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# Pharmacological Evaluation and Detection of Anacardic Acid in Callus Culture and Various Plant Parts of *Anacardium occidentale* L.

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**ABSTRACT**

*Anacardium occidentale L.* is much known for its commercially valuable kernals and also bioactive compounds like polyphenols which serve as natural antioxidant having an ability to quench cytotoxic free radicals that may prevent many diseases. The present study was carried out to evaluate the presence of anacardic acid, total phenolic compound, antioxidant activity and antibacterial activity in methanolic extract of callus and various plant parts such as flower, young leaves, shoot and cotyledon. Total phenolic contents were measured by the Folin-Ciocalteau method using gallic acid as standard compound. The maximum phenolic content was found in the methanol extract of flower (260.8 ± 5.543 mg/g) whereas the methanol extract of cotyledon (16.2 ± 3.284 mg/g) showed the lowest total phenolic content. The action of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) on radical scavenging effect of the extracts was determined spectrophotometrically using ascorbic acid as standard compound. All extracts exhibited a DPPH radical scavenging activity and among the extracts, *A. occidentale* flower demonstrated greater antioxidant potential with a low $IC_{50}$ (206.312 ± 0.38µg/ml) in comparison with those of the other extracts. Total phenolic contents showed positive correlations with the DPPH radical scavenging activity (r = 0.979; $p<0.01$) and negative correlations with $IC_{50}$ (r = -0.984; $p<0.01$). The antibacterial capabilities of various extracts were also investigated against two common human pathogens of clinical importance, *Escherichia coli* and *Staphylococcus aureus*. Presence of anacardic acid in various extracts was also estimated by using HPTLC method. Pre-coated aluminum silica gel plate $60F_{254}$ was used as stationary phase and chloroform: ethyl acetate (9:1) was used as mobile phase.

**Keywords:** *Anacardium occidentale,* 2, 4-D, BAP, Callus, Total phenolic contents, Folin-Ciocalteu reagent, Antioxidant activity, Antibacterial activity, DPPH, HPTLC.

## INTRODUCTION

*Anacardium occidentale* is a tropical tree indigenous to Brazil, a member of the family *Anacardiaceae*, which is now widely grown in other tropical countries like India and is a multi-purpose plant. [1] Different parts of the tree such as root, leaves, cashew nut, cashew apple, liquid from nut shell, cashew testa and cashew kernel are used for various purposes. Among these, commercially important two parts are cashew nut for diet and the liquid from nut shell (CNSL) for various industrial and medical applications. [2-3] Anacardic acid is having very much demand in the international market. [4] Apart from this, a large number of other phenolic compounds are founds in very small quantities in the shell. [5] Phenol is seen throughout the

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plant system though it is mainly concentrated in the nut shell. [6-7]

Anacardic acid is a highly valuable natural polyphenolic compound used in medicine and industry. In medical field, it has been successfully used to treat warts, ringworms and even elephantiasis and also used in beauty therapy to remove the skin of the face in order to grow a new skin. The biological activities of anacardic acid are widely reported and it has been reported to possess anti-viral, anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidantal [8-10] anti-carcinogenic [11] activities etc. In industrial field, it is used as a raw material for brake lining compounds of automobiles, synthetic resins, heat and water proof paints, corrosion resistant varnishes, insulating enamels for the electrical industry, plywood adhesives, different types of surface coatings and plastic industry. [12]

*In vitro* cultures have the potential to form secondary metabolites and to exhibit bioactivity comparable to the original plant. [13] The plant polyphenols may act as potent antioxidants and can prove beneficial for maintenance of optimal health. [14] The antioxidant activities and phenolic content of this plant have been reported mainly in the nuts and stem barks. [15] Recent studies have reported the antioxidant activities in some extracts of the leaves of this plant. [16]

In view of the role of plant extracts in curing many infections, the present study was designed to give a detailed picture on the analysis of various plant extracts against selected pathogenic microbes that cause human skin disorders. It also aims to estimate total phenolic contents, evaluation of antioxidant activity, estimation of anacardic acid by HPTLC and to correlate between total phenolic content and antioxidant activity. This will help to find new sources of safe and inexpensive natural antioxidants to use them in food or nutraceutical and pharmaceutical preparations to replace synthetic antioxidants.

## MATERIALS AND METHODS

### Plant material

The *A. occidentale* plant parts such as flower, young leaves, shoots, cotyledons from mature seeds were procured from the mother stock trees growing at the Kerala State cashew development corporation, Mundakkal, Kollam, Kerala, India and callus from the cotyledons derived from the mature seeds. The collected plant parts were brought into the laboratory, cleaned and air-dried for 4 days and later grinded to fine powder.

### Callus induction

Cotyledonary explants were taken from mature seeds of *A. occidentale* for callus induction. Callus initiation was studied with the 2, 4-D and BAP alone and with different combinations in the MS medium. [17] After inoculation the cultures were maintained at a constant room temperature of 24 ± 2°C and 55 ± 5% relative humidity in the culture room under dark conditions for 4 weeks before the first subculture. Sub culturing was done regularly every four weeks until callus establishment. Callus obtained after 4 weeks of culture was further evaluated for phenolic content, antioxidant activity, antibacterial activity and HPTLC analysis.

### Preparation of plant extracts and callus extract

The plant materials (flower, young leaves, cotyledons and shoot) of *A. occidentale* were collected washed and air dried. The dried samples were ground to coarse powder form. Powdered samples were extracted with methanol at room temperature for 24 hours at a ratio of 1: 100 (g: ml). Homogenized samples were centrifuged at 10,000 rpm for 15 min and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature. Residue was dissolved in methanol and stored at 4-8°C in a refrigerator for further analysis.

A known quantity of two-month old callus was taken and oven dried at 60°C to constant weight. The callus was finely grinded and was extracted with methanol at room temperature for 24hrs at a ratio of 1: 100 (g: ml). Homogenized samples were centrifuged at 10,000 rpm for 15 min and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature. Residue was dissolved in methanol and stored at 4-8°C in a refrigerator for further analysis.

### Determination of total phenolic content

The total soluble phenolic compounds in the different extracts of *A. occidentale* were determined with Folin-Ciocalteu reagent using gallic acid as a standard. [10] Extracts were diluted to the concentration of 1 mg/ml in methanol and 0.5 ml of the diluted extract was mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $Na_2CO_3$ (7.5%). After 15 min at 45°C, the absorbance was measured at 630 nm versus blank sample on spectrophotometer (shimadzu-UV-3600). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

### Determination of antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl free radical

The ability of the extract to scavenge 1,1-dyphenyl-2- picrylhydrazyl (DPPH) free radicals was assessed by the modified method. [18] DPPH (20 mg) was dissolved in methanol (250 ml) to obtain the concentration of 80µg/ml. The stock solution of the plant extract was prepared in methanol to achieve the concentration of 10mg/ml. Dilutions were made to obtain concentrations of 18, 16, 14, 12 and 10µg/ml. Diluted solutions (1 ml each) were mixed with DPPH (1 ml). After 30 min in darkness at room temperature (23°C), the absorbance was recorded at 517 nm. The DPPH solution in methanol was prepared daily before the absorbance measurements. DPPH is a purple coloured stable free radical. When reduced, it becomes

the yellow coloured diphenylpicryl hydrazine. Ascorbic acid was used as a standard. [18] Percent inhibition was calculated using the following expression % Inhibition = $(A_0–A_1/A_0)$ x 100 Where $A_0$ and $A_1$ stand for absorption of the control sample and absorption of tested extract solution respectively. The control samples contained all the reagents except the extract. $IC_{50}$ value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the % inhibition versus concentration sigmoidal curve, using a non-linear regression analysis.

**Determination of antibacterial activity**

The antibacterial activity of the methanol extracts was screened against gram-negative bacteria; *Escherichia coli* (MTCC 198) and gram-positive bacteria; *Staphylococcus aureus* (MTCC 96) obtained from the Microbiology Laboratory of the CEPCI Laboratory & Technical Division, Kollam, Kerala. Antibacterial activities of the different extracts were investigated by the disc diffusion method [20-21] using Mueller-Hinton agar plates previously inoculated with 18 hour old Nutrient broth culture for the bacteria. The sterile disc, 5 mm in diameter impregnated with known concentration of the plant extract (1 g/ml) were placed on the agar surface with flamed forceps and gently pressed down to ensure complete contact of the disc with the agar surface. Antibiotic discs of Streptomycin (10µg/ml) were also used to compare their antibacterial activity with that of methanol extracts. All the experiments were performed in aseptic conditions. Bacterial plates were incubated at 37°C for 24 hour. The zone of inhibitions produced by inhibitory action of different plant extracts and standard antibiotic discs were taken as the antibacterial activity.

**HPTLC estimation of anacardic acid**

HPTLC analysis was performed on Camag HPTLC. Methanol extracts were applied on pre-coated silica gel plate $60F_{254}$ (Merk, Germany) using the Linomat V applicator (Hamilton, USA). Anacardic acid from Sigma Aldrich was used as the standard compound (100µg/ml). The separation was carried out in twin trough chamber using the solvent system as mobile phase. The bands were applied over the HPTLC plate with following settings: Band length 8 mm, distance between track 15 mm, slit dimension - 6.00 mm × 0.45 mm × micro, scanning speed- 20 mm/s; data resolution- 100µm/step. Remaining parameters were left as default settings. Qualitative analysis was done by using TLC scanner 3 Camag HPTLC systems at wavelength 305 nm by comparing the peak area values of sample with that of standard using the Wincats software.

**Statistical analysis**

Data were expressed as means ± standard deviation (SD) of three replicate determinations. All statistical analysis was carried out using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver.17, 2008). To determine whether there were any differences among the means, one way analysis (ANOVA) and the Duncan's New Multiple range test were applied to the result at 0.05 level of significance ($p$<0.05). The Pearson correlation analysis was performed between antioxidant activity and total phenolic contents.

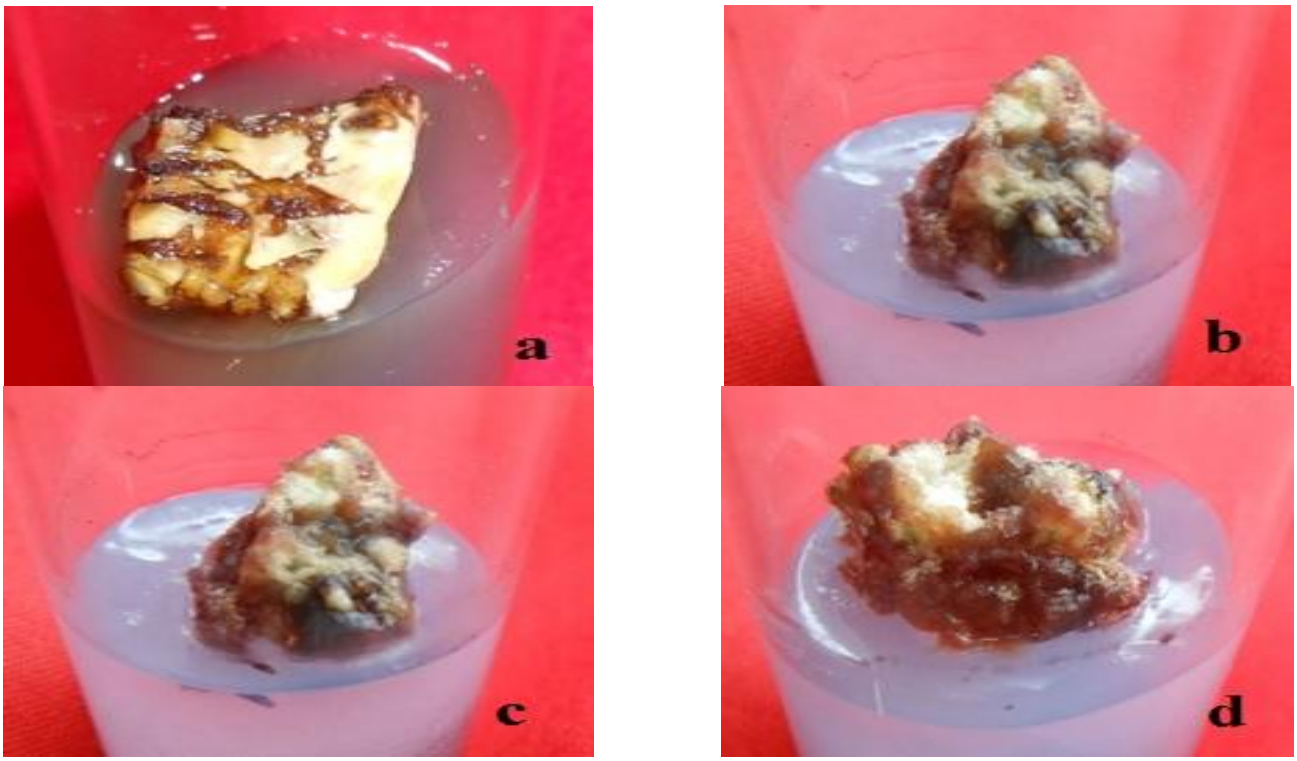


**Fig. 1: Induction and proliferation of callus from cotyledon explant of *A. occidentale***
**a. Direct callus initiation after one week of culture; b. Callus after 2 weeks of culture; c. Callus after 3 weeks of culture; d. Callus after 4 weeks of culture.**

**Table 1: The callus induction from the cotyledon explants of *A. occidentale* on ms media**

| S. No | Growth hormones (mg/L) 2,4-D | BAP | Days to callus initiation | Callus score | Explants forming callus (%) | Callus texture | Callus colour |
|---|---|---|---|---|---|---|---|
| 1. | 0 | 0 | 0 | NC | NC | NC | NC |
| 2. | 10 | - | 20 | + | 19.99 ± 3.33a | compact | dark brown |
| 3. | 25 | - | 19 | + | 26.66 ± 3.33ab | compact | dark brown |
| 4. | 50 | - | 14 | ++ | 39.99 ± 3.33c | semi friable | whitish yellow |
| 5. | - | 10 | NC | NC | NC | NC | NC |
| 6. | - | 25 | NC | NC | NC | NC | NC |
| 7. | - | 50 | NC | NC | NC | NC | NC |
| 8. | 25 | 10 | 18 | ++ | 27.77 ± 5.09b | compact | dark brown |
| 9. | 25 | 25 | 13 | ++++ | 69.99 ± 3.33ef | compact | light brown |
| 10. | 50 | 10 | 12 | +++ | 62.21 ± 5.09e | compact | dark brown |
| 11. | 50 | 25 | 14 | +++ | 44.44 ± 5.09cd | compact | dark brown |
| 12. | 50 | 50 | 13 | +++ | 66.66 ± 3.33f | compact | pale yellow |
| 13. | 75 | 75 | 15 | +++ | 47.66 ± 5.13d | semi friable | pale yellow |

NC = no callus formed; + = callus only formed at the edge of the explant; ++ = callus formed more at the edge; +++ = callus covered the surface; ++++ = callus covered more on the surface. For each treatment the means within the column by different letters are significantly different at $p<0.05$. Each value is expressed as the means ± SD

**Table 2: Antioxidant activity of investigated callus and plant extracts of *A. occidentale***

| Test compound (methanolic extract) | $IC_{50}$ (µg/ml) |
|---|---|
| Cotyledon | $630.98 \pm 0.78^{f}$ |
| Callus | $605.48 \pm 0.81^{e}$ |
| Shoot | $522.57 \pm 0.96^{d}$ |
| Young leaves | $324.87 \pm 0.47^{c}$ |
| Flower | $206.31 \pm 0.38^{b}$ |
| Ascorbic acid (control) | $7.64 \pm 1.34^{a}$ |

For each treatment the means within the column by different letters are significantly different at $p<0.05$. Each value is expressed as the means ± SD (n=3).

**Table 3: Pearson's correlation coefficients between the variables**

| | Total phenolic content | DPPH radical scavenging activity (%) | $IC_{50}$ values |
|---|---|---|---|
| Total phenolic content | | 0.979** | -0.984** |
| DPPH radical scavenging activity (%) | 0.979** | | -0.989** |
| $IC_{50}$ values | -0.984** | -0.989** | |

**Correlation is significant at the 0.01 level (2 tailed)

**Table 4: Zones of inhibition produced by methanol extracts of *A. occidentale* and standard disc**

| Bacteria | Zone of inhibition(mm) | | | | | |
|---|---|---|---|---|---|---|
| | Methanol extracts | | | | | Standard disc |
| | Cotyledon | Young leaves | Flower | Shoot | Callus | Streptomycin |
| *Staphylococcus aureus* | 9 | 15 | 10 | 7 | 10 | 25 |
| *E. coli* | 10 | 17 | - | - | - | 23 |

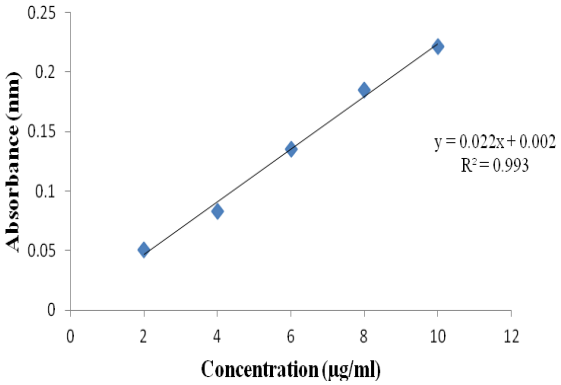


**Fig. 2: Standard calibration curve of gallic acid at concentrations of 2, 4, 6, 8 and 10 µg/ml.**
**Spectrophotometric detection was at 630 nm.**

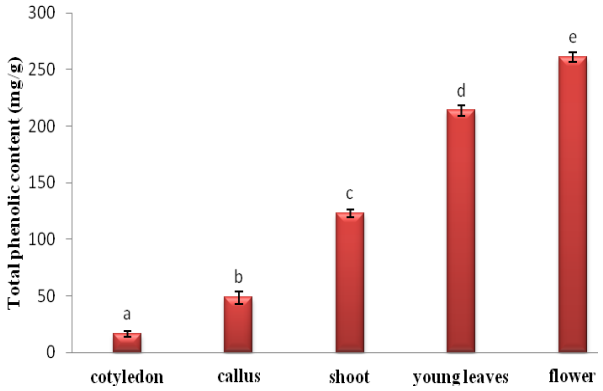


**Fig. 3: Total phenolic content of callus and different plant parts of *A.occidentale*; values with different letters are significantly different ($p<0.05$), (n=3, error bars represent standard deviation)**

## RESULTS AND DISCUSSION

### Callus induction from cotyledon explants

In the present investigation, it was observed that 2, 4-D alone was poor in inducing callus from cotyledon explants. It only produced 39.99 ± 3.33% of callus response in MS medium (Table 1). Besides that, 2, 4-D when used in combination with BAP produced increased percentage of callus induction than when it used alone. Maximum callusing (69.99 ± 3.33%) was noticed on the cotyledon explants grown on MS basal medium supplemented with 25 mg/2, 4-D and 25 mg/l BAP (Table 1) and it was not significantly different ($p<0.05$) with other hormonal concentrations such as 2, 4-D (50 mg/l) + BAP (10 mg/l) and 2, 4-D (50 mg/l) + BAP (50 mg/l) respectively. The callus was compact in texture and light brown in colour and covered more on the surface after 4 weeks of culture (Fig. 1d). These results are in agreement with the findings of previous researchers. [22-23]

### Total phenol contents of the extracts

In the present study, five different extracts were explored for their antioxidant properties and related phenolics content. Total phenol compounds, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve (Fig. 2). The calibration curve showed linearity for gallic acid in the range of 2-10µg/ml, with a correlation

coefficient ($R^2$) of 0.993 (Fig. 2). The result of the present study showed that the amount of total phenolic content differ significantly ($p$<0.05) among the various extracts (Fig. 3). Total phenolic content values were ranked as: flower (260.8 ± 5.543 mg/g) > young leaves (213.76 ± 5.43 mg/g) > shoot (122.9 ± 3.874 mg/g) > callus (48.6 ± 6.71mg/g) > cotyledon (16.2 ± 3.284 mg/g) (Fig. 3). Presence of total phenolic content was reported in cashew nut [24], cashew apple [25], leaves [26-27] and stem bark. [28-29] The present study also confirmed that *in vitro* developed callus had the potential to form phenolic compounds. The results also revealed that methanol extract of flower had the highest total phenolic content (260.8 ± 5.543 mg/g) while the methanol extract of cotyledon (16.2 ± 3.284 mg/g) showed the least total phenolic content (Fig. 3).

**DPPH Radical Scavenging Activity**

All the methanolic extracts from plant parts and callus were selected for their free radical scavenging capacities using DPPH free radicals along with ascorbic acid as standard. The absorbance values were measured at wavelength 517 nm for different concentration of extracts and the control. These values are used to calculate the percentage inhibitions of DPPH radicals against the samples. The $IC_{50}$ values of various extracts were calculated from the percentage inhibitions at various concentrations. It has been observed that the percentage of scavenging effect on the DPPH radical was increased with the increase in the concentration of all the extracts from 10 to 18µg/ml. Among the five extracts and standard tested for antioxidant activity, the methanolic extracts of flower showed the highest percent of inhibition from 34.28 ± 0.36 at 10µg/ml to 34.92 ± 0.58 at 18µg/ml while the methanolic extracts of cotyledon showed the least (0.306 ± 0.03% at 10µg/ml and 0.952 ± 0.13% at 18µg/ml), which is comparable with the standard antioxidant activity of ascorbic acid (68.26 ± 1.01% at 10 µg/ml and 69.11 ± 0.96% at 18µg/ml) (Fig. 4).

The antioxidant activity of callus and different plant part extracts of *A. occidentale* is also expressed in terms of $IC_{50}$ (µg/ml) values (Table 2) and it ranged from 206.31 ± 0.8µg/ml to 630.98 ± 0.78µg/ml. The result revealed that callus and cotyledon showed weak antioxidant activity, with $IC_{50}$ values of 605.48 ± 0.81 and 630.98 ± 0.78µg/ml respectively. While the methanolic extracts of flower showed the highest antioxidant activity, with $IC_{50}$ value of 206.31 ± 0.38µg/ml. The $IC_{50}$ value for standard ascorbic acid was 7.64 ± 1.34µg/ml. The $IC_{50}$ value decreased with the increase of antioxidant activity of each explant type and *vice versa*. It could be noticed that the samples that had the highest phenolic content had also the highest antioxidant activity and the lowest $IC_{50}$ value, which ensure that the phenolic compounds in *A. occidentale* are responsible for its antioxidant activity. As the strong correlation between the results, they showed that phenol compounds largely contribute to the antioxidant activity of this plant. Studies have demonstrated the radical scavenging properties of plant phenolic compounds and confirm the relationship between phenolic compounds, phenolic compounds and antioxidant activity. [30]

The Pearson's correlation coefficients between the variables are presented in Table 3. As shown in the table, total phenolic content showed strong positive significant correlations with the DPPH radical scavenging activity (r = 0.979; $p$<0.01) and high negative correlations with $IC_{50}$ (r = -0.984; $p$<0.01). Several investigations of the antioxidant activity of plant extracts have confirmed a high linear correlation between the values of phenol concentration and antioxidant activity [31] and many researchers have demonstrated that phenolic compounds are one of the most effective antioxidants in *A. occidentale*. [26, 32-33]

**Antibacterial activity**

In the present study, the antibacterial activity was found higher in methanol extracts of young leaves against gram-negative and gram-positive bacteria (Table 4). It was observed that antibacterial activity of the standard discs was higher than those of the methanol extracts of the plant parts. The standard antibiotic disc i.e., streptomycin (10µg/ml) showed 25 mm zone of inhibition against *Staphylococcus aureus* and 23 mm against *E. coli* (Table 4). All the extracts showed inhibitory action against *Staphylococcus aureus* as given in Fig. 5 whereas only cotyledon and young leaves showed inhibitory action against *E. coli* (Fig. 6). *In vitro* developed callus also showed inhibitory action against *Staphylococcus aureus* (Fig. 5). The antibacterial activities of cashew leaves, barks and flower were also reported. [34-36] However the polyphenolic compounds in the cashew nuts, pericarp or seed coats of nuts have been scantly reported and no studies have explored the antibacterial activity of cotyledon and callus derived from cotyledon. The antimicrobial properties of *A. occidentale* are derived from the presence of a polyphenol known as anacardic acid and other compounds such as Tatrols and Tanins. [37]

**Estimation of anacardic acid by HPTLC**

Qualitative estimation of anacardic acid is carried out by HPTLC using solvent systems. The mobile phase consisted of chloroform: ethyl acetate (9:1) (v/v). Aluminum-backed TLC plates pre-coated with 0.2 mm layer of silica gel 60 $F_{254}$ (20 cm × 10 cm) was used as stationary phase. The present study revealed that all the plant material showed the presence of anacardic acid. Regression analysis and statistical data were automatically generated by the winCATS software. Calibration curve and HPTLC chromatogram of standard anacardic acid were presented in fig.7&8. HPTLC chromatogram of callus and various plant parts of *A. occidentale* was also obtained (Fig. 9). HPTLC has become a potential tool for identification, authentication and quality control of phytochemicals. [38]

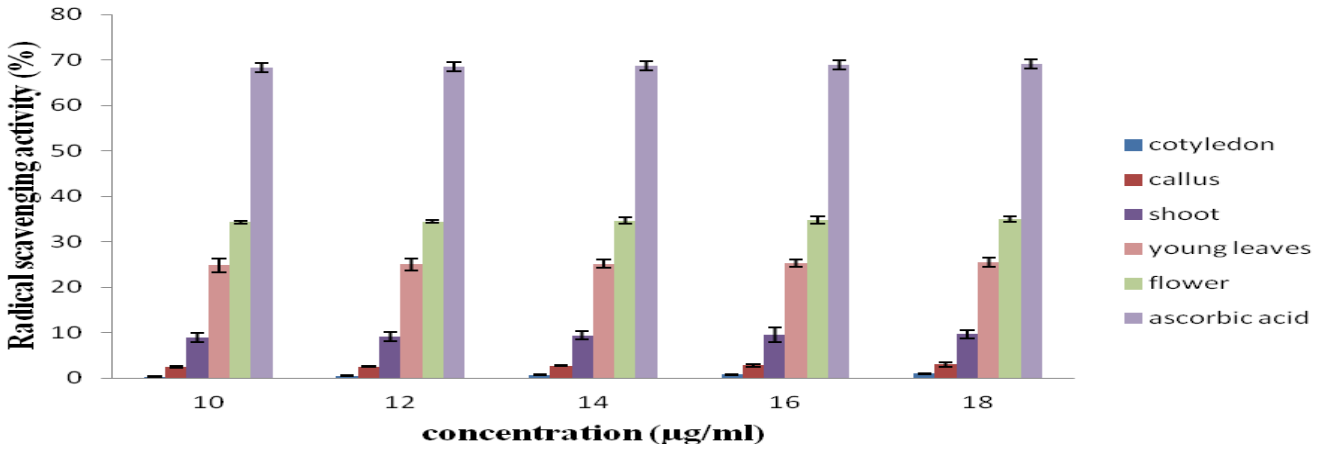


**Fig. 4: Free radical scavenging activities of various extracts of *A.occidentale* measured using the DPPH assay. Ascorbic acid was used as a reference control in this experiment. Each assay was performed in triplicate, error bars represent standard deviation.**


**Fig. 5: showing the zone of inhibition for *Staphylococcus aureus***
**a) Cotyledon b)Young leaves c) Flower d) Shoot e) Callus**


**Fig. 6: showing the zone of inhibition for *E. coli***
**a) Cotyledon b)Young leaves c) Flower d) Shoot e) Callus**

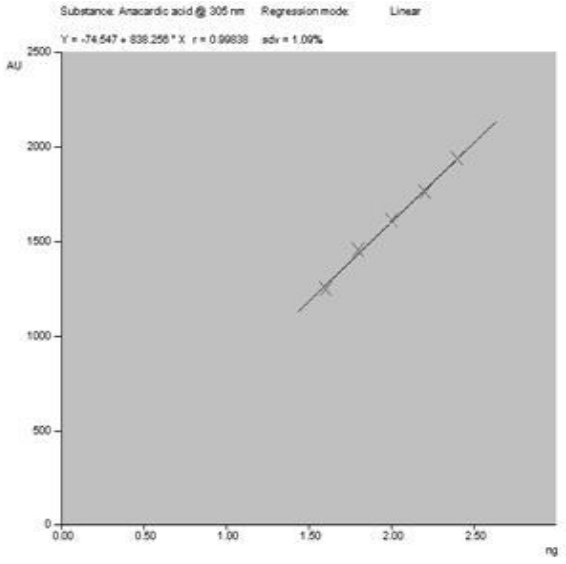


**Fig. 7: Calibration curve of standard anacardic acid**

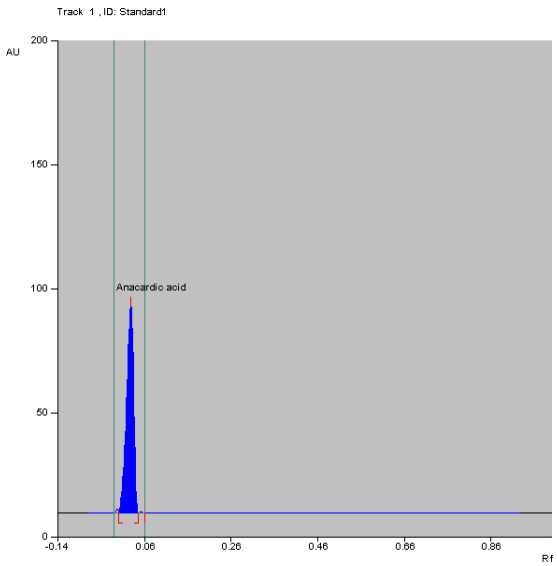


**Fig. 8: HPTLC chromatogram of standard anacardic acid**

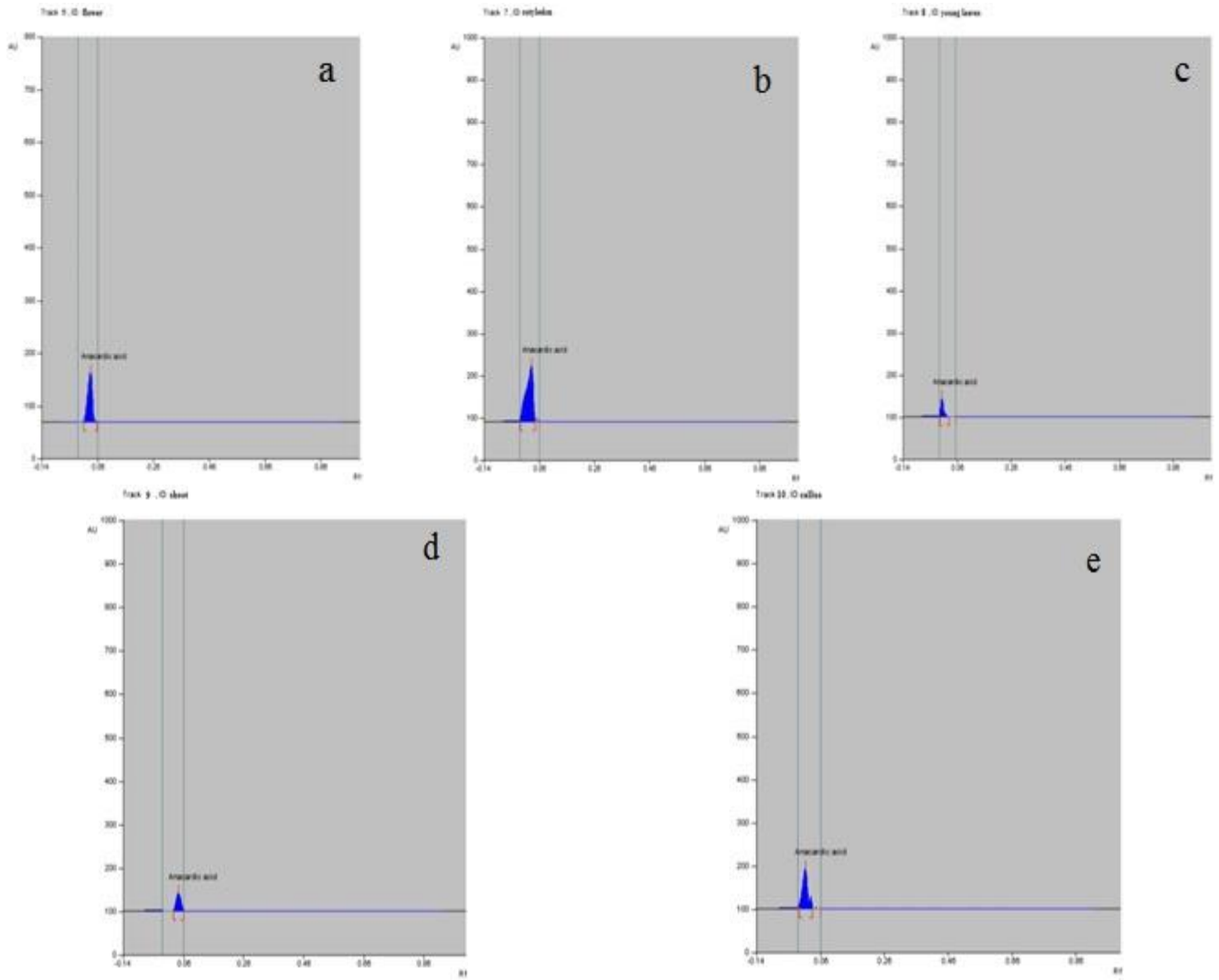


**Fig. 9: Showing the HPTLC chromatogram of a) Flower b) Cotyledon c) Young leaves d) Shoot e) Callus**

The presence of anacardic acid in cashew nut shell liquid, cashew nut and cashew fruit was also reported. [39-41] The presence of anacardic acid in flower, young leaves and *in vitro* resulting callus were seems to be a new report by using HPTLC technique. The study also indicated that the antioxidant activity and antibacterial activity of the plant parts may be due to the presence of anacardic acid in these parts.

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