

The compound Ea 2 exhibited its peaks in IR spectra at 3390.3, 2922.2, 2851.3, 1738.8, 1458.1 and 1027.6 as shown in the Fig.4.14. The compound was determined to have the molecular weight 268.22 and the molecular formula $C_{15}H_{28}N_2O_2$. The proton and carbon NMR spectra of the compound (Fig.4.15a and b) showed the values are as follows:

$^{1}H_1$ NMR: 3.48 (C=C−C−H), 2.20(−OH), 2.0 (CH_3), 1.25 (−CH_3), 0.89 (−CH3),

^{13}C NMR ($CDCl_3$): 206.8 (C=O), 31.95 (CH_2OH), 30.91 (OCH_3), 29.72 (CH_2OH), 22.7 (CH_3CO), 14.1 (RCH_3).

The probable structure of the compound Ea 2 is shown in Fig 4.16.

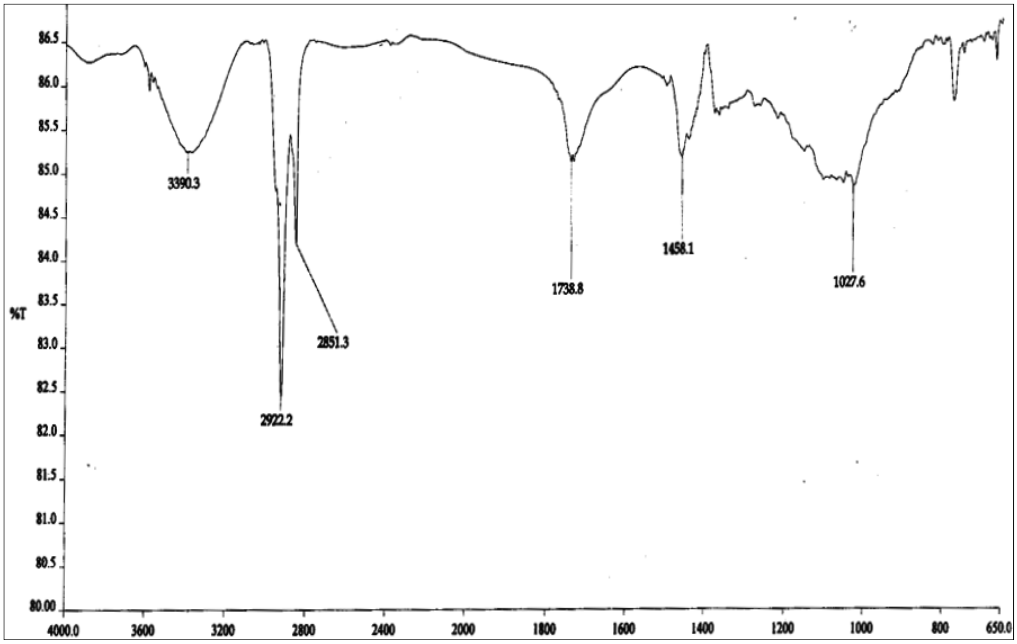

Fig.4.14. FTIR spectra of the compound Ea 2

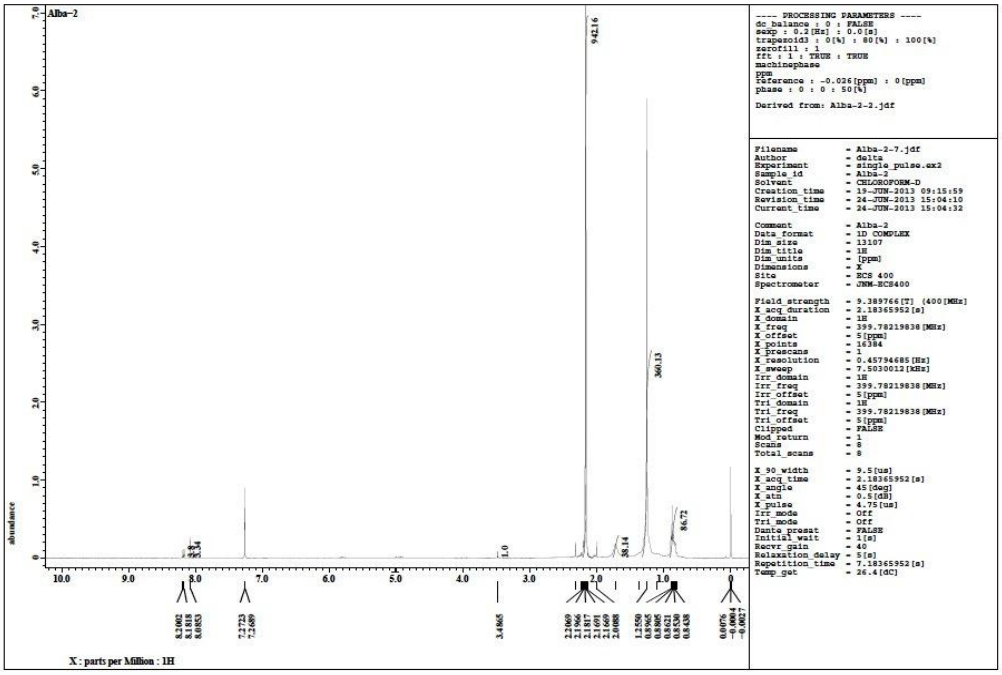

Fig.4.15.a) Proton NMR spectra of the compound Ea 2

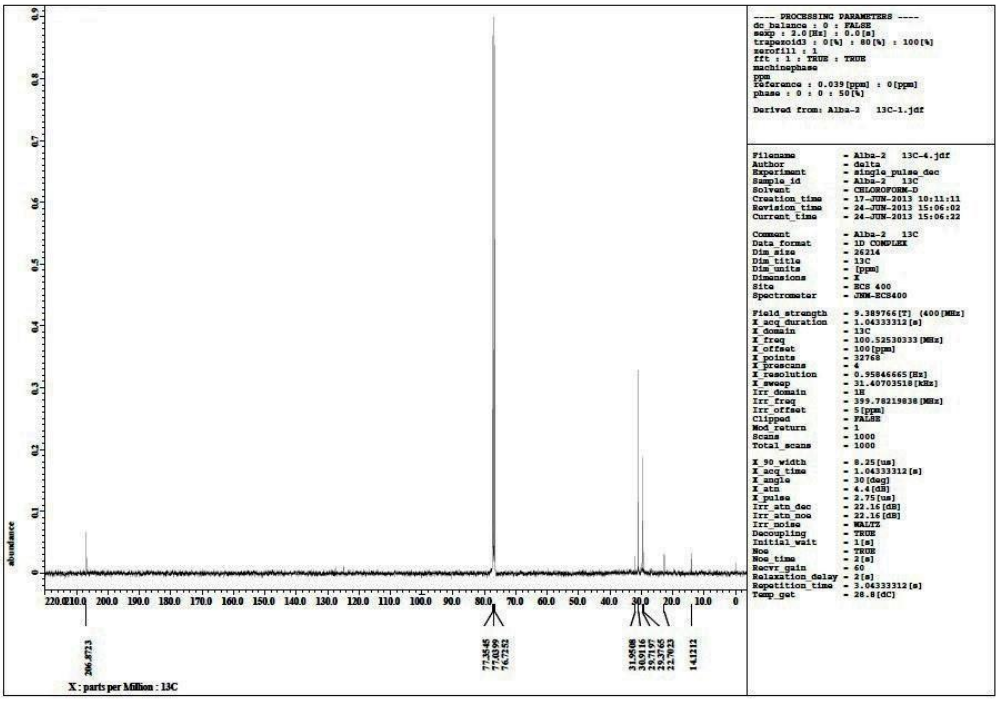

Fig.4.15.b) Carbon NMR spectra of the compound Ea 2

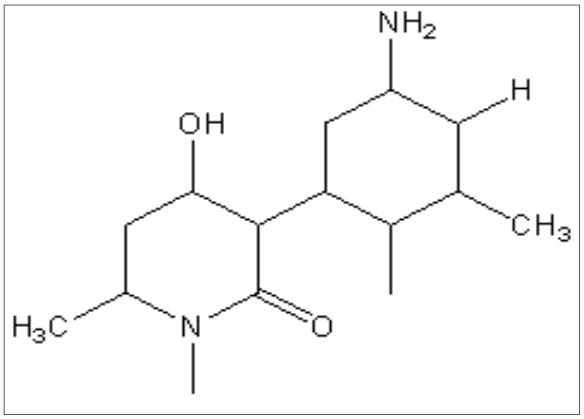

Fig.4.16. Probable structure of the compound Ea 2

4.4.2. *A. barbadensis*

The purified compound Av 3 isolated from *A. barbadensis* showed its absorption peaks at 3397.5, 2940.8, 1642.5, 1412.5, 1251.1, 1059.7 (Fig.4.17) and the molecular formula of the compound was found to be $C_{13}H_{18}O_4$ with mass value 238.12. The proton and carbon NMR spectra of the purified compound (Fig.4.18a and b) are as follows:

$^{1}H_1$ NMR: 4.97 (aromatic OH), 4.13 ($-NH_2$), 3.46 ($-CH_3$), 2.6 ($-CH_3$), 2.17 ($-CH_3$), 2.04 (OH), 1.26 ($-CH_3$)

^{13}C NMR: 207.6 (C), 171.5 (aromatic ring), 60.5 (CH_3-CH), 50.3 ($-CH_2$), 30.8 ($-CH_3$), 20.9 (CH_3CO), 14.1 (CH_3)

The probable structure of the compound Av 3 is shown in Fig.4.19.

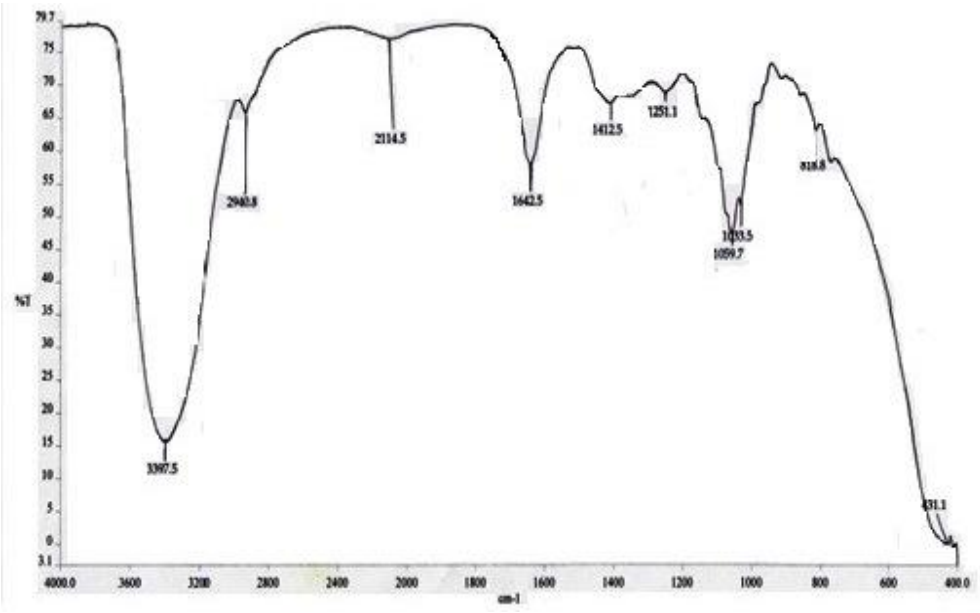

Fig.4.17. FTIR spectra of the compound Av 3

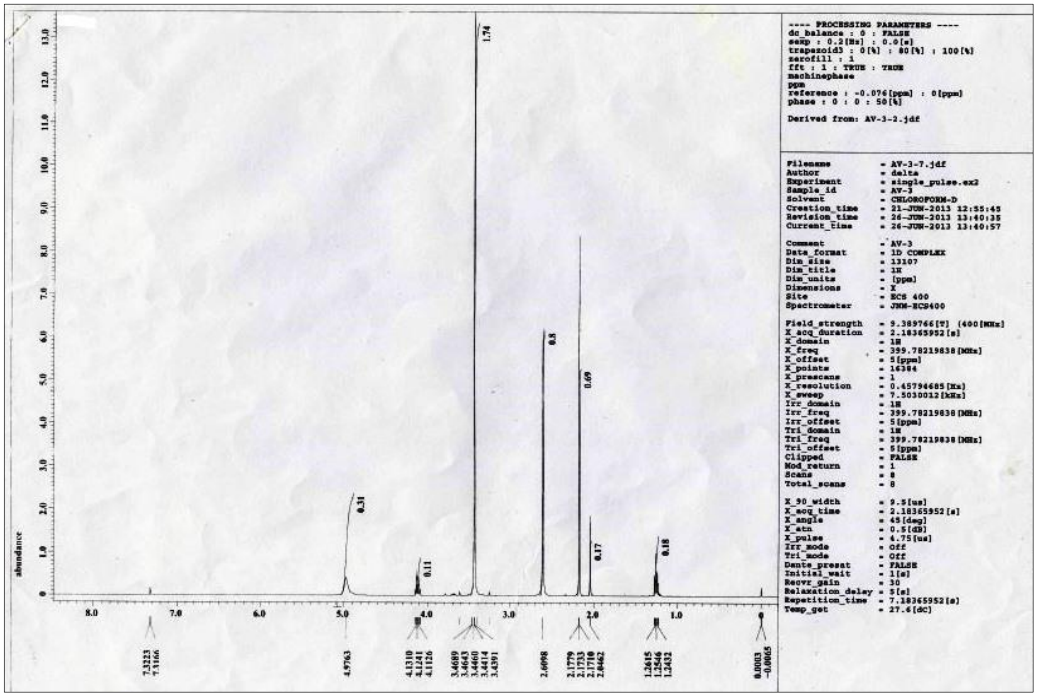

Fig.4.18a) Proton NMR spectra of the compound Av 3

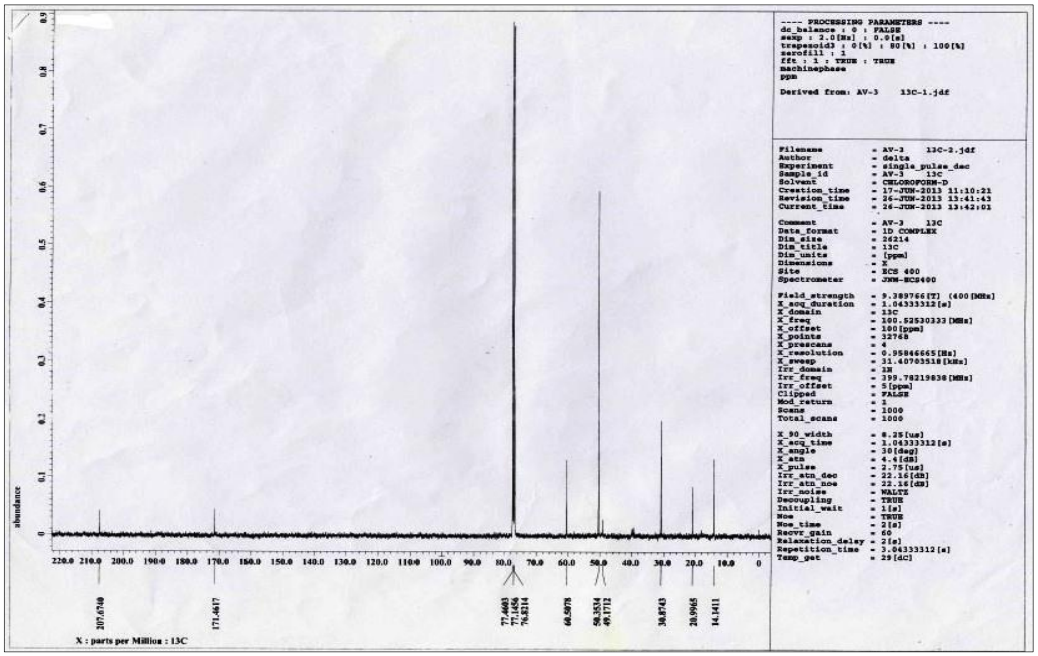

Fig.4.18b) Carbon NMR spectra of the compound Av 3

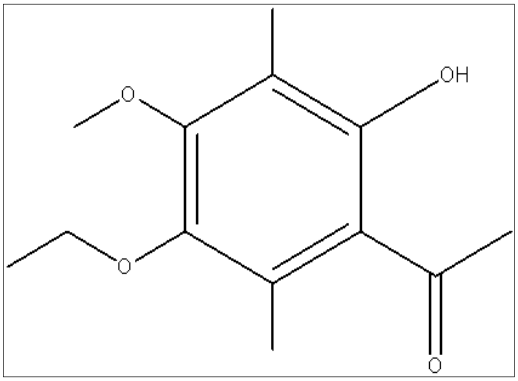

Fig.4.19. Probable structure of the compound Av 3

The compound Av 4 , isolated from *A. barbadensis* showed its IR peaks at 3405, 2941,2113, 1642, 1420, 1104 and 1054 as shown in Fig.4.20.The molecular formula of the purified fraction was found to be $C_{14}H_{14}O_5$ and the mass value calculated to be 262.26. The proton and carbon NMR spectra of the compound obtained from the Fig.4.21a and Fig.4.21b are presented below:

^{1}H NMR: 1.08, 1.22, 1.89, 2.27, 2.50, 3.17, 3.45, 3.65, 3.78, 3.83, 4.28, 4.48, 4.85, 5.32, 5.63, 5.98, 6.17, 6.59, 8.24

^{13}C NMR: 167.2, 192.1, 104.3, 98.4, 92.5, 82.8, 79.5, 78.9, 77.03, 75.1, 73.4, 72.2, 70.8, 63.4, 61.5, 60.8, 40.63, 39.7, 38.9.

The probable structure of the compound Av 4 is shown in Fig.4.22.

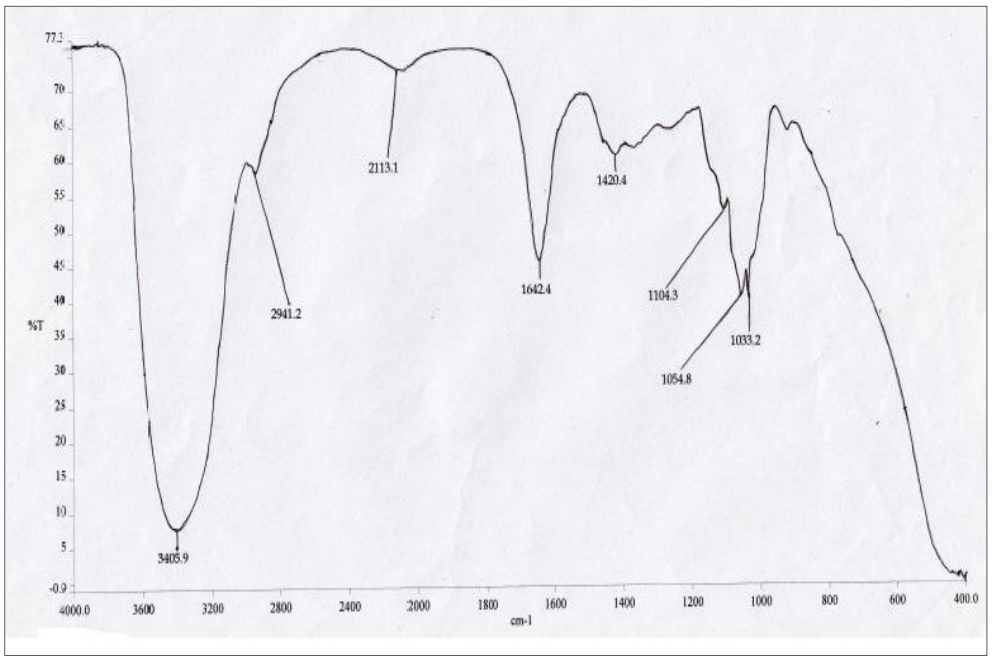

Fig.4.20. FTIR spectra of the compound Av 4

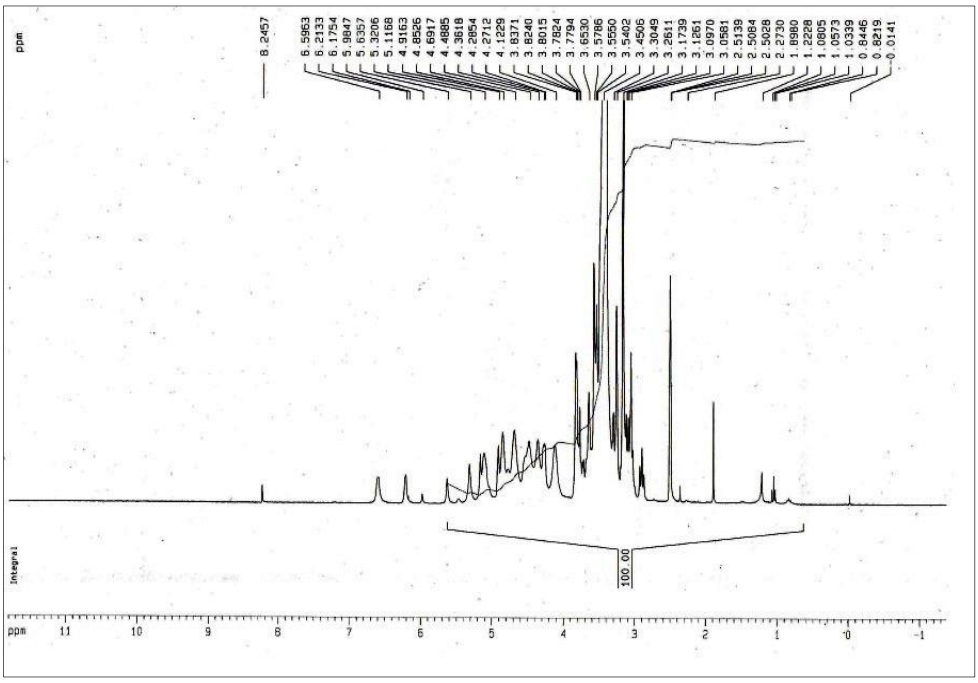

Fig.4.21.a) Proton NMR spectra of the compound Av 4

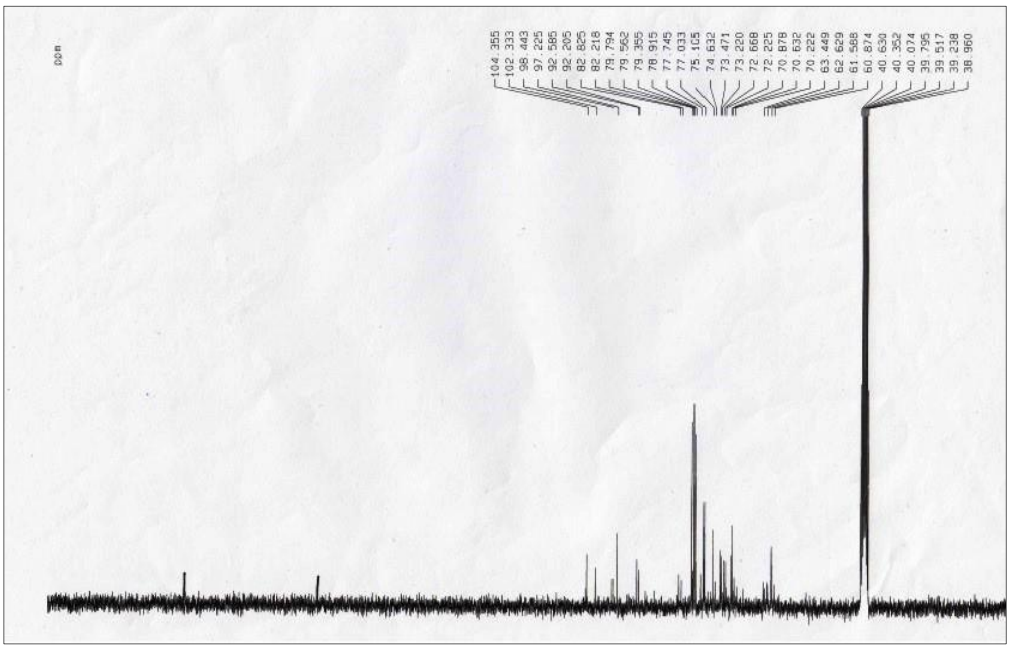

Fig.4.21.b) Carbon NMR spectra of the compound Av 4

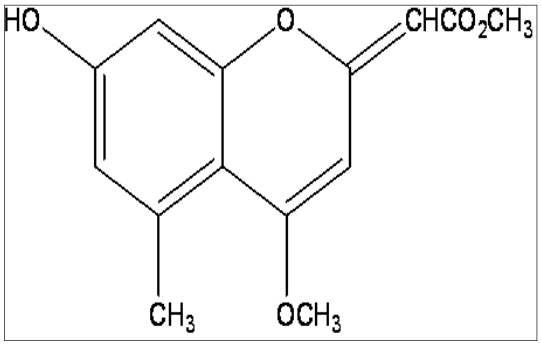

Fig.4.22. Probable structure of the compound Av 4

4.5. Biochemical assessment

4.5.1. Microbial assay

4.5.1.1. Antibacterial

All the purified phyto-compounds were subjected to antibacterial activity test and the same are shown in Fig.4.23 and data obtained are presented in Table 4.5. Compound Ea 1 and Ea 2 isolated from *E. alba*, showed strong antibacterial activity against both gram positive and gram negative bacterial strains. Compound Ea 1 showed inhibitory activity against all the tested strains and maximum zone of inhibition was observed against *B. subtilis* (13 mm) followed by *P.aeruginosa* (12 mm). The compound Ea 2 also showed good antibacterial activity against all the bacterial strains except *K. pneumoniae.* The highest zone of inhibition of Ea 2 was observed against *B. subtilis* (13 mm) followed by *S.aureus* (11 mm).

Among the compounds isolated from *A. barbadensis*, the compound Av 3 showed moderate antibacterial activity against the tested bacterial strains. The maximum inhibitory activity of Av 3 was observed against *E. coli* (12 mm) followed by *B. subtilis* (11mm). However, almost no inhibitory activity was observed against *K. pneumonia*. The compound Av 4 was observed to have equal antibacterial activity against the bacterial species *B. subtilis* and *E. coli* with the inhibition zone being 12mm, followed by *P. aeruginosa*. However, against *K. pneumonia*, the compound Av 4 showed good inhibitory activity. The MIC and MBC values were calculated for the phyto-compounds against the tested bacterial strains and the data were presented in Table 4.6 (a and b). Minimum MIC value 93.7µg/ml was

observed for Ea 1 followed by 117.1µg/ml for Av 4 against *B.subtilis*. The standard antibacterial drug streptomycin (1mg/ml) was taken as the positive control for the experimentation.

Table 4.5. Antibacterial activity of Ea1, Ea 2, Av 3 and Av 4

Phytocompounds	Zones of Inhibition (mm)				
	B.subtilis	*S.aureus*	*P.aeruginosa*	*E.coli*	*K.pneumoni*
Ea 1	13	10	12	12	11
Ea 2	13	11	7	6	-
Av 3	11	10	10	12	-
Av 4	12	10	11	12	9

(N.B. The values presented here are average of three repetitions of study)

Table 4.6a. MIC values of Ea 1, Ea 2, Av 3 and Av 4

Phyto-compounds	*B.subtilis* (µg/ml)	*S.aureus* (µg/ml)	*P.aeruginosa* (µg/ml)	*E.coli* (µg/ml)	*K.pneumini* (µg/ml)
Ea 1	93.7	217.1	187.5	193.6	202.9
Ea 2	153.5	211.5	429.2	487.1	976.3
Av 3	184.1	243.3	272.1	188.2	886.2
Av 4	117.1	274.3	245.5	152.8	643.6

(N.B. The values presented here are average of three repetitions of study)

Table 4.6b. MBC values of Ea 1, Ea 2, Av 3 and Av 4

Phyto-compounds	*B.subtilis* (µg/ml)	*S.aureus* (µg/ml)	*P.aeruginosa* (µg/ml)	*E.coli* (µg/ml)	*K.pneumini* (µg/ml)
Ea 1	187.5	434.2	375	387.2	405.8
Ea 2	307	423	858.4	974.2	1952.6
Av 3	368.2	486.6	544.2	376.4	1772.4
Av 4	234.2	548.6	491	305.6	1287.2

(N.B. The values presented here are average of three repetitions of study)

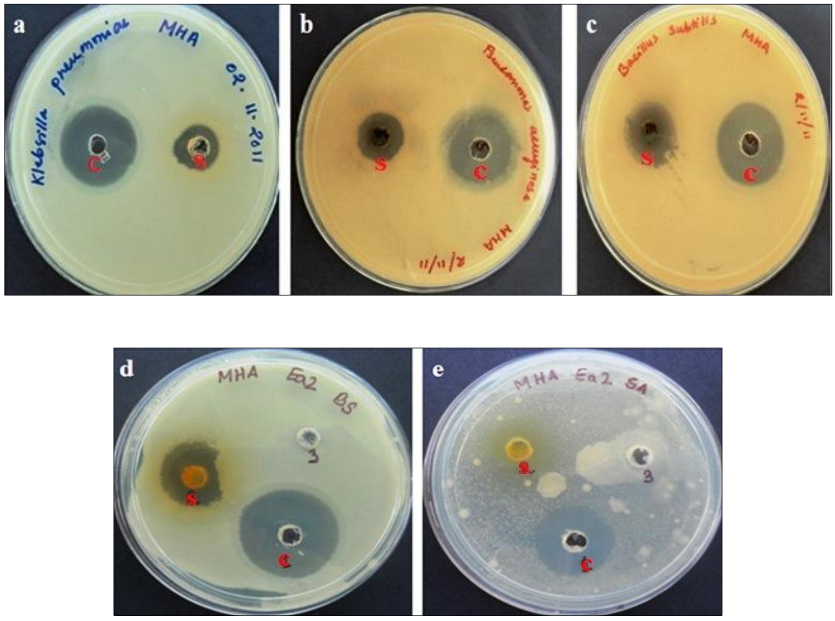

Fig.4.23A**:** Antibacterial activity of Ea 1 against *K. pneumonia* (a), *P.aeruginosa* (b), *B.subtilis* (c); antibacterial activity of Ea 2 against *B.subtilis* (d) and *S.aureus* (e)

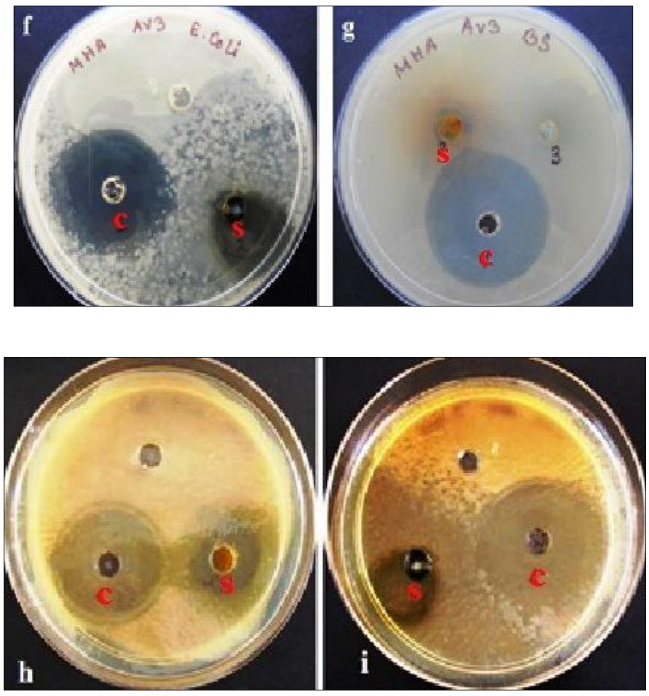

Fig.4.23B**:** Antibacterial activity of Av 3 against *E.coli* (f) and *B.subtilis* (g); antibacterial activity of Av 4 against *P.aeruginosa* (h) and *E.coli* (i)

4.5.1.2.Antifungal

Antifungal activity of the isolated phyto-compounds was assessed against *C. albicans* (MTCC 227) and *F. oxysporium* (MTCC 284). The zone of inhibition of the compounds against the tested fungi varied from 16.0 - 10.0 mm (Table 4.7). Among the isolated compounds, Ea1 showed 11 mm zone of inhibition against *F. oxysporium* (MTCC 284) (Fig. 4.24) and 14 mm against *C. albicans* (MTCC 227). The other *E. alba* isolated compound Ea 2 showed moderate antifungal activity against *F.oxysporium* (MTCC 284) only.

In the case of *A. barbadensis*, the compound Av 4 exhibited strong antifungal activity with the inhibition zone of 13 mm and 14 mm against *F. oxysporium* (MTCC 284) and *C. albicans* (MTCC 227), respectively. On the other hand, Av 3 showed very less or no inhibitory effect against both the tested fungal strains. All the isolated phyto-compounds showed better inhibitory activity against *C. albicans* (MTCC 227) than *F. oxysporium* (MTCC 284).

Table 4.7. Antifungal activity of Ea 1, Ea 2, Av 3 and Av 4

Phyto-compounds	Zone of inhibition (mm)	
	F. oxysporium	*C. albicans*
Ea 1	11	14
Ea 2	10	11
Av 4	13	14

(N.B. The values presented here are average of three repetitions of study)

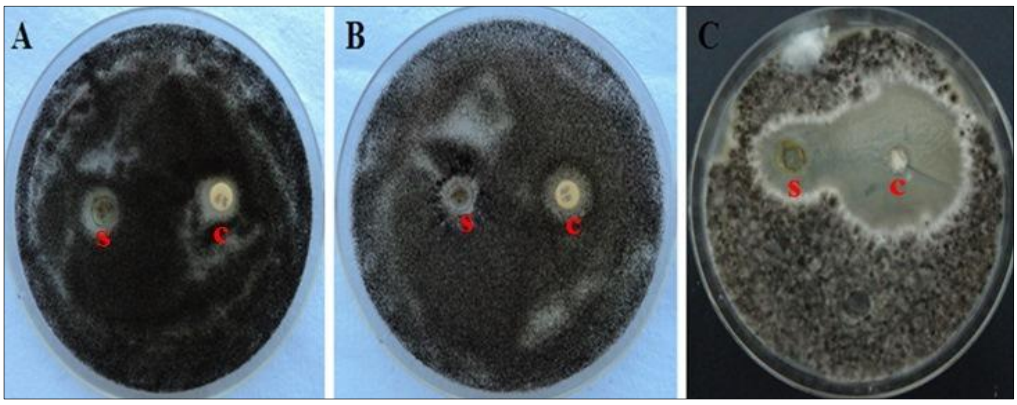

Fig.4.24a. Antifungal activity of compound Ea 1(A), Ea 2 (B) and Av 4 (C) against *F.oxysporium*

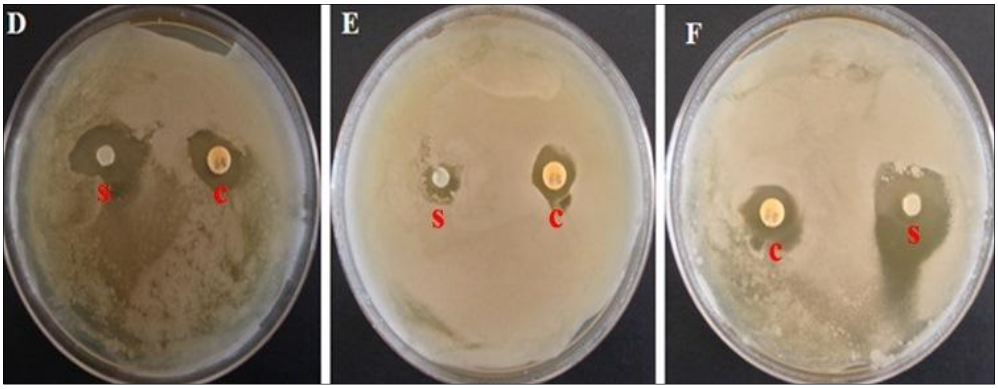

Fig.4.24b. Antifungal activity of compound Ea 1 (D), Ea 2 (E) and Av 4 (F) against *C.albicans*

4.5.2. Antioxidant assay

DPPH free radical scavenging activity of the compounds Ea 1, Ea 2, Av 3 and Av 4 are presented in Fig.4.25. Quercetin and gallic acid were taken as the standards for the study. The compound Ea 1 and Ea 2 showed strong DPPH free radical scavenging activity with 89.19% and 51% at 100 mg/ml concentration. The

compound Ea 1 showed better scavenging property as compared to the compound Ea 2.

The compound Av 4 showed strong scavenging property, nearly 88.95% at a concentration of 100 mg/ml whereas, the compound Av 3 showed 15.67% scavenging activity at the same concentration. In this study, both the standard and tested compounds showed a dose-dependent effect which increased with the increasing concentrations. The percentage of scavenging activity was presented graphically against the concentrations. IC_{50} (Inhibition concentration 50%) values were calculated by the linear regression analysis on the basis of the graphical data. IC_{50} values of all the tested compounds were calculated for the DPPH scavenging assay and the same are presented in Table 4.8.

Table 4.8. IC_{50} values of the purified compounds

Compound	**IC_{50} value**
Gallic acid	14.99±0.98
Quercetin	12.3±0.41
Ea 1	23.64±0.79
Ea 2	81.13±0.88
Av 3	134.02±0.85
Av 4	27.49±0.77

(N.B. The values presented here are average of three repetitions of study)

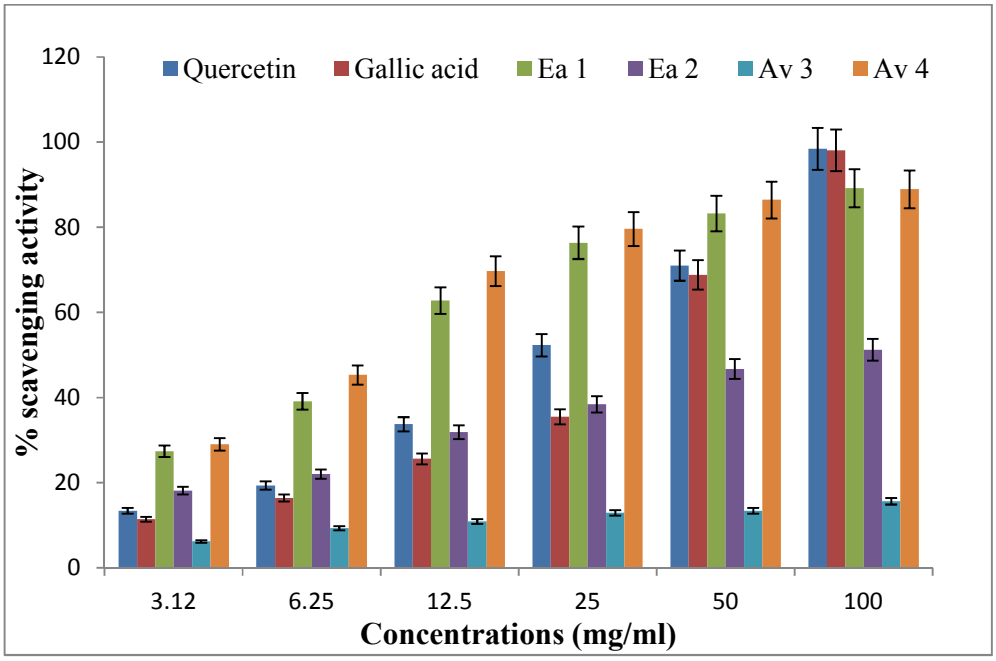

Fig.4.25. Free radical scavenging activity of isolated phyto-compounds

4.5.3. Cytotoxicity assay

The present study analyzed the cytotoxic effect of Ea 1, Ea 2, Av 3 and Av 4 using MTT assay. All the phyto-compounds failed to inhibit the proliferation of murin macrophage cell line (RAW 264.7) in comparison to standard drug Kanamycin (10-100 mg/ml). The experiment was evaluated in a dose dependent manner and no cytotoxic effect was observed upto 100mg/ml concentration. The LD_{50} value of all the compounds were calculated and presented in Table 4.9. The result of the study is presented in Fig.4.26.

Table 4.9. LD_{50} value of four phyto-compounds

Compound	LD_{50}
Kanamycin	14.23± 0.65
Ea 1	458.19±0.77
Ea 2	577.3±0.85
Av 3	299.38±0.92
Av 4	514.92±0.94

(NB. The values presented here are average of three repetitions of study)

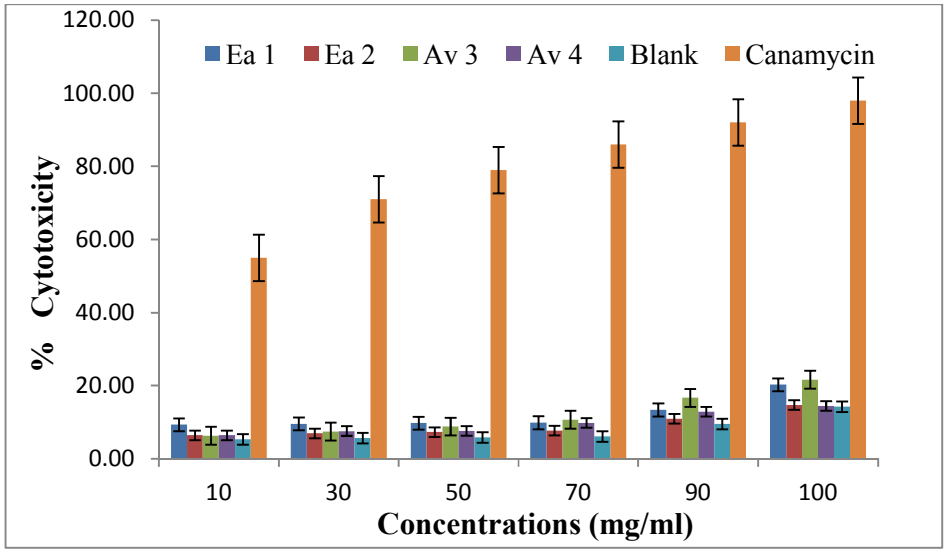

Fig.4.26. Cytotoxicity of the phyto-compounds in RAW 264.7

4.6. Acute dermal irritation study of the purified compounds

No dermal responses, including erythema or edema, were found in rabbits on being treated with the phyto-compounds Ea 1, Ea 2, Av 3 and Av 4 as compared to the positive control 0.8% (*w/v*) formaldehyde. PII was calculated to be 0 in both control and phyto-compound treated group of rabbits. In the case of the

group treated with 0.8% (*w/v*) aqueous solution of formaldehyde, all rabbits exhibited severe erythema within 72 hours. PII was calculated to be 10.58 in the group, indicating severe irritation. Cage-side observation did not reveal any observable signs of systemic toxicity in any of the treated groups of rabbits. The results of acute dermal toxicity of the purified compounds are presented in Table 4.10.

Table 4.10a. Acute dermal toxicity of 0.8% formalin (positive control) and 10% DMSO (negative control)

Skin irritation	0.8% Formalin			DMSO		
Observation time (hr.)	24	48	72	24	48	72
Total score	18	13	11	0	0	0
Mean score	6.11	5.21	7.33	0	0	0
Total of mean score	31.76			0		
PII	10.58			0		
Remarks	Severely irritating			Non-irritant		

Table 4.10b. Acute dermal toxicity study of Ea 1, EA 2, Av 3 and Av 4

Skin irritation	Ea 1			Ea 2			Av 3			Av 4		
Observation time (hr.)	24	48	72	24	48	72	24	48	72	24	48	72
Total score	0	0	0	0	0	0	0	0	0	0	0	0
Mean score	0	0	0	0	0	0	0	0	0	0	0	0
Total of mean score	0			0			0			0		
PII	0			0			0			0		
Remarks	Non irritant			Non-irritant			Non-irritant			Non-irritant		

4.7. Induction of alopecia in Wistar albino rats

Alopecia was induced in the experimental wistar albino rats by standardizing the dose of warfarin at 1.7mg/kg/day and medication was given orally for 2 months. After 2 months, hair fall started and patchy areas of hair loss were observed. With the increase in number of days, the number of fallen hair inside the cage was also found to increase. Hair fall was observed near eye area, legs and stomach of the body. Hair falling from the warfarin treated animals was collected and weighted. From each group of wistar rats, 30 hairs were collected and weighted. All the obtained data were depicted in Table 4.11 and the hair fall in treated rats are shown in Fig.4.27.

Table 4.11. Average lengths and weights of the hair fall during induction of Alopecia

Group no.	**Treatment**	**Number of Hairs fall (days)**				**Average length (cm)**	**Average weight (mg)**
		5th	**15th**	**25th**	**30th**		
1	Control	6	11	23	37	2.3±0.91	3.1±0.8
2	Minoxidil	7	9	17	25	2.7±0.65	4.4±0.77
3	Ea 1	4	13	21	28	2.3±0.76	4.3±0.73
4	Ea 2	5	12	23	30	2.5±0.31	3.01±0.11
5	Av 3	3	11	25	34	2.5±0.54	2.6±0.82
6	Av 4	5	14	19	32	3.1±0.81	3.8±0.67

(N.B. The values presented here are average of three repetitions of study)

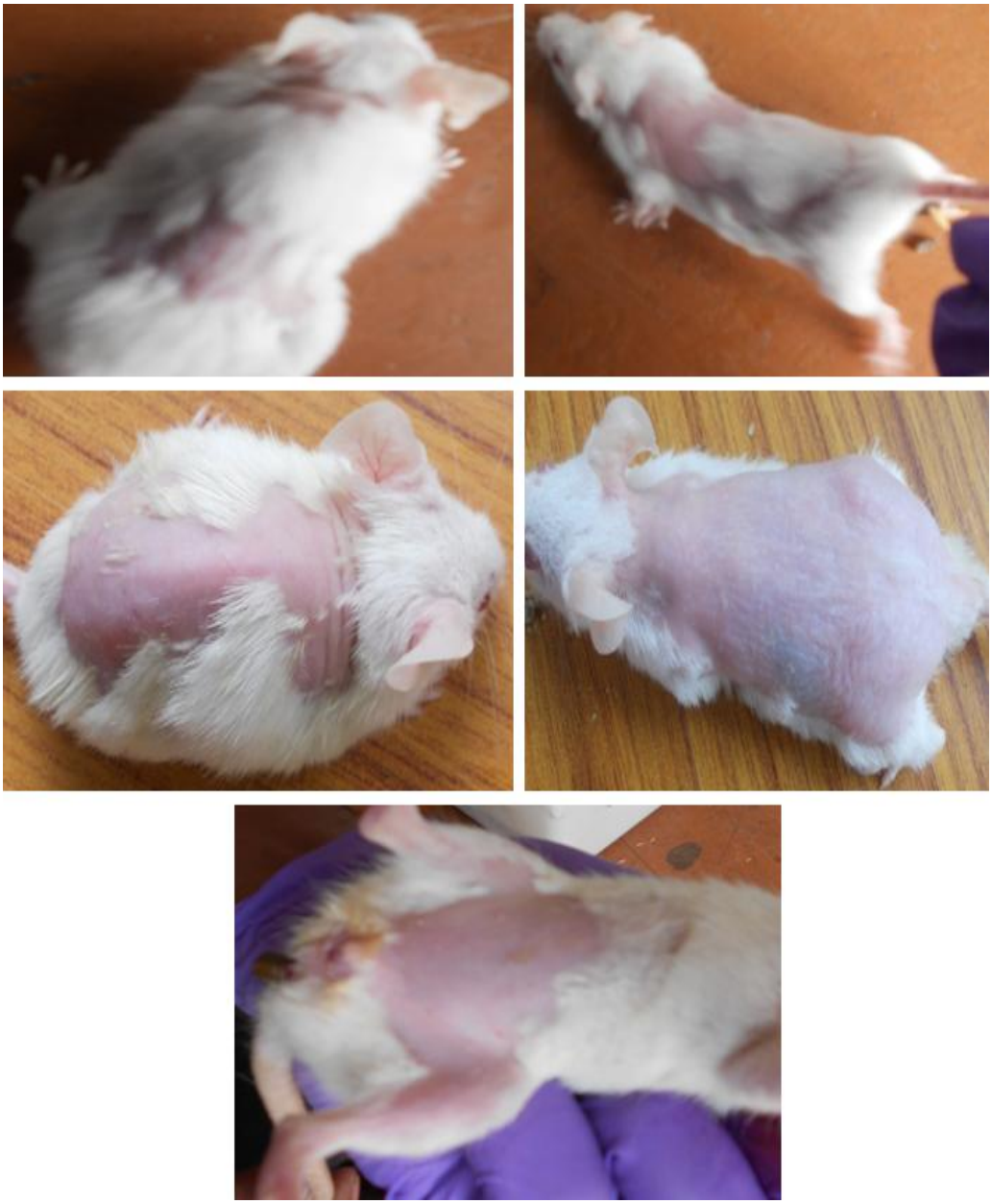

Fig.4.27. Induction of alopecia in wistar rats

4.8. Qualitative study of hair regeneration

The initiation of hair follicles was observed in the Ea 1 treated wistar rats on 4 ± 0.82 days and the completion of hair growth was observed on 19 ± 0.51 days. However, the initiation of hair follicles took more time in the case of Av 4 and minoxidil treated wistar rats i.e. 6 ± 0.58 and 7 ± 0.58 days, respectively and the complete hair growth was observed on 23 ± 0.83 and 24 ± 0.62 days, respectively. The quality of hair was soft and silky in the case of Ea 1 and minoxidil treatments. The average length of the minoxidil treated wistar rat hairs was 1.92±0.37 cm and the average weight 4.3±0.97 mg, for 10 numbers of hairs collected from the group. In the case of Ea 1 and Av 4 treated group, the average length of hairs was 2.58±0.84 and 1.77±0.46 cm with average weight 5.3±1.12 and 4.1±1.04 mg, respectively for 10 numbers of hairs collected from each group. Visual observation on hair growth was recorded on 13th day with minoxidil treated positive control group. Hair growth was visually observed on 7th day after Ea 1 treatment whereas Av 4 treated wistar rats showed hair growth on 9^{th} day. The complete hair growth was observed on 21st day in the case of Ea 1 and 24^{th} day in the case of Av 4 compounds. But in the case of minoxidil, complete hair growth was observed on 25^{th} day following the treatment. Regeneration of hair in treated with minoxidil and phyto-compounds are showed in Fig.4.28 and the data obtained from the study are presented in Table 4.12.

Table 4.12.a) Density of hair follicles

Treatment	Follicular Density (no./cm^2)		
	7 days	10 days	15 days
Minoxidil	8±1.69	21±0.82	29±0.31
Compound Ea 1	14±0.76	27±0.43	34±0.26
Compound Av 4	9±2.13	20±0.66	31±0.11

(N.B. The values presented here are average of three repetitions of study)

Table 4.12.b) Hair growth initiation and completion time

Treatment	Hair growth (days)	
	Initiation Time	Completion Time
Minoxidil	7 ± 0.58	24 ± 0.62
Compound Ea 1	4 ± 0.82	19 ± 0.51
Compound Av 4	6 ± 0.58	23 ± 0.83

(N.B. The values presented here are average of three repetitions of study)

Table 4.12.c) Length and weight of hair in treated groups

Treatment	Length of hair (cm)	Rate of elongation (cm/day)	Weight of hair (mg)	Rate of increment (mg/day)
Minoxidil	2.7±0.37	0.11	4.3±0.97	0.18
Compound Ea 1	3.1±0.84	0.16	5.3±1.12	0.27
Compound Av 4	2.9±0.46	0.12	4.1±1.04	0.17

(N.B. The values presented here are average of three repetitions of study)

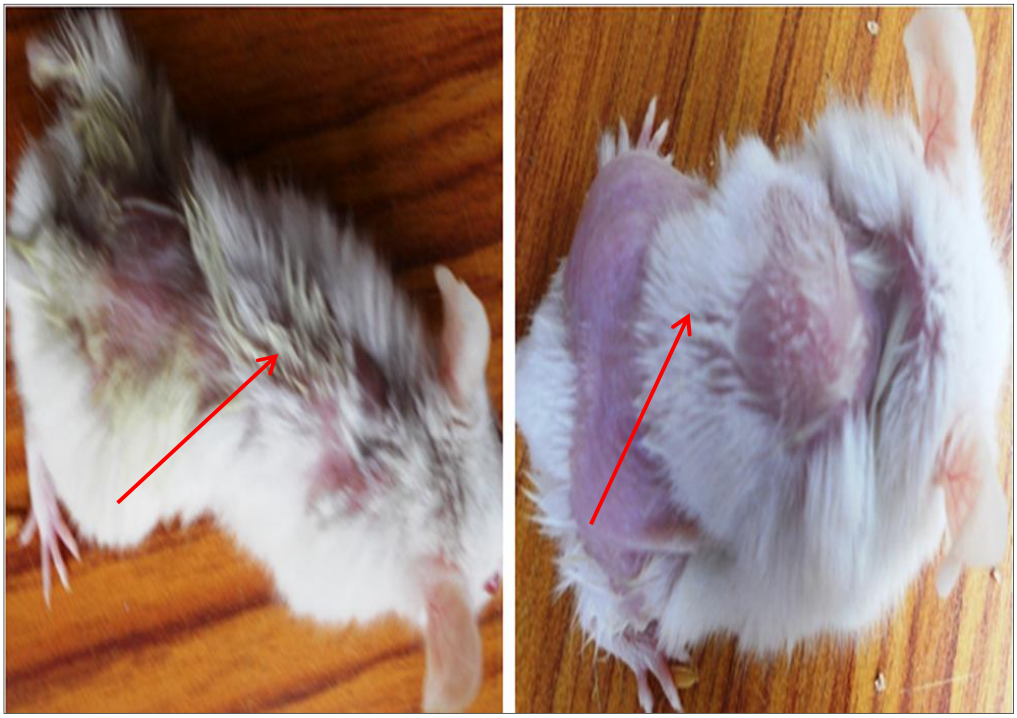

Fig.4.26.A) Hair regeneration in wistar rats treated with compound Ea 1

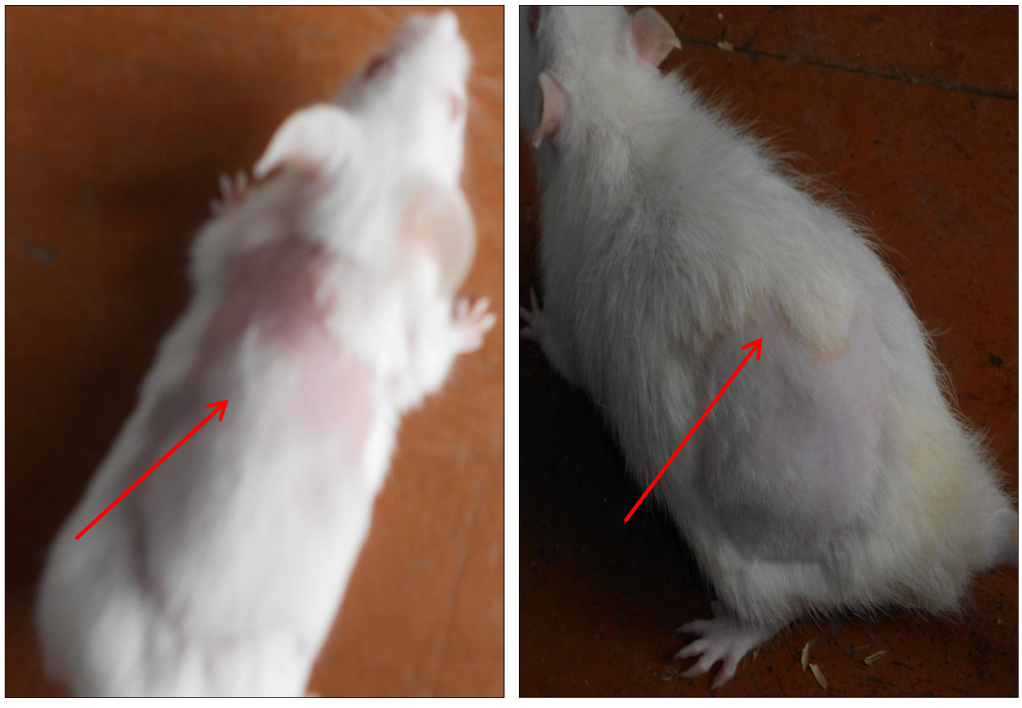

Fig.4.26.B) Hair regeneration in wistar rats treated with compound Av 4

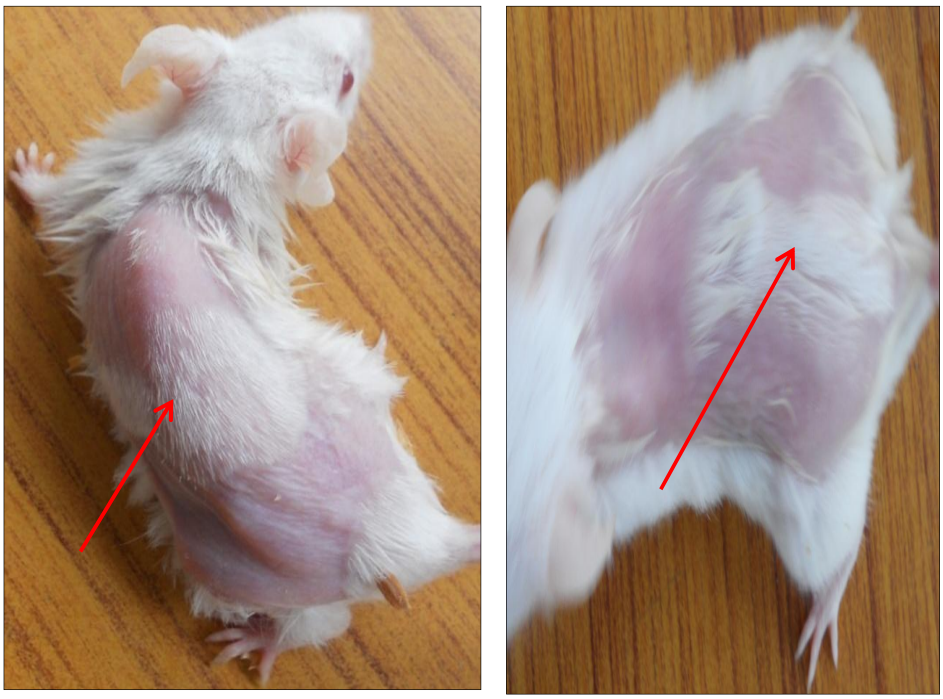

Fig.4.26.C) Hair regeneration in wistar rats treated with 2% Minoxidil solution

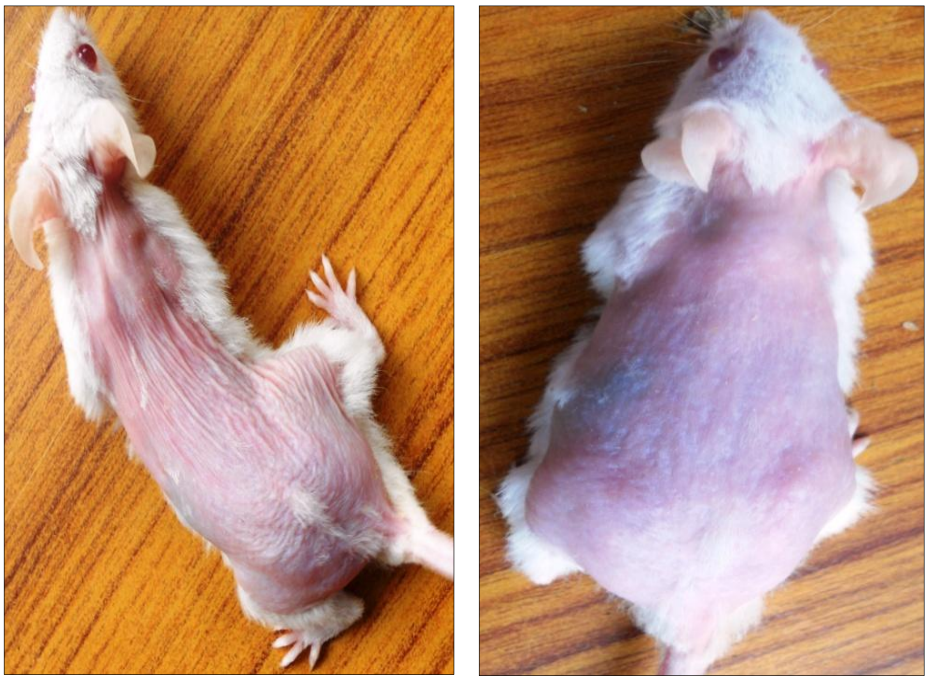

Fig.4.26.D) Control group wistar rats without any treatment

4.9. Haematology and serum biochemical assessment

No significant changes were observed in the body weights of wistar rats after treating with the phyto-compounds Ea 1 and Av 4. WBC and neutrophil counts significantly increased in the case of the animals treated with Ea 1 and Av 4, whereas the lymphocyte counts got decreased. On the other hand, RBC, HB and Hct were increased in the treated groups as compared to the control group.Also, THR was significantly increased in case of positive control and phyto-compound treated groups. Data obtained from the study are presented in Table 4.13. In the case of biochemical analysis of serum of both control and treated animals, no significant changes were detected after 15 days of topical application with the phyto-compound Ea 1 and Av 4. Serum biochemical data analysis in respect of Ea 1 and Av 4 treated animals are presented in Table 4.14.

Table 4.13. Hematology Study of Ea 1 and Av 4 treated animals

Items	Control	Minoxidil	Ea 1	Av 4
WBC (K)	8.4± 0.73	5.4±0.41	13.62±0.55	12.16±0.96
Lymphocyte (%)	36.4±2.87	44.4±3.11	35.22±1.35	33.19±0.98
Monocyte (%)	12.4±0.25	20.6±0.84	17.8±0.62	14.3±1.03
RBC (M)	8.41±0.92	10.12±0.81	13.17±0.79	11.3±0.89
Hb (g/dl)	12.8±2.71	13.9±3.43	14.6±4.12	13.7±4.71
Hct (%)	43.4±5.87	54.4±4.63	48.01±6.01	47.6±5.21
MCV (fL)	51.7±7.29	53.8±6.28	48.93±5.93	57.3±5.44
MCH (pg)	15.2±1.49	15.7±3.11	14.3±1.91	16.4±1.72
MCHC (g/dL)	29.4±4.31	29.2±4.57	29.01±5.01	28.7±4.66
Gra (%)	51.2±0.87	35±0.47	43.26±0.65	37.8±1.21
RDW (g/dl)	10.6±0.07	10.6±0.06	12.1±0.03	11.9±0.04
MPV (fl)	6.7±0.11	6.2±0.19	6.4±0.17	6.7±0.13
Pct (%)	0.24±0.05	0.41±0.03	0.36±0.05	0.45±0.07
PDW	7.6±2.11	7.6±1.21	8.2±0.97	8.9±0.81
THR (m/mm^3)	363±13.19	655±19.71	603±23.44	593±32.01

(N.B. The values presented here are average of three repetitions of study)

Table 4.14. Serum biochemistry of Ea 1 and Av 4

Items	Control	Minoxidil	Ea **1**	Av **4**
GLUC	105±17.51	114±19.34	121±20.03	118±16.71
UREA	64.8±5.79	66.3±9.37	69.2±8.99	66.1±6.74
TP	6.38±0.82	7.6±0.75	6.92±0.69	6.54±0.55
UA	1.22±0.05	1.41±0.06	1.01±0.09	1.24±0.12
Chol	37.4±4.11	41.7±5.12	33.7±3.87	34.5±2.93
CRE	0.7±0.04	0.8±0.03	0.5±0.02	0.7±0.02
SGPT	99±8.6	63±6.0	76.2±7.7	93±9.1
SGOT	159±9.5	113±11.52	141±10.97	160±18.36

(N.B. The values presented here are average of three repetitions of study)

4.10. Histological study of the treated and control skin

Histological study of both control and phyto-compound (Ea 1 and Av 4) treated animals were analyzed using the standard protocol. A considerable difference in the hair follicle initiation was observed in the treated wistar rats in the case of minoxidil and phyto-compounds. Initiation of hair follicle was observed in case of Ea 1 treated group on 4^{th} day whereas it was observed on 6^{th} day in case of Av 4 treated group and 9^{th} day in case of minoxidil treated group of wistar rats. Histological study also revealed the regeneration of hair follicles in the case of treated and control groups. Digital photomicrographs were taken from representative areas at a magnification of 10x, 20x and 40x. All the observations in the case of control, standard and phyto-compound treated animals were shown in Fig.4.29.

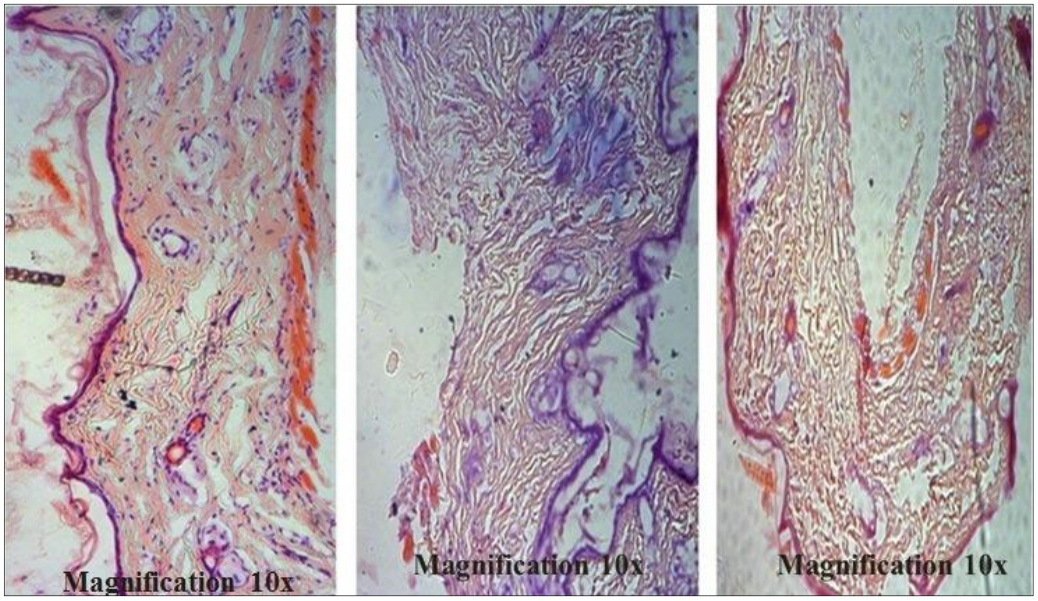

Fig.4.27.a) Histology of control group wistar rat skin

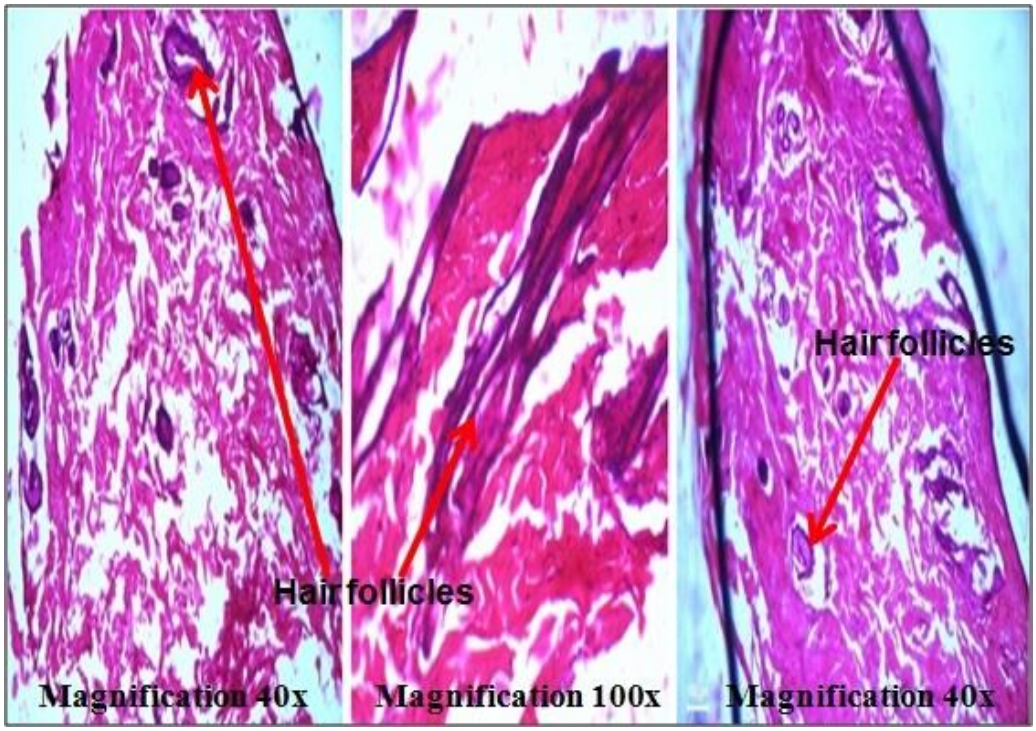

Fig.4.27.b) Histology of minoxidil treated wistar rat skin

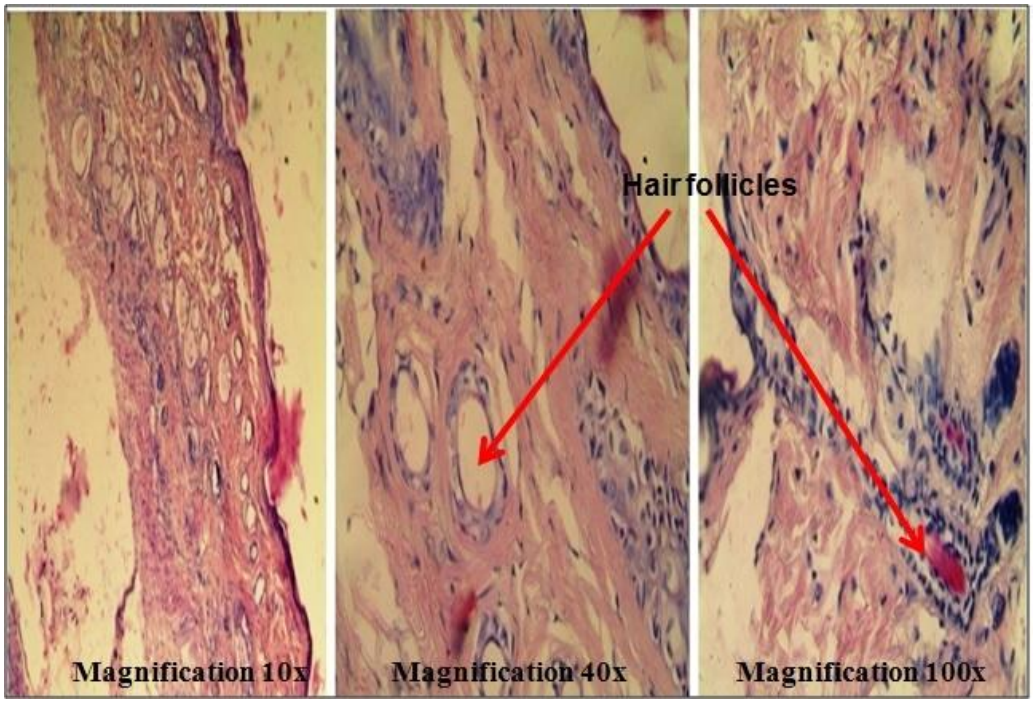

Fig.4.27.c) Histology of Ea 1 treated wistar rat skin

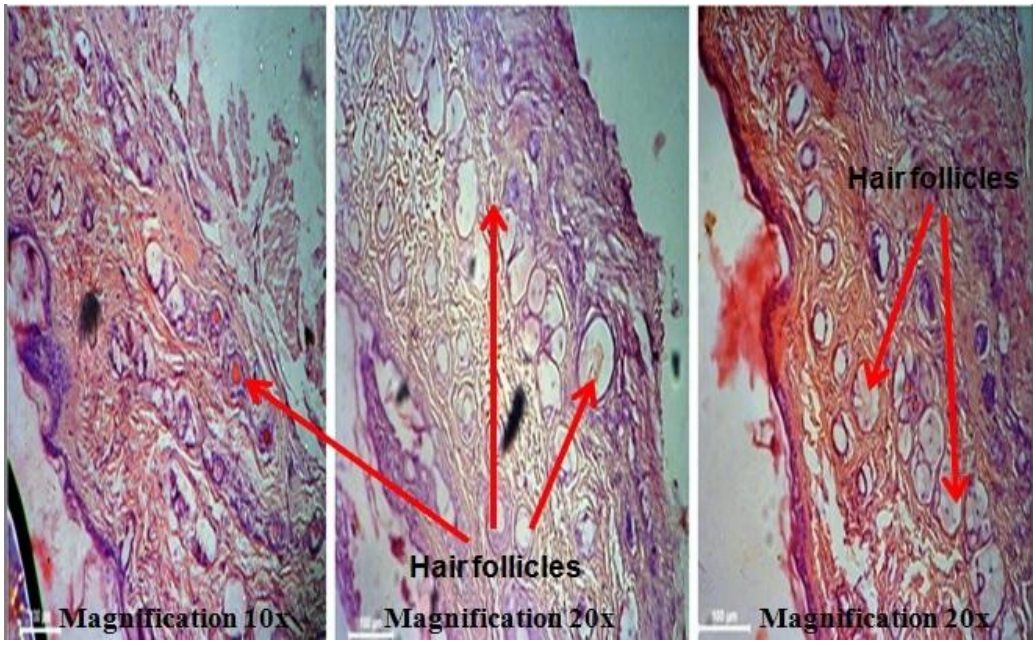

Fig.4.27.d) Histology of Av 4 treated wistar rat skin