# Selective Antimicrobial properties of *Phyllanthus acidus leaf* extract against *Candida albicans, Escherichia coli* and *Staphylococcus aureus* using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method

R.C. Jagessar*[a], A.Mars[a], G. Gomes[b]

*[a]Lecturer and Supervisor, Department of Chemistry, University of Guyana, Turkeyen campus, Faculty of Natural Sciences, South America.

[b]Microbiologist, Department of Biology, Faculty of Natural Sciences, John's Campus, University of Guyana, South America.

[a]Final Year Research student, Department of Biology, University of Guyana.

raymondjagessar@yahoo.com

---

ABSTRACT:

*The antibacterial and antifungal activities of Phyllanthus acidus was investigated against S.aureus (gram+ve), E.coli (gram-ve) and C.albicans using the Stokes disc diffusion, the Pour plate, Well diffusion and Streak plate methods. The solvent type extracts were obtained by three extractions with hexane, $CH_2Cl_2$, EtOAc and $CH_3CH_2OH$ respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.035g in 0.01L(10 mL)of the respective solvents. These were tested in varying volumes of 0.2-0.6 ml/plate. The solvents were used as control whereas ampicillin and nystatin were used as references for bacteria and fungal species respectively. The solvents had no effect on the microorganisms whereas ampicillin and nystatin inhibited microbial growth. Phyllanthus acidus showed antimicrobial inhibitory activity at 0.18mg/10mL plate of medium with activity most prominent with the ethanol extracts and negligible with the hexane. This study suggests that the ethanol extracts of Phyllanthus acidus ,can be used as herbal medicines in the control of E.coli and S.aureus following clinical trials.* [Nature and Science. 2008;6(2):24-38]. ISSN: 1545-0740.

**Keywords**: Antimicrobial; *Phyllanthus acidus; S.aureus; E.Coli*; *C.albicans*; Stokes Disc diffusion; Well diffusion; Streak plate; dilution method; herbal medicines.

## 1.0. Introduction:

This paper discusses the antimicrobiological (antibacterial and antifungal) activity of leaves of *Phyllanthus acidus* also known as *gooseberry* from the coastal plain of the Guyana flora and its possible use as an herbal cream/herbal medicine. Its antimicrobial properties were investigated against *S.aureus* (gram+ve), *E.coli* (gram-ve) and *C.albicans* strains using the Stokes disc diffusion sensitivity technique, Well diffusion, Streak plate and a dilution method. An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity) or parasites (antiparasitic activity).

Guyana has a rich biodiversified flora whose crude extracts, both organic and aqueous can be investigated for their antimicrobial activity. In addition, their role as global $CO_2$ sinks (in the context of global warming) is noted. Also, the extracts of the specified plants parts of the same species, fractionated for natural products whose antimicrobial activity can also be correlated with that of crude extracts. Following this, clinical trials can lead to the formulation of an herbal plant cream or herbal medicine. A few herbal medicine shops have now been established in Guyana. Plants extracts and fractionated plant extracts have been used for their antimicrobial properties[1-15]. Besides used as an herbal cream, following clinical trials, crude plant extracts can be chromatographed leading to the isolation and purification of new and known bioactive natural products/phytochemicals, whose medicinal activity can also be investigated.

For example, two new *ent*-trachylobanes (1) and (2) were isolated from *Xylopia langsdorffiana* and their *in vitro* cytotoxicity assay investigated against a permanent lung fibroblast cell line derived from Chinese hamsters (V79) and rat hepatocytes using MTT[15-16] method and gave $IC_{50}$ values of 224 and 231 uM respectively.

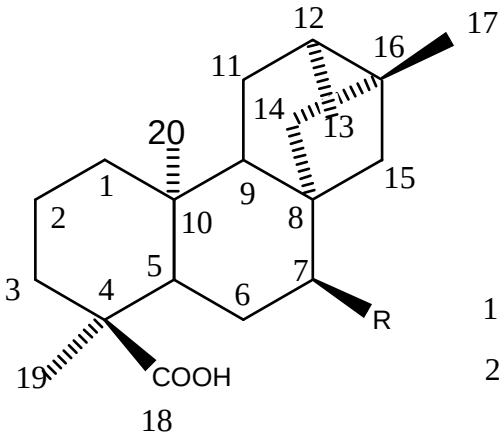



Fig. 1.0. Structure of *en*trachylobanes (1) and (2).

There is an urgent need to revolutionalsed and intensify research in herbal medicine and isolated drug discovery, considering the threat to mankind of incurable diseases as HIV AIDS and other new emerging disease such as SARS, bird flu (H5N1) etc. Plants are a good source of herbal medicine and natural products/ phytochemicals[1-17] . Thus, research in herbal medicine needs to be intensified. Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from isolated natural products from plants rather than to the creativity and imagination of contemporary organic chemists. For example, the drug taxol, paclitaxel, one of the most powerful anticancer drug known , first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer[6 14.] Paclitaxel is a mitotic inhibitor used in cancer chemotherapy.

.

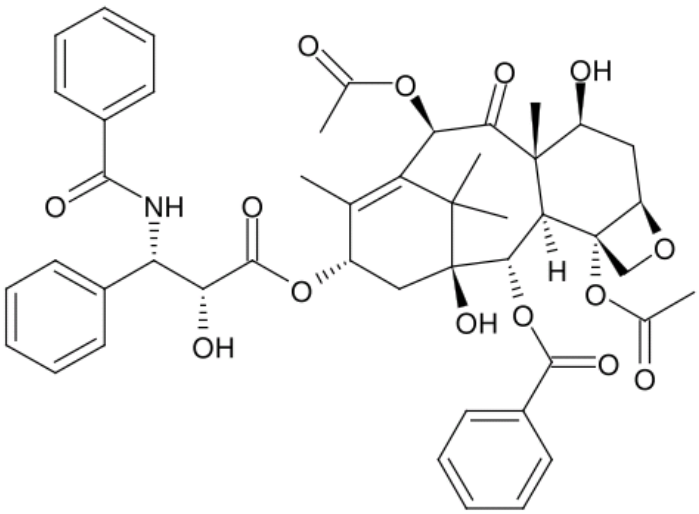



Fig 2.0. Paclitaxel, “Taxol” (3)

In Guyana, there are many medicinal folklore practises but most are without scientific research. Its our scientific endeavour, to correlate antimicrobial activity of *Phyllanthus acidus* with its folklore practices. In Guyana's traditional medicine, an infusion of the herb is taken for the relief of dysentery and also as a blood purifier (bitter tonic to reduce blood sugar level). Also, an infusion or tea for women who are dieting and wish to remain slim. However, little is known of the antimicrobial properties of *Phyllanthus acidus.* As part of a project to investigate extracts and chromatographic fractions from plants of the Guyana's flora[7-15, 17] for antimicrobial activity, we report here, the antimicrobial properties of *Phyllanthus acidus*.

*Phyllanthus* is the largest genus in the family *Phyllantheae*. *Phyllanthus* has a remarkable diversity of growth forms including annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous. *Phyllanthus acidus* is an annual erect little branched herb, 10-50 cm high [18-20]. Its completely green including the flowers. Leaves are simple, oblong, acute or obtuse, slightly oblique to 14 mm long and 6 mm broad and bear the inconspicuous flowers in pairs in their axils. Each pair of flowers comprises one male and one female. The capsule is a flattened globose about 2 mm in diameter. The classification of the plant is given in Table 1.0:

Table 1.0 Classification of *Phyllanthus acidus*.

| | |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Tracheobionta |
| Superdivision | Spermatophyta |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Subclass | Rosidae |
| Order | Euphorbiales |
| Family | Euphorbiaceae |
| Genus | *Phyllanthus* |
| Species | *Phyllanthus acidus* |

The microbes studied are *Eschericia coli, Staphylococcus aureus and Candida albicans. Escherica. coli* can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia[21]. *Staphylococcus aureus* can cause furuncles (boils), carbuncles (a collection of furuncles)[22]. In infants, *Staphylococcus aureus* can cause a severe disease Staphylococcal scalded skin syndrome (SSSS). Staphylococcal *endocarditis* (infection of the heart valves) and pneumonia may be fatal. *Candida Albicans* is a diploid fungus (a form of yeast) and is a casual agent of opportunistic oral and genital infections in humans[23-24].

**2.0. Procedure**:

**2.1**: **Collection of Plant materials**: The leaves of the above plant was collected from the University of Guyana. The detached plant leaves were subjected to aerial drying for three weeks, removed and placed in separate conical flasks. It was then extracted with the required solvents.

**2.2 Extraction:** Using selective solvent extraction**,** the leaves were first extracted thrice in hexane over a period of five days[1-13].Water was removed from the accumulated extract by stirring over anhydrous $Na_2SO_4$ and extract filtered. Solvents were removed in *vacuo* using a rotor vapor. The extracts was placed in sample vials and allow to evaporate. Further drying was done in a dessicator to remove residual solvents. Extracts were stored in capped vials and were weighed. The above procedure was repeated with the same leaves but with different solvents of increasing polarity: $CH_2Cl_2$, EtOAc, and then $CH_3CH_2OH$. At the end of drying process, plant extract was either viscous oils, solid or paste.

**2.3. Antimicrobial activity tests**

**2.3.1. Making up extract solution**

Approximately 0.035g of dried crude extract of *Phyllanthus acidus* was weighed and transferred to a 10 ml volumetric flask. The respective solvent was then added to make up the 10 ml solution (0.035g in 0.01L).

**2.3.2. Microorganisms:**

Micro organisms: *Staphylococcus aureus, Escherichia coli and Candida albicans* were obtained from the Georgetown Public Hospital (GPH) microbiology laboratory and was stored in a refrigerator at the Food and Drug microbiology lab.

**2.3.3. Agar Preparation**:

Two types of agar were used, nutrient agar to make up the medium for bacteria and PDA (Potato Dextrose Agar) to make up the medium for fungi.

**2.3.4. Potato dextrose agar (PDA)** [25]

The potato *was peeled* and 100g was measured, finely chopped and boiled to a mash in distilled water.The dextrose was measured (12.5g) and placed in a 1L measuring cylinder. Agar was measured (12.5g) and added to the measuring cylinder (with the dextrose).The potato mash was stirred and strained into the cylinder. Hot distilled water was added to make up 500mL.The contents was continuously poured and stirred until consistency was achieved. The content was then poured into a conical flask, plugged with cotton wool, over which aluminium foil was tightly wrapped. The flask was then autoclaved at 121 $^0$C for 24hrs. The pH range was between 6.5-7.0.

**2.3. 5. Reference and Control**:

The references were antibiotic in nature. *Ampicillin* and *Nyastatin*. *Ampicillin* was choosen as the reference for all bacterial species used: *E.Coli* and *S.aureus*. *Nyastatin* was used as the reference for the fungus, *Candida.albicans.* The Control experiment consists of a plate of solidifying agar onto which was inoculated pure solvent with microorganism mixed in a 1:1 portion [25].

**2.3.6. Aseptic conditions:**

The aseptic chamber which consists of a wooden box (1m x 1m x 0.5m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from a lamp).

**2.3.7. Mother plates:**

These were made by culturing *C.albicans* on PDA. A sterilized 6 mm cork borer was used to cut agar discs in the plate.

**2.3. 8. Nutrient Agar:**

Nutrient agar was purchased from the International Pharmacy Association in Guyana. 14g of nutrient agar was suspended in 500ml of distilled water in a 1L flask, stirred, boiled to dissolve and then autoclaved for 15 minutes at 121$^0$C. The pH range was between 7.0-8.0. The plates were poured in a sterile environment and allowed to cool for 2 hours. Under aseptic conditions, the micro organisms were streaked onto separate

plates and the discs were applied with a forceps.They were labeled and placed in an incubator at 37 $^0$C for 24 and 48 hours for bacteria and fungi respectively.

**2.3.9. Disc diffusion:** Stokes Disc diffusion sensitivity technique [25].

Using Stokes Disc diffusion sensitivity testing technique[25], an inoculum containing bacterial or yeast cells was applied onto nutrient agar plates. On each plate, a reference antibiotic was also applied.The reference antibiotic disc contained 200mg of antibiotic/ml. The discs were made by cutting discs (5-6mm) from a filter paper with a perforator, placing 5 of these discs in a vial and adding 0.2mL of each extract solution. These were left to dry. Discs were also made for the controls: ampicillin for the bacteria and nystatin for the fungus. Each disc was impregnated with the anticipated antimicrobial plant extract at appropriate concentration of 200 mg/ml using a microlitre syringe. This was then placed on a plate of sensitivity testing nutrient agar which was then incubated with the test organism:Bacteria/fungi. Incubation was done at 37ºC for 24 hr and 48 hr for the bacteria and *Candida albicans* species respectively. The antimicrobial compound diffuses from the disc into the medium. Following overnight incubation, the culture was examined for areas of no growth around the disc (zone of inhibition). The radius of the inhibition zone was measured from the edge of the disc to the edge of the zone.The end point of inhibition is where growth starts. Larger the inhibition zone diameter, greater is the antimicrobial activities. It is anticipated through the antimicrobial activity of plant extract, no area of growth will be induced around the disc. Bacteria or fungal strains sensitive to the antimicrobial are inhibited at a distance from the disc whereas resistant strains grow up to the edge of the disc. Discs applied to the plates already streaked with bacteria and the fungus.

**2.3.10. Well Diffusion Plate Method Diffusion plate (Well diffusion)[25]:**

A fungus (*Candida albicans*) was inoculated into a test tube containing three ml of distilled water (medium), using a flamed loop. Drops of fungus/water culture was mixed with the warm, melted, autoclaved PDA and poured into separate plates under aseptic conditions.The plates were covered and allowed to cool. As soon as the agar was partly solidified, the plates were inverted and left for 2h. When cooled, a well was made at the centre of the plate. The well was made by using a 6 mm cork borer or puncher that was sterilized with alcohol and flame. Plant extracts dissolve in solvent at final concentration of 0.035g/0.01L was pipette into the different wells in a sterilized environment at different volumes (0.2-0.4-0.6ml) in separate plates, using a micro liter syringe. The four solvents (hexane, $CH_2Cl_2$, EtOAc and $CH_3CH_2OH$) at different volumes were used as control whereas nystatin dissolved in dichloromethane at same concentration with plant extract (0.035g/0.01L) at different volumes (0.2-0.4-0.6ml) was used as the reference.The plates were labelled, covered, inverted and placed in a fume hood (no incubator was available) for 48h.

**2.3.11. Streak Plate Method:**

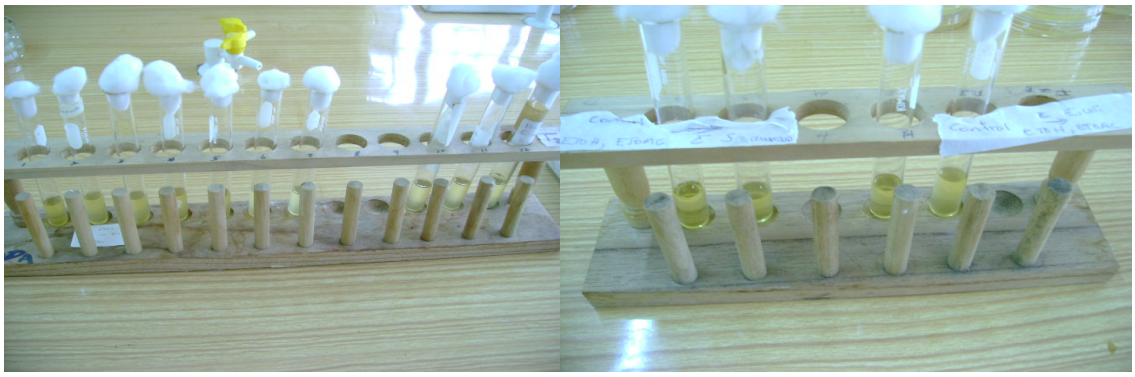
Nutrient agar was prepared as described above and 10 ml was poured into plates. Plant extracts dissolved in solvent at a final concentration of 0.035g/0.01L were pipette into three sterilized plates under asceptic conditions at different volumes (0.2-0.4-0.6 ml), using a micropipette. The plates were allowed to cool and then the bacteria were streaked onto the surface of the solidified agar/plant extract medium. A flame loop was used to inoculate the bacteria from their cultures These plates were left for 24 hours in a dessicator.The plates with inhibition were used in further experiments. A reference experiment was setup using an antibiotic (ampicillin capsule) at the same concentration as plant extracts (0.035g/0.01L) at different volumes (0.2-0.4-0.6ml). Controls were also setup using solvents: hexane, $CH_2Cl_2$ and EtOAc at the different volumes.

**2.3.12. LB (*Luria-Bertani)* broth :**

*Luria –Bertani* broth (LB broth) is a rich medium used to culture bacteria such as *E.Coli and S.aureus.* To make it, tryptone (10g), yeast extract (5g) and sodium chloride (10g) were measured and placed in a 1L cylinder. Distilled water was added to make up the 1L solution and the mixture was poured and re-poured until the contents were dissolved. The pH of the solution was adjusted to 7.4 using sodium hydroxide. 3mL each of LB broth was placed in 56 test tubes. The tubes were plugged with cotton wool foil and wrapped over each top. The tubes were placed into a beaker and autoclaved at 121 $^0$C for 2h. These tubes were used in the dilutions experiments.

**2.3.13. Dilution Method:**

This method was used to test the plant extracts for antimicrobial activities against bacteria by investigating whether there was turbity or not. Turbidity represents microbial growth, while no turbidity represents inhibition of microbes. One set of tubes containing LB (*Louria Bertinieia*) broth was inoculated with *Staphylococcus aureus* and the second set was inoculated with *Escherichia coli* using a loop, flame and alcohol. Under aseptic conditions, the plant extracts (dissolved in solvent at concentration 0.035g/0.01L) and which showed inhibition in the streak plate were added to the one set of test tubes containing *E. Coli* and the other set, *S.aureus* with LB broth(medium) in differing volumes (0.2-0.4-0.6ml). Two sets of four tubes each were treated with the four solvents (hexane, $CH_2Cl_2$, EtOAc and $CH_3CH_2OH$). One set was inoculated with *S.aureus* and the other with *E.coli,* Fig 3.0. Cotton wool was used to plug test tubes. The tubes were observed after 24 hrs.



(a) Actual experiment (b) Control Experiment

Fig. 3.0. Dilution tubes after 24 h: (a) *Phyllanthus acidus* extract with *S.aureus & E.coli in* LB broth *(b) Control solvent added to LB broth.*

**2.3.14. Retention Factor**: $R_f$ = Distance moved by sample

Distance moved by solvent front.

In general, the most polar compound has the lowest $R_f$ value.

**2.3.15. Thin Layer Chromatography (TLC):**

A baseline was drawn on the TLC plate. A spot of the plant extract was placed on the baseline with use of the pipette and allowed to dry. The plate was placed in the developing jar with the solvent. When taken out of the jar, the solvent front was drawn. The plates were then held in the iodine jar for a few seconds, shaken and taken out. They were examined under the UV/Vis lamp and the spots were circled with a pencil. The plate was further examined under UV lamp and any new spots were marked. The spots were labelled and their distances from the baseline were measured. The distance between the baseline and the solvent front was also measured. The $R_f$ values were calculated.

**2.3.16. Results**:

Mass of dried leaves used for *Phyllanthus acidus species was* 8.55g respectively. These extracts were in the concentration of 0.035g in 10ml of solvent except for *Phyllanthus acidus* with ethanol which was 0.5g in 25ml. This works out to 0.0003mg/uL and 0.02mg/uL of crude extract respectively. The ampicillin and nystatin controls were in concentration of 250mg in 10ml.

**Disc diffusion:**

Table 2.0. Antimicrobial activity of Plant extract as shown by the inhibition zone diameter**.**

| **Area of inhibition. ($mm^2$) using** *E.Coli* | **Area of inhibition. ($mm^2$) using** *S.aureus* | **Area of inhibition. ($mm^2$) using** *Candida albicans* | **Plant Extracts** *Phyllanthus acidus* | **Reference compound (Ampicillin) ($mm^2$)** | **Control Experiment** |
|---|---|---|---|---|---|
| | | | | | No zone of inhibition |
| **< 5** | **< 5** | **< 5** | Hexane extract | **27** | No zone of inhibition |
| **< 5** | **< 5** | **< 5** | $CH_2Cl_2$ extract | **28** | No zone of inhibition |
| **20** | **15** | **18** | EtOAc extract | **28** | No zone of inhibition |
| **22** | **21** | **20** | $CH_3CH_2OH$ extract | **30** | No zone of inhibition |

Table 3.0. Results of Well diffusion method for plant extracts *Phyllanthus acidus* against *C.albicans.*

| **Extract** | **Volume of Extract (mL)** | | **Diameter of Zone of Inhibition ($mm^2$)** |
|---|---|---|---|
| *Phyllanthus acidus* with Hexane | 0.2 | No zones of inhibition visible, scattered colonies. | **-** |
| | 0.4 | " " | **-** |
| | 0.6 | " " | **-** |
| *Phyllanthus acidus* with $CH_2Cl_2$ | 0.2 | Zones of inhibition visible. | **67** |
| | 0.4 | " " | **79** |
| | 0.6 | " " | **79** |
| *Phyllanthus acidus* with EtOAc | 0.2 | Zones of inhibition visible. | **-** |
| | 0.4 | " " | **69.5** |
| | 0.6 | | **79** |
| *Phyllanthus acidus* with $CH_3CH_2OH$ | 0.2 | | **75** |
| | 0.4 | | **80** |
| | 0.6 | | **84** |
| **Reference(Nystatin)** | 0.2 | Zones of Inhibition visible | **50** |
| | 0.4 | " " | **51** |
| | 0.6 | " " | **51** |

| | | | |
|---|---|---|---|
| **Controls** ($CH_2Cl_2$, EtOAc, $CH_3CH_2OH$) | 0.2 | No zone of inhibition | - |
| | 0.4 | No zone of inhibition | - |
| | 0.6 | No zone of inhibition | - |
| | | | |

**Streak Plate Method:**

Table 4.0. Results obtained from Streak plate method for the bacteria's *Escheria coli* and *Staphylococcus aureus* against different volumes of dissolved plant extracts at a final concentration of 0.035g/0.01 L and controls. Inhibition or no growth of microbes were represented by a positive sign (+), while the negative sign (-) represents no inhibition or growth of microbes**.**

| Plant extract dissolved in solvent | Volume of dissolved plant extract used in (ml) at concentration 0.035g/0.01L | Inhibition or no growth of microbe, *Escherichia coli* | Inhibition or no growth of microbe *Staphylococus aureus* |
|---|---|---|---|
| *Phyllanthus acidus* dissolved inn hexane | **0.2** | - | - |
| | **0.4** | - | - |
| | **0.6** | - | - |
| *Phyllanthus acidus* dissolved in $CH_2Cl_2$ | **0.2** | - | + |
| | **0.4** | - | + |
| | **0.6** | - | + |
| *Phyllanthus acidus* dissolved in Et(OAc) | **0.2** | + | - |
| | **0.4** | + | - |
| | **0.6** | + | - |
| *Phyllanthus acidus* dissolved in $CH_3CH_2OH$ | **0.2** | + | + |
| | **0.4** | + | + |
| | **0.6** | + | + |
| **Reference**(Ampicillin with same concentration as dissolved plant extracts(0.035g/0.01L). | | | |
| | **0.2** | + | + |
| | **0.4** | + | + |
| | **0.6** | + | + |
| Hexane | **0.2** | - | - |
| | **0.4** | - | - |
| | **0.6** | - | - |
| $CH_2Cl_2$ | **0.2** | - | - |
| | **0.4** | - | - |
| | **0.6** | - | - |
| EtOAc | **0.2** | - | - |
| | **0.4** | - | - |
| | **0.6** | - | - |

**Dilution Method:**

$T_0$ = No Turbidity = Inhibition
$T_1$ = Lightly Turbid = Moderately Inhibited
$T_2$ = Moderately Turbid = Lightly Inhibited
$T_3$ = Very Turbid = No Inhibition

Table 5.0. Table showing degree of turbidity of dissolved *Phyllanthus acidus* extracts at a concentration of 0.035g/0.01L at different volumes against *Escheria coli* microbe.

| Plant extract dissolved in solvents at concentration of 0.035g/0.01L | Volume of dissolved plant extract (ml) 0.2 ml | Volume of dissolved plant extract (ml) 0.4ml | Volume of dissolved plant extract (ml) 0.6 ml |
|---|---|---|---|
| *Phyllanthus acidus* with hexane | **$T_3$** | **$T_3$** | **$T_3$** |
| *Phyllanthus acidus* with $CH_2Cl_2$ | **$T_3$** | **$T_3$** | **$T_3$** |
| *Phyllanthus acidus* with EtOAc | **$T_2$** | **$T_1$** | **$T_o$** |
| *Phyllanthus acidus* with $CH_3CH_2OH$ | **$T_0$** | **$T_0$** | **$T_0$** |

Table 6.0. Table showing the degree of turbity of dissolved plant extract at concentration 0.035g/0.1 L at different volumes against *Staphylococcus aureus* microbe.

| Plant extract dissolved in solvents at concentration of 0.035g/0.01L | Volume of dissolved plant extract (ml) 0.2 ml | Volume of dissolved plant extract (ml) 0.4ml | Volume of dissolved plant extract (ml) 0.6 ml |
|---|---|---|---|
| *Phyllanthus acidus* with hexane | **$T_3$** | **$T_3$** | **$T_3$** |
| *Phyllanthus acidus* with $CH_2Cl_2$ | **$T_0$** | **$T_0$** | **$T_0$** |
| *Phyllanthus acidus* with EtOAc | **$T_0$** | **$T_0$** | **$T_0$** |
| *Phyllanthus acidus* with $CH_3CH_2OH$ | **$T_0$** | **$T_0$** | **$T_0$** |

Table 7.0**.** Table showing the degree of turbity of dissolved plant extract at concentration 0.035g/0.1 L at different volumes against reference ampicillin and control.

| Reference (Ampicillin at same concentration as dissolved plant extract 0.035g/0.01L | Volume of Reference or control (ml) 0.2 ml | Volume of Reference or control (ml) 0.4ml | Volume of Reference or control (ml) 0.6 ml |
|---|---|---|---|
| | $T_2$ | $T_1$ | $T_0$ |
| **Control Experiment** | | | |

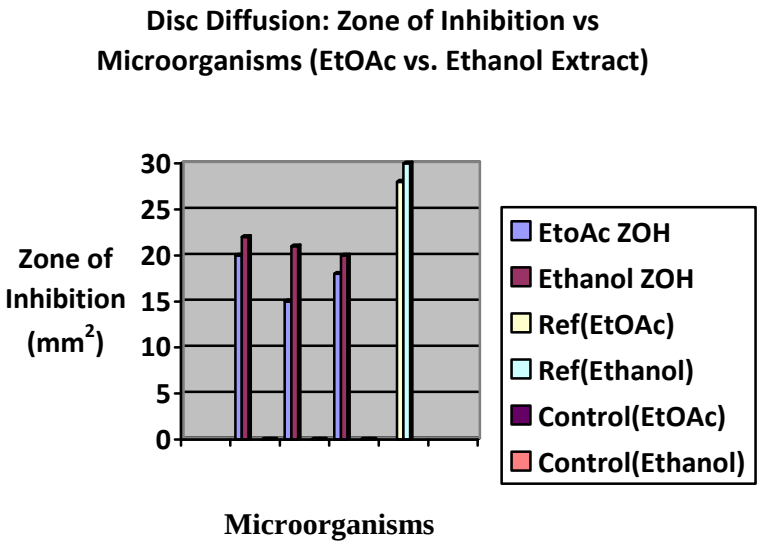
| | | | |
|---|---|---|---|
| Hexane | $T_3$ | $T_3$ | $T_3$ |
| $CH_2Cl_2$ | $T_3$ | $T_3$ | $T_3$ |
| EtOAc | $T_3$ | $T_3$ | $T_3$ |
| $CH_3CH_2OH$ | $T_3$ | $T_3$ | $T_3$ |

Table 8.0. showing the results of TLC for all the extracts.

| **Solvents** | **Plants** | **No of spots visible by UV** | **$R_f$ value** |
|---|---|---|---|
| Hexane | *Phyllanthus acidus (Hexane Extract)* | 4 | 0.022<br>0.087<br>0.739<br>0.978 |
| $CH_2Cl_2$ | *Phyllanthus acidus* ($CH_2Cl_2$ Extract) | 2 | 0.026<br>0.051 |
| EtOAc/$CH_2Cl_2$, 90: 10, v/v) | *Phyllanthus acidus* (EtOAc Extract) | 3 | 0.048<br>0.333<br>0.414 |

**Graphs**: Bar graphs are shown in Fig. 4.0 (a) and 4.0 (b) whereas corresponding line graph for Fig. 4.0 (a) and 4.0 (b) are shown in Fig. 5.0 (a) and Fig. 5.0 (b).

(a)




(b)

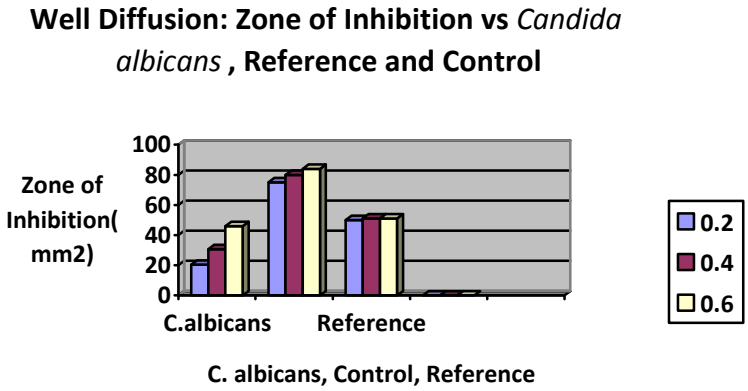
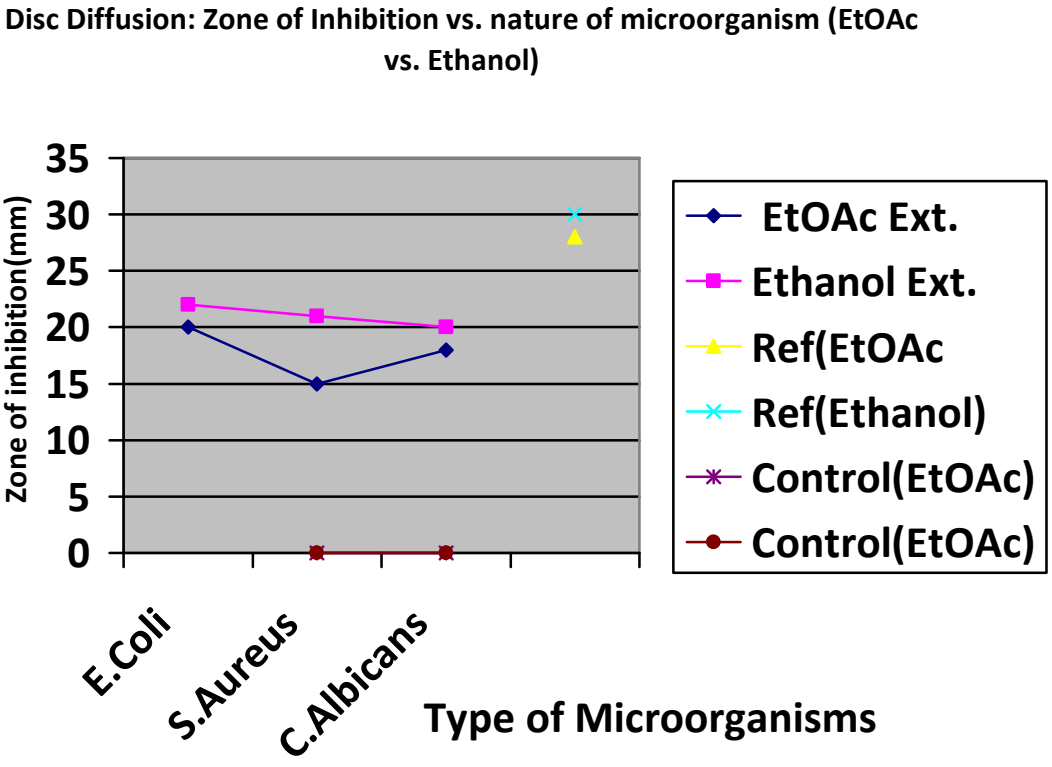



Fig. 4.0 (a) and 4.0 (b).




(b)

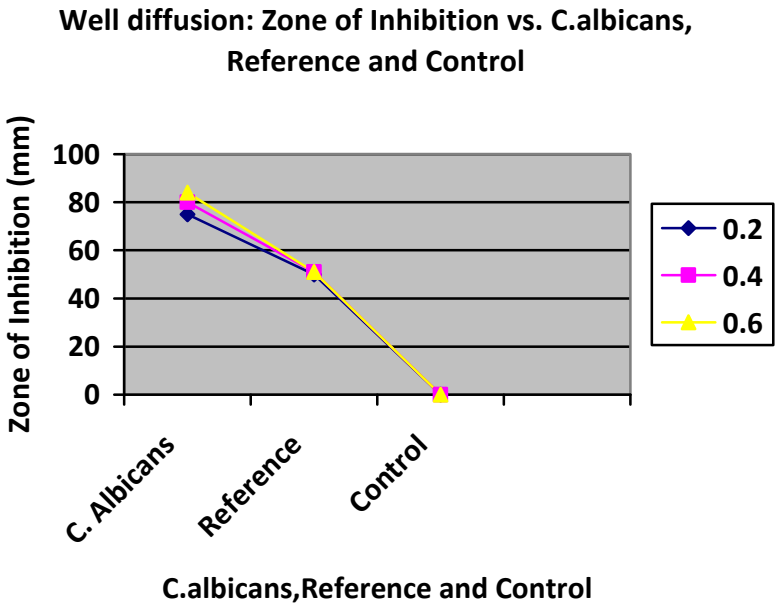



**Fig. 5.0 (a) and 5.0 (b)**

**2.3.17. Discussion**: All four methods: Stokes disc diffusion, Well diffusion, Streak plate and a dilution method were successful in determining *Phyllanthus acidus* antimicrobial activities. Several trends are noted. Antimicrobial activity follow the sequence: $CH_3CH_2OH$ extract > EtOAc extract > $CH_2Cl_2$ extract > hexane extract. For example, for the disc diffusion method, zone of inhibition of 22 $mm^2$, 21 $mm^2$ and 20 $mm^2$ were obtained for *E.Coli*, *S.aureus* and *C.albicans* for the $CH_3CH_2OH$ extract, whereas for the corresponding Hexane extract, zone of inhibition of 5 $mm^2$ were obtained for all three microbes. In another method, the Well diffusion, zone of inhibition of 84 $mm^2$ was obtained when the well was filled with 0.6 ml compare to the hexane extract for which the zone of inhibition was <5 $mm^2$. These results strongly suggest that *Phyllanthus acidus* antimicrobial active constituents are localized in the EtOAc and $CH_3CH_2OH$ extracts. For all methods used, the control experiments which necessitate the use of pure distilled solvent alone, rather than pure plant extract induced negative result i.e no zone of inhibition or in the case of the dilution method, turbidity in test tubes containing LB broth with bacterial microbes. As anticipated, our reference antibiotic compound, ampicillin for bacteria and Nyastatin for fungi displayed positive results. For the disc diffusion method, a larger zone of inhibition was observed for the reference compound in comparison to the plant extract. The opposite was noted for the Well diffusion method . For example, for the disc diffusion method, ampicillin induced zone of inhibition of 30 $mm^2$ for the $CH_3CH_2OH$ extract whereas for the turbidity experiment no inhibition was seen. For the Well diffusion method, nyastatin induced zone of inhibition of 51 $mm^2$ at 0.6 ml. All these results suggest that *Phyllanthus acidus* antimicrobial properties are due to the plant active constituent rather than to the distilled solvents. It should be noted that for each solvent extracts, extracts were added in increasing volume (0.2-0.4-0.6) ml to the microbial medium.

The salient feature for each method can be discussed. Disc diffusion indicates that the plant extract induced a larger zone of inhibition against *E.Coli* as compared against *S.aureus*. For example, for the EtOAc extract, zone of inhibition of 20 $mm^2$ and 15 $mm^2$ were obtained for *E.Coli* and *S.aureus* respectively. For the streak plate method, a similar trend was noted: *E.Coli* showed inhibition whereas *S.aureus* showed negligible inhibition (< 5$mm^2$).

The Well diffusion method was used primarily against *C. albicans* microbe. The Well diffusion indicated that a larger zone of inhibition was observed compared with the Stokes Disc diffusion method. For example, for the EtOAc extract, a zone of inhibition of 79 $mm^2$ was observed at a volume of 0.6 ml.

Compared with the Stokes Disc Diffusion method, zone of inhibition of 18 $mm^2$ were observed. This suggest that the Well diffusion method is a much more sensitive method than the Disc diffusion.

The streak plate method indicated both the hexane and $CH_2Cl_2$ extract induce no inhibition against *E.Coli* and *S.aureus*. However, selective inhibition was observed for the EtOAc extract. Positive inhibition or no growth of microbe was observed against *E.Coli* at volume of 0.2 to 0.6 ml whereas negative inhibition was observed against *S. aureus* at the same volume range.

The Dilution method was used to test the dissolved plant extracts for antimicrobial activity against bacteria: *E.Coli* and *S.aureus*. Plant extract that showed positive results for the streak plate was used. Results were recorded in terms of turbidity. In general, no turbidity indicates inhibition. The use of LB broth as a rich medium to foster or stimulate the growth of the bacteria is noted. Yeast extract and tryptone provide vitamins and amino acids respectively for the bacteria to grow. The result indicates that for *E.Coli* microbe no inhibition (very turbid mixture, $T_3$) was observed for the hexane and $CH_2Cl_2$ extract. However, inhibition was observed for the EtOAc extract at a volume of 0.6 ml and for the $CH_3CH_2OH$ extract from volume 0.2 to 0.6 ml. Interestingly, for the dilution method against *S.aureus*, inhibition were seen for $CH_2Cl_2$, EtOAc and $CH_3CH_2OH$ extract at increasing volume from 0.2 to 0.6 ml. The reference compound ampicillin and the controls showed inhibition and non inhibition respectively as anticipated.

. Fig. 4.0 (a) represent for the Disc diffusion method using the $CH_3CH_2OH$ and EtOAc extract, a bar graph plot of the zone of inhibition vs. type of microorganism, Fig. 4.0 (b) represent for the Well Diffusion method, a bar graph plot of the zone of inhibition vs. Candida albicans. Fig. 5.0 (a) and 5.0 (b) represent plots for the corresponding line graphs. For the disc diffusion technique, a larger zone of inhibition was observed for the $CH_3CH_2OH$ extract as compared with the EtOAc extract. The largest zone of inhibition was observed for *E.Coli* in both cases. For the Well diffusion method, as the volume of plant extract increased in the well so too is the zone of inhibition for the $CH_2Cl_2$, EtOAc and $CH_3CH_2OH$ extract.

TLC analyses in various solvent system for each solvent type extract revealed the presence of spots that range from two to four, Table 8.0. Each spot is probably due to a pure natural product or phytochemical. Each also has a specific $R_f$ value. The number of spots and $R_f$ value for each spot is recorded in Table 8.0. For example for *Phyllanthus acidus* EtOAc, extract using the solvent system, EtOAc/$CH_2Cl_2$ (90:10, v/v), three spots at $R_f$ values of 0.048, 0.333 and 0.414 were seen..

**Conclusions**:

It is clearly seen that *Phyllanthus acidus* has antimicrobial properties. However, antimicrobial activity is selective and solvent dependent with the $CH_3CH_2OH$ extract, the most potent and hexane the least. In general, the order of antimicrobial activity follow the sequence: $CH_3CH_2OH$ extract > EtOAc extract > $CH_2Cl_2$ extract > hexane extract. Thus, the $CH_3CH_2OH$ and EtOAc extract of *Phyllanthus acidus* can be used as the active constituent of an antimicrobial cream or following clinical trials as herbal medicines. Future work such as isolation and purification of bioactive constituents should target the $CH_3CH_2OH$ and EtOAc extract of *Phyllanthus acidus*.

**Acknowledgements**: This research was carried out by a final year research student, A.Mars under my constant supervision and also that of Dr. G. Gomes, microbiologist at John's campus, University of Guyana in the county of Berbice.We thank the University of Guyana, Faculty of Natural Sciences, Chemistry Department for the provision of laboratory space for the extraction process and equipment to carry out this research. Special thanks also extend to Food and Drugs, Kingston, Georgetown for partial use of the microbiology laboratory there at the initial microbial stage of this project. Also, the Georgetown Public hospital (GPH) for the provision of microorganisms essential for research. This research was supported via a grant to Dr. R.C.Jagessar from the Research and Publication agency of the University of Guyana, South America and the Royal Society of Chemistry, England.

**Correspondence:**

Dr. R.C.Jagessar, BSc, PhD, PDF

Lecturer and Supervisor,
Department of Chemistry,
Faculty of Natural Sciences
University of Guyana,
Turkeyen,
Greater Georgetown
South America
Email: raymondjagessar@yahoo.com

Feb 9, 2008 9:25 PM

**References:**

1. Kandil O, Redwan N.M, Hassan A.B, Amer A.M.M, El-Banna H.A, "Extracts and fractions of *Thymus capitatus* exhibit antimicrobial activities", *Journal of Ethnopharmacology* 1994, 44, 19-24.

2. Barre J.T, Bowden B.F, Coll J.C, Jesus J, Fuente V.E, Janairo G.C., Ragasa C.Y . "A bioactive triterpene from *Lantana camara*, *Phytochemistry* 1997, 45: 321-324.

3. Batista. O. , Duarte A, Nascimento J, Simones, M.F. "Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus heranthus*, *J. Nat. Products*. 1994 57: 858-861.

4. Rojas A, Hernandez L, Pereda-Miranda R, Mata. R "Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants". *J. Ethnopharmacology*. 1992, 35: 275-283.

5. Silva O, Duarte A, Cabrita. J, Pimentel. M, Diniz. A, Gomes. E. "Antimicrobial activity of Guinea-Bissau traditional remedies". *J.Ethnopharmacology*. 1996, 50: 55-59.

**6.** "Chemistry in Britain", December, 2003; pg 27-29.

7. Jagessar, R.C. "The isolation and structural elucidation of natural products from a marine organism and a plant species, a research project in collaboration with the University of the West Indies, St. Augustine campus", University of Guyana library, 2004.

8. Jagessar, R.C. Mohamed, A. Research abstracts "Extractions and isolation of natural products from *Momordica Charantia*", 15th Annual conference of the Caribbean Academy of Sciences, Guadeloupe, 2006.

9. Jagessar, R.C., Narine W. Research abstracts "The making of perfumes from the essential oils of local flowers", 14th annual conference of the Caribbean Academy of Sciences, Mount Hope, Trinidad, 2004.

10. Jagessar R.C., Cox. M. F. , Research abstracts, "Phytochemical screening of the stems, twigs, roots and bark of *Conacarpus erectus"*, 14th annual conference of the Caribbean Academy of Sciences, Mount Hope, Trinidad, 2004.

11. Jagessar, R.C, Mohamed A., Research abstracts "Antimicrobial activities of selected plants", 1st International conference on the status of Biological Sciences in the Caribbean and Latin America Societies, Buddy's International hotel, Providence, Guyana, 2007.

12. Jagessar. R.C, Mohamed N., Research abstracts "Antimicrobial activities of selected tropical plants", 1st International conference on the status of Biological Sciences in the Caribbean and Latin America Societies, Buddy's International hotel, Providence, Guyana, 2007.

13. Jagessar, R.C, Mohamed N, Paper presentation "Antimicrobial activities of selected tropical plants", 1st International conference on the status of Biological Sciences in the Caribbean and Latin America Societies, Buddy's International hotel, Providence, Guyana, 2007.

    Jagessar R.C, Research Proposal for funding from the Royal Society of Chemistry, England and the University of Guyana,"Extraction, Isolation and structural elucidation of Natural products from the Guyana flora", 2006 and 2007.

14. Tavares J.F., Queiroga K.F. Silva, M.V.B. Diniz, M.F., Filho, J.M.B. da Cunha, E.V.L. Simone C.A, Junior, J.X.A. Melo P.S, Haun M, Da Silva M.S, *J. Nat. Prod*. **2006**, 69, 960-962.

15. Melo P.S., Duran. N, Haun. M, Human Exp. *Toxicol*. 2002, **21**, 281-288.

16. Jagessar R. C., Mohamed A. Gomes G. "Antibacterial and antifungal activity of leaf extracts of *Luffa operculata*, *vs Peltophorum Pterocarpum*, against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*" *Nature and Science*, 5(4), 2007, 81-93.

17. Profile for *Phyllanthus acidus*(Tahitan gooseberry tree) USDA PLANTS.html.

18. F: / *Phyllanthus acidus*.html

19. F:/ *Phyllanthus*- Wikipedia, the free encyclopedia.html

20. http://en.wikipedia.org/wiki/Escheria_coli.

21. http://en.wikipedia.org/wiki/Staphylococcus_aureus

22. http://en.wikipedia.org/wiki/Candida_albicans

23. http://hcd2.bupa.co.uk/fact_sheets/html/fungal_skin_infections.html

24. Murray P.R., Baron E.J, Pfaller M.A, Tenover F.C, Yolke, R.H. 1995. "Manual of Clinical Microbiology", 6th ed. Mosby Year Book, London.