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RESEARCH ARTICLE

Phytochemical screening and antibacterial activity of leaf extracts of *Anacardium occidentale L.*

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Abstract: The study analyzes the phytochemical constituents and the antibacterial activity of methanol extracts of young and mature leaves of *Anacardium occidentale* L. Phytochemical screening and Total Phenol Content (TPC) of leaf extracts were done using standard protocols. Antibacterial activity was assessed by Disc diffusion method. Minimum Inhibitory Concentration (MIC) was tested by Tube method and Minimum Bactericidal Concentration by agar plate method. The screening tests showed the presence of alkaloids, flavonoids, phenolic compounds and tannins in both young and mature leaves extract. But, saponins and steroidal compounds were present only in young leaf extracts. Further, the total phenolic content of the extracts was estimated and results found that most of the phenolic compounds can be extracted from mature leaves in methanol (246±3.4 mg/g). The antibacterial activity was assessed against Es*cherichia coli, Vibrio cholerae* and *Streptococcus pneumoniae*. The mature leaf (MM) extract showed inhibitory effect against all three test organisms with a zone of inhibition of 10mm (*E. coli)*, 13mm (S. pneumoniae) and 15mm (V. *cholerae*). While young leaf extract (YM) showed inhibition against *E. coli* and V. *cholerae* with a zone of inhibition of 12 and 13mm respectively but was not effective against P. *aeruginosa*. Minimum Inhibitory Concentration (MIC) of both extracts was also evaluated. The MIC against *E. coli* was 187.5ug/ml and 325ug/ml for mature and young leaf extracts respectively. This study compares the phytochemical profile and antibacterial activity of young and mature leaf extracts and reveals that mature leaves are better source of pharmaceuticals. This study sheds an insight into the preferable stage of leaves to be used for pharmaceutical purposes and raw consumption.

Keywords: *Anacardium occidentale L.*, Antibacterial activity, Minimum Bactericidal Concentration, Minimum Inhibitory concentration, Phytochemical screening, Phytochemical screening of *A. occidentale L.*

Introduction

Anacardium occidentale L. is an evergreen tree of Brazilian origin belonging to the family Anacardiaceae, found widespread in the tropical countries around the world (Morton 2003, de Brito *et al*. 2018). The tree species is well-known for its seed, cashew nut. It has a wide range of medicinal uses and is economically important as well. All the parts of the tree is useful in one or the other way; its leaves being used in salads, for treating tooth ache, malaria etc (Ayepola *et al*. 2009). Similarly, the fruit and bark juice are important in treating cough and cold, reduce sugar levels (Jaiswal *et al.* 2017, Sokeng *et al*. 2007) snake bite and so on (Hollands *et al*. 2016). The medicinal value of leaves of *A. occidentale L*. is also reported. The different plant parts have showed antimicrobial activity against a number of pathogenic organisms (Dahake *et al*. 2009, Sija *et al*. 2015).

The presence of secondary metabolites imparts medicinal properties to the tree species. The most important among the secondary metabolites are phenolic acids which includes cardol, cardanol and anacardic acid (Schultz *et al*. 2006). The value of anacardic acid as a medicine is studied in abundance (Chan *et al*. 2017, Hamad and Egid, 2015). It is extracted mainly from cashew nut shell but is present in all parts of the plant. It is an important commercially as well. Anacardic acid is a major component of CNSL (Cashew Nut Shell Liquid) and exhibits anticancerous, antimicrobial activity, acts as an inhibitor of histone acetyl transferase (HATs), lipoxygenase, xanthine oxidase, tyrosinases, ureases and an inhibitor of inflammation (Wu *et al*. 2011, Schultz *et al*. 2017, Cui *et al*. 2008, Sun *et al*. 2006). Apart from phenolic acids, cashew tree is a rich source of

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various other secondary metabolites.

Taking into account the vast uses of *A. occidentale* both commercially and medicinally, the present study makes a comparison of the different secondary metabolites present in young and matures leaves. Also, the antibacterial activity of the extracts against pathogenic bacteria is also studied. Further, quantification of anacardic acid in young and mature leaves extract in methanol is evaluated.

Materials and methods

Plant material

Leaves of *Anacardium occidentale* were collected from a tree (Figure 1) in the premises of Kerala State Cashew Development Corporation Limited (KSCDC), Mundakkal, Kollam during November, 2020. Both mature and young leaves were collected to make a comparative study of the phytochemical constituents, Total phenolic content, antimicrobial activity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Leaves were shade dried and ground to a coarse powder in mixer grinder.

Sample extraction

Young and mature leaf powder (1g) were weighed and 100ml of methanol was added to the powder. The mixture was kept for extraction in shaker for 24hours at 37°C and filtered with Whatman No. 1 filter paper to remove all debris. The filtrate was concentrated by keeping in water bath for an hour. The concentrated filtrate was completely dried in hot air oven at 40°C and dissolved in methanol and stored in refrigerator for further analysis.

Phytochemical screening of secondary metabolites

Tests for alkaloids

Dragendorff's Test

To 2 ml of extract, few drops of dilute hydrochloric acid and 1 ml of Dragendorff's reagent was added and left for few minutes. A positive result is indicated by the presence of an orange red precipitate (Manimekalai and Chitra 2021)

Figure 1. *Anacardium occidentale L.*

Test for steroidal compounds

Salkowski's test

To 1ml of the extract, 2 ml of chloroform and few drops of acetic anhydride were added. To this, equal amount of concentrated sulfuric acid was added. The formation of bluish green color shows a positive result (Manimekalai and Chitra 2021).

Test for phenolic compounds

To 2ml of extract a few drops of freshly prepared ferric chloride solution was added. The formation of a dark blue colour indicated the presence of phenolic compounds (Manimekalai and Chitra 2021).

Test for flavonoids

Reaction with sodium hydroxide

1ml of dilute sodium hydroxide (10%) solution was added to 3ml of extract. The mixture was checked for production of yellow color which indicates presence of flavonoids (Tafinta *et al*. 2020)

Test for Saponins

Froth test

0.5g of each extract was dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 20seconds.It was then allowed to stand for 20minutes. Honeycomb froth above the surface that stays after 20minutes is taken as positive result (Manimekalai and Chitra 2021).

Test for tannins

Lead Acetate Test

5ml of each types of extract and few drops of freshly prepared 1% lead acetate were mixed together. Yellow precipitate shows a positive result (Saha *et al*. 2020)

Estimation of Total Phenolic Content

Total phenolic content of the methanol extracts of young and mature leaves of *Anacardium occidentale* was determined using the Folin-Ciocalteau reagent method (Manimekalai and Chitra 2021). To the extract, 2.5ml of Folin-Ciocalteau reagent (Sigma Aldrich) and 2 ml of freshly prepared 7.5% Na_2CO_3 solution was added. The mixture was incubated at 45°C for 15 minutes. The Optical Density (OD) was measured at 765nm. The reaction mixture without extract was taken as blank. Gallic acid was used as standard.

Antimicrobial Screening against Pathogenic microbes

Antimicrobial activity of leaf extracts of *Anacardium occidentale* against three pathogenic microorganisms was evaluated by disc diffusion method. The activity was tested against *Escherichia coli* (MTCC 443), *Streptococcus pneumoniae* (MTCC 1936) and *Vibrio cholera* (MTCC 3906). Muller Hinton Agar (MHA) (38g/L) was used for testing antibacterial activity. To the sterile MHA plates, each of the pathogenic organisms to be tested was spread plated using sterile cotton swab. Ampicillin discs (25mcg) were placed on the spread plate agar. Sterile discs (Hi MEDIA) were placed on the agar and 100µL of each extracts were added drop by drop, soaking the sterile discs. The plates were incubated at room temperature in a bacteriological incubator for 24hrs. The plates were evaluated for antimicrobial activity against the organisms. The clearance zone or zone of inhibition (ZI) was measured. Activity index (AI) was calculated using the formula given below.

$$AI = \frac{\text{ZI of extract}}{\text{ZI of antibiotic}}$$

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory Concentration (MIC) is the lowest concentration of extract that can inhibit the growth of microorganisms. MIC against *E. coli* was evaluated. MIC was determined by broth dilution method (Jahan *et al*., 2011). The antimicrobial agents (methanol extracts of young and mature leaves) were added in different concentrations to test tubes containing nutrient broth (13g/L). The tubes were inoculated with *E. coli* culture and incubated for 24 hours at room temperature. Test tubes without the extracts were considered as control. MBC determines the least concentration of the antimicrobial agent that could cause microbial death. It was analyzed by plating the culture broth which showed inhibition of microbial growth in MIC determination on nutrient agar. The concentration at which no colony formation occurs was considered to be MBC.

Results

Phytochemical Screening

Phytochemicals are also considered therapeutic against various diseases when ingested. They have antioxidant, antimicrobial activity and

Table 1: Phytochemical compounds in mature and young leaf extracts

Phytochemical compounds	Young leaf extracts	Mature leaf extracts
Alkaloids	+	+
Steroidal compounds	+	-
Flavonoids	+	+
Phenolic compounds	+	+
Saponins	+	-
Tannins	+	+

Note: '+' indicates the presence and **'-'** indicates the absence of phytochemical compounds in the leaf extracts.

Table 2: Antimicrobial activity (Zone of inhibition in mm) against pathogenic microbes

Bacteria	YM	MM	Standard (Ampicillin)
E. coli	10	12	22
S. pneumoniae	-	13	16
V. cholerae	13	15	20

Note: '-' indicates the absence of inhibition zone; ZI – Zone of Inhibition

are effective against a wide range of diseases in humans like cancer, diabetes, heart related diseases, liver damage, osteoporosis etc (Thakur *et al*. 2020). In ancient medicinal history, plant parts have been widely used as medicines and consumed raw. The phytochemical compounds present in young and mature leaf extracts are shown in Table 1**.** The phytochemical composition of leaf extracts of *A. occidentale* imparts protection to the plant against microbial attack, stress tolerance, infections by pests and the diseases affecting humans. Table 1. illustrates the comparison of phytochemicals present in young and mature leaves. The results obtained from screening tests revealed the presence of alkaloids, steroids. Flavonoids, phenolic compounds, saponins and tannins in young leaf extract whereas saponins and steroidal compounds were absent in mature leaf extract.

Estimation of total phenolic content

The Total Phenolic Content (TPC) was decided using Folin-Ciocalteu method. The results showed that the phenol content in both extracts

Figure 2: Total Phenolic Content of methanol extracts of young and mature leaves with a level of significance $p<0.01$ (n=3)

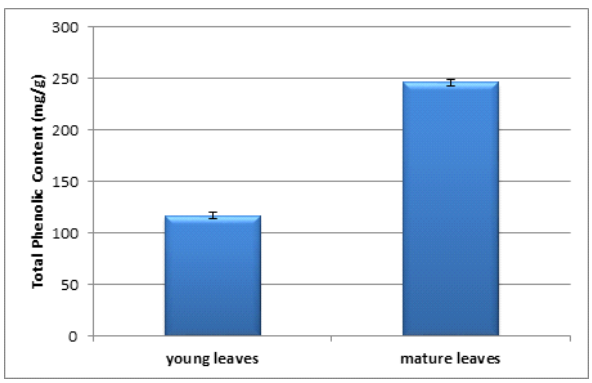


Table 3: MIC and MBC values of young and mature leaf extracts

Extract	MIC (µg/ml)	MBC (µg/ml)
Young leaves	325	500
Mature leaves	187.5	250

varied significantly ($p<0.01$). The amount of phenolic content was found to be 246±3.4 mg/g and 117±4.4 mg/g (expressed in Gallic Acid Equivalence) in mature and young leaf extracts respectively (Figure 2). A standard curve of gallic acid is shown in Figure 3. The presence of phenolic acids confer the antimicrobial activity of leaf extracts of *A. occidentale* (Cowan 1999).

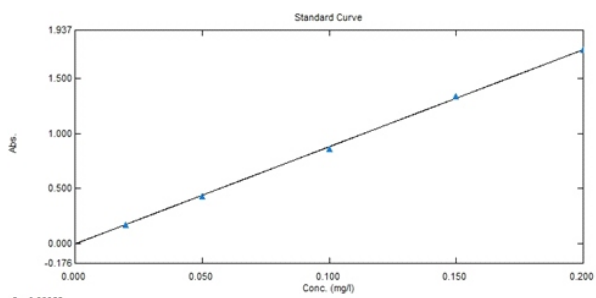
Antimicrobial activity

The antimicrobial activity of methanol extracts of young and mature leaves were studied by disc diffusion method using sterile ampicillin disc as positive control. Mature leaf extract showed activity against *E. coli, S. pneumoniae and V. cholerae* while young leaf extract showed activity against *E. coli and V. cholera* as shown in Figure 4. The Zone of Inhibition (ZI) values are given in Table 2. The Activity index (AI) is also reported as given in **Figure 5**.

Determination of MIC and MBC

The activity of the antimicrobial agents (MM and YM) can be best determined by finding out its MIC and MBC. Though MIC determines the least concentration that inhibits the growth of the test organism, plating of the broth onto agar plates might result in colony formation of the organism.

Figure 3: Standard curve of Gallic acid



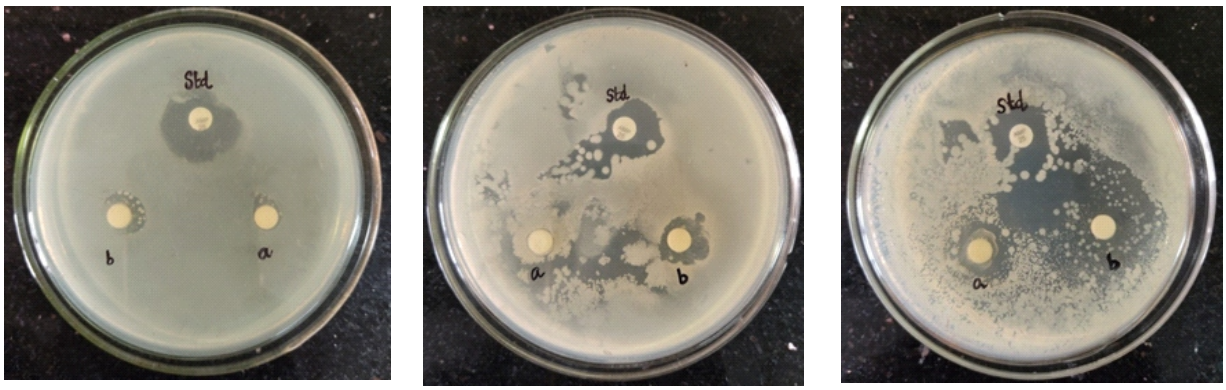


Figure 4. Antimicrobial activity of YLE and MLE against *E. coli, S. pneumoniae* and *V. cholerae*
Note: Std – Standard (Ampicillin); a – Young leaf Extract (YLE); b – Mature leaf extract (MLE)

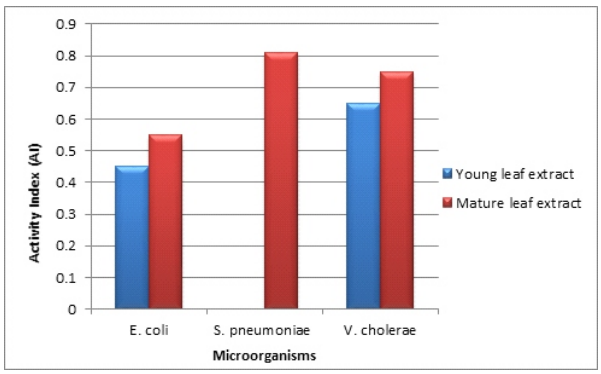


Figure 5: Activity Index (AI) of methanol extracts of young and mature leaves against different microorganisms

Thus, finding out the concentration of the antimicrobial that causes death of the test organism is necessary which is determined by MBC. In this study, MIC and MBC was measured against *E. coli* (test organism) by broth dilution method. The methanol extract of mature leaves showed the least MIC against the test organism. The value was found to be 187.5µg/ml. While methanol extracts of young leaves inhibited the growth of test organism at an MIC of 325µg/ml. From the above results it is clear the mature leaves of *A. occidentale* are better antimicrobial agents. MBC was found to be 500 and 250 µg/ml for young and mature leaf extract respectively (Table 3).

Discussion and conclusion

Phytochemicals are gaining importance in this era where human beings are often affected by numerous diseases. Now research is focused on its health benefits for humans. These compounds are also known for its ability to protect plants against microbial attack and defense against disease causing pests. In this study, a comparison of the phytochemical and antibacterial potential of young and mature leaves of *A. occidentale* was carried out. From the results, it is clear that mature leaves are better protective and medicinal agents compared to young leaves. This may be attributed to the high level of phenol content in mature leaf extracts. The presence of secondary metabolites was confirmed in ethanol, methanol, ethyl acetate, chloroform and hexane extracts of leaves and bark (Abubakar and Rabiatu 2016). Alkaloids, steroids, tannins, flavonoids and glycosides were present in all the extracts. The presence of saponins in leaf extract was also revealed (Tafinta *et al.* 2020). In this study it clearly indicates the absence of saponins in mature leaf extract. The estimation of total phenol content of both extracts revealed that higher amount of polyphenols is present in mature leaf extract. In a study, the total phenol content of stem bark, leaves and cashew nut was determined and found that phenol content in leaves was 620.71 ± 7.28 mg/100g dry mass. Stem bark had higher and cashew nut had lower phenol content compared to leaves (Madjitoloum *et al*. 2018). In another study, TPC of chloroform extract of leaves of *A. occidentale* was found to be 564.60mg GAE (Gallic Acid Equivalence)/g dry extract (Chotphruethipong *et al*. 2017). Antibacterial activity of cashew leaf extract against *E. coli* and *Staphylococcus aureus* was reported (Liangpanth and Wirongrong 2018). 0.5% methanol extract had no effect against *E. coli* and exhibited a zone of inhibition of 9.5±0.7mm against *S. aureus*. While 1% methanol extract was effective against both the test organisms. It was also reported that methanol

extract of young leaves of cashew had greater inhibitory effect against *E. coli* and *S. aureus* than cotyledon, flower, shoot and callus (Sija *et al*. 2015). The efficiency of antibacterial activity of ethanol and acetyl acetate extract of leaves of *A. occidentale* was studied (Chabi *et al*. 2014). The MIC values of ethanol extract of leaves against reference organisms ranged from 0.039-0.625 mg/ml and that of acetyl acetate leaf extracts ranged from 0.313 - 0.625mg/ml. The MBC values ranged from 0.313 -20mg/ml for ethanol leaf extract and 0.625-10mg/ml for acetyl acetate leaf extract. Another study on antibacterial activity of cashew leaves showed that the extract was effective against bacterial and fungal cultures (Onuh *et al*. 2017). Methanolic leaf extract of *A. occidentale* reported the highest zone of inhibition of 32.67±0.67 mm for *S. aureus* (Omotayo *et al*. 2020). MIC against *E. coli* was higher (32mg/ml) compared to the result obtained in this work. MIC results reported in a study showed that cashew leaf extract in 70% methanol was 0.353mg/ml against *E. coli* (Jinah *et al*. 2020) which is similar to the MIC of young leaves obtained in this study. The MBC value according to their study was 2.821mg/ml. Antibacterial activity of ethanol and aqueous leaf extracts of cashew was studied (Liangpanth and Wirongrong 2018). Their results revealed an MIC of 0.0625g/l against *E. coli* and *S. aureus* and 0.125g/l against *Klebsiella pneumoniae*. MBC according to them was 0.25g/l and above.

Recently many research works have been carried out evaluating the phytochemical profile and antibacterial activity of medicinally valued plants namely *Azadirachta indica (*Hikaambo *et al*. 2022), *Ocimum basilicum* (Sharaf *et al*. 2022), *Rubus hyrcanus Juz.* (Yousefbeyk *et al*. 2022), *Ricinus communis L.* (Rahman *et al*. 2022), *Adansonia digitata* (Bashir *et al*. 2022). But no such works have been reported recently in *Anacardium occidentale L.* Further, the antimicrobial activity of the extract makes it a suitable material for developing bio-based antimicrobial drug.

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