An evaluation of the Antibacterial and Antifungal activity of leaf extracts of *Momordica Charantia* against *Candida albicans, Staphylococcus aureus* and *Escherichia coli.*

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ABSTRACT: The antibacterial and antifungal activities of Momordica.charantia, 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, dichloromethane, ethyl acetate and ethanol respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.03g in 10 mL of the respective solvents. These were tested in varying volumes of 100-600 uL/plate (i.e. concentrations of 0.03-0.18 mg/10 mL agar). 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. Momordica.Charantia 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 M Momordica.Charantia,can be used in the control of E.coli and S.aureus induced diseases as herbal medicines following clinical trials. [Nature and Science. 2008;6(1):1-14]. ISSN: 1545-0740.

Key words: *Momordica Charantia*, Antimicrobial, *S.aureus, E.Coli*, *C.albicans*, Stokes Disc diffusion, Pour plate, Well diffusion, Streak plate, herbal medicines.

1.0. Introduction:

This paper discusses the microbiological properties of leaves of *Momordica Charantia, bitter melon* from the coastal plane of the Guyana flora and its possible use as an 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, Pour plate, Well diffusion and Streak plate.

Guyana has a rich flora biodiversity whose crude extracts, both organic and aqueous can be investigated for antimicrobial activity in addition to their role as global CO_2 sinks(in the context of global warming). Also, the specified plants parts of the same species be screened for natural products whose antimicrobial activity can also be correlated with the 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[1-14]. Besides used as an herbal cream, following clinical trials, crude plant extracts can be subjected to chromatographic separation, leading to the isolation and purification of new and known bioactive natural products/phytochemicals, whose medicinal activity can also be investigated. For example, the diterpene (1) and (2) were isolated from the bark of *Xylopia aethiopica* and was investigated against the enzymes prolyl endopeptidase (PEP) and α-thrombin and was found to have inhibitory activity against them [15].

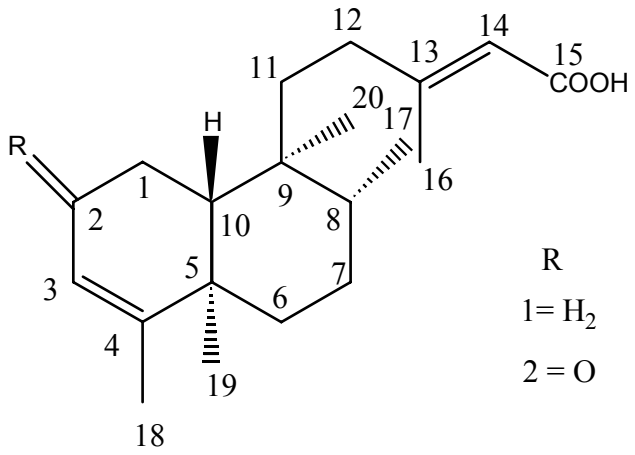

Fig. 1.0. Structure of Diterpene (1) and (2).

Research in herbal medicine and isolated drug discovery need to be continued, considering the threat of new emerging disease such as SARS, bird flu, not to mention the killer HIV AIDS. Plants are a good source of herbal medicine and natural products/ phytochemicals[1-15]. Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from natural products isolated from plants rather than to the creativity and imagination of contemporary organic chemists. For example, the drug taxol (a diterpenoid), first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer[6,14]. In Guyana, there are many medicinal folklore practises but most are without scientific research. For example, drinking water extract of *Momordica Charantia* is a good remedy for diabetes. Thus, there exist an urgent need to correlate folklore herbal practices with scientific evidences. With an increasing emphasis on scientific research, Guyana stands well in this area. Its our scientific endeavour, to correlate antimicrobial activity of *Momordica Charantia* with its folklore practices.

Momordica Charantia commonly called bitter melon belongs to the family *cucurbitaceae* and grows in tropical areas, including parts of the Amazon, East Africa, Asia, and the Caribbean, and is cultivated throughout South America as a food and medicine[16-18]. It's a slender, climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf axils. The fruit looks like a warty gourd, usually oblong and resembling a small cucumber. All parts of the plant, including the fruit, taste very bitter. In Guyana traditional medicine, a leaf tea is used for diabetes, to expel intestinal gas, to promote menstruation, and as an antiviral for measles, hepatitis, and feverish conditions. It is used topically for sores, wounds, and infections and internally and externally for worms and parasites.

Escherica. coli can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia[19]. *Staphylococcus aureus* can cause furuncles (boils), carbuncles (a collection of furuncles)[20]. 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[21].

Fig. 2.0: Momordica Charantia

2.0. Procedure:

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

2.2 Extraction: The leaves were first extracted in hexane thrice 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: dichloromethane, ethyl acetate, and then ethanol.

2.3. Antimicrobial activity tests

2.3.1. **Making up extract solution**

A pproximately 0.03g of dried crude extract of *Momordica Charantia* was weighed and transferred to a 10 mL volumetric flask. The respective solvent was then added to make up the 10 mL solution.

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. **Potato dextrose agar (PDA)** [23]

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 (12.5 g) 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.

2.3. 4. 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 [24].

2.3.5. Aseptic conditions:

The aseptic chamber 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).

2.3.6. Mother plates:

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

2.3. 7. Nutrient Agar:

Nutrient agar was purchased from the International Pharmacy Association in Guyana. 500ml of nutrient agar was made by placing 14g of the powdered mixture in a 1L flask, stirred, boiled and then autoclaved for 15 minutes at 121^{0}C. 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.

2.3.8. **Colonies Counting**:

Colonies were estimated with the assistance of a colony counter. The number was estimated for 1cm^2 and then calculated for the entire plate. The plate radius was determined.

2.3.9. Retention Factor: R_f = Distance moved by sample

$$\frac{\text{Distance moved by sample}}{\text{Distance moved by solvent front.}}$$

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

2.3.10. Disc diffusion: Stokes Disc diffusion sensitivity technique [23].

Using Stokes Disc diffusion sensitivity testing technique[24] , 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 10mg of antibiotic/disc. 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

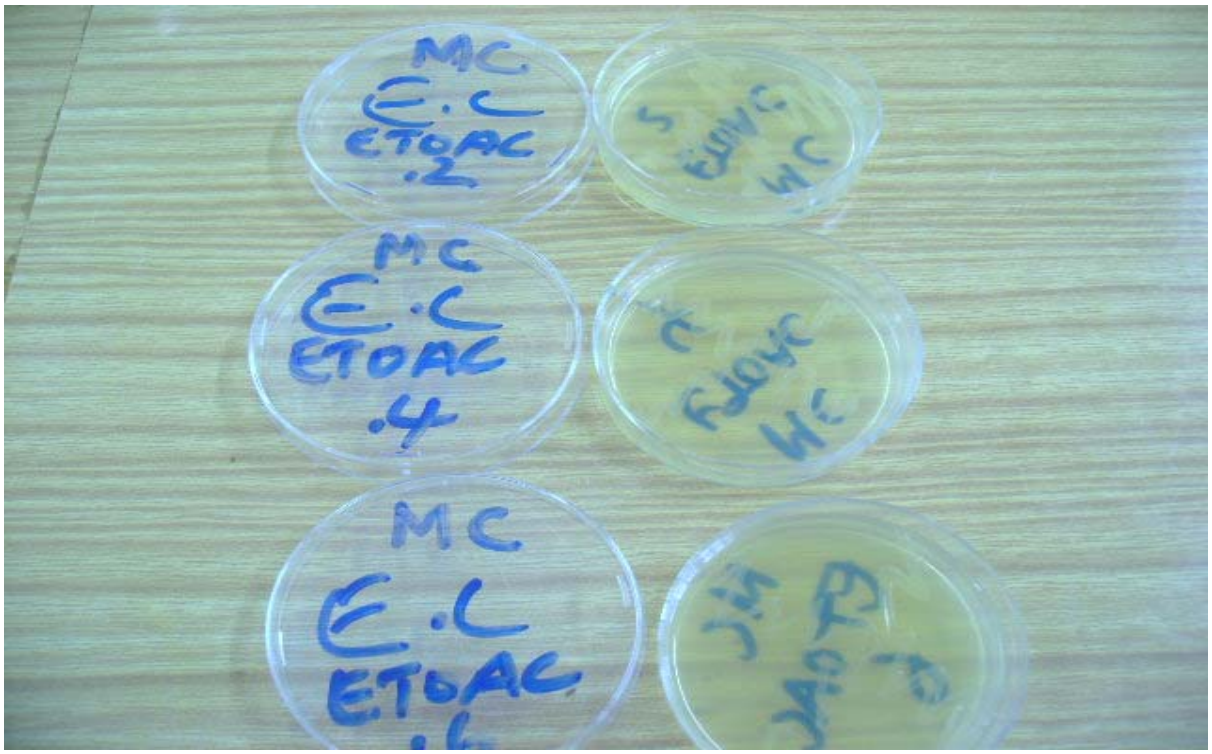

Fig. 3.0. Streak plate after 24 h: The ethyl acetate extract of *Momordica. charantia against E. coli*.

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2.3.11. Diffusion plate (well diffusion):

The fungus (*Candida albicans*) was mixed with the warm, melted, autoclaved PDA and poured into 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 6mm cork borer that was sterilized with alcohol and flame. The extracts were applied to different wells in volumes of 100-600uL using a micro liter syringe. The four solvents (hexane, dichloromethane, ethyl acetate and ethanol) were used as control whereas nystatin was used as the reference.The plates were labelled, covered, inverted and placed in a fume hood (no incubator was available) for 48h.

2.3.12. Pour Plate Method [23].

After the nutrient agar was placed in the autoclave at 120^{o}C for one and half hour, it was taken out and left to semi cool in a sterilized environment. 0.1mL of each solvent type extract and control were measured and placed in separate sterile glass plates plates (100mm diameter). 10mL of nutrient agar was then poured into the 100 mm plate, with an even depth of 4mm on a level surface shaken and allowed to cool. A sterile glass rod was used to uniformly stir the mixture into the nutrient agar which was left to solidify in the glass plate. The microorganisms were then streaked onto the plates and placed in an incubator at 37 ^{0}C for 24 and 48 hours for bacteria and fungi species respectively.

The inoculated plates were incubated in an inverted position (lid on bottom) to prevent collection of condensation on the agar surface. The plates were examined for the appearance of individual colonies growing throughout the agar medium. The number of colonies were counted so as to determine how effective the plant extract were against bacterial and fungi.

2.3.13. Streak plate for bacteria:

Nutrient agar was prepared as described above and 10mL was poured into plates. The plates were treated with the extracts and reference compound ampicillin in varying volumes of 100-600uL.The plates were allowed to cool and then the bacteria were streaked onto the surface.These plates were left for 24 hours.The plates with inhibition were used in further experiments.

2.3.14. 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.They were examined under the UV/Vis lamp and the specks were circled with a pencil. The plates were then held in the iodine jar for a few seconds, shaken and taken out.The plate was further examined under UV lamp and any new specks were marked. The specks were labeled and their distances from the baseline were measured. The distance between the baseline and the solvent front was measured.The R_f values were determined.

2.3.15. **Results**:

Mass of dried leaves used for , *Momordica .charantia species was* 8.55g respectively. The physical state of the dried extract are shown in Table 1.0.

Table 1.0 Shows physical properties of the dry extracts.

Solvent	**Plant**	**Dry extract**
Hexane	*Momordica.charantia*	Yellow, brown, oily.
Dichloromethane	*Momordica.charantia*	Crystalline, black..
Ethyl acetate	*Momordica.charantia*	Soft, black ..
Ethanol	*Momordica.charantia*	Brown, powdery.

These extracts were in the concentration of 0.03g in 10ml of solvent except for *M.charantia* 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**	**Reference compound (Ampicillin) (mm^2)**	**Control Experiment**
			Momordica Charantia		No zone of inhibition
< 5	**< 5**	**< 5**	Hexane extract	**27**	No zone of inhibition
< 5	**< 5**	**< 5**	Dichloromethane extract	**28**	No zone of inhibition
< 10	**< 5**	**< 5**	EtOAc extract	**28**	No zone of inhibition
21	**18**	**20**	Ethanol extract	**30**	No zone of inhibition

Table 3.0. Results of the Well diffusion for plant extracts against *C.albicans.*

Zones of inhibition (mm^2)	**Extract**	**Volume of Extract**	**Observations**
0	*Momordica.charantia* with Hexane	100-600 uL	No zones of inhibition visible, scattered colonies.
0	*Momordica.charantia* with ethyl acetate	100-600 uL	No zones of inhibition visible, scattered colonies.
0	*Momordica.charantia* with dichloromethane	100-600 uL	No zones of inhibition visible, scattered colonies.
75x45	*Momordica.charantia* with ethanol	600 uL	Complete zones of inhibition.
20x30 = 600 30x50 = 1500 50x70 = 3500	Nystatin	200 uL 400 uL 600 uL	Zones of inhibition
Controls	Diffusion well with four solvents		Scattered colonies
Reference(Nystatin)		600 uL	Complete zones of inhibition

Pour Plate:

Table 4.0 showing the number of visible colonies when viewed under a colony counter.

Bacterium	**Extract**	**Volume**	**# of colonies**
S.aureus	*Momordica charantia* with ethanol	300 uL	80
S.aureus	*Momordica charantia* with ethanol	400 uL	60
S.aureus	*Momordica.charantia* with ethanol	500 uL	55
S.aureus	*Momordica.charantia* with ethanol	600 uL	50
E.coli	*Momordica.charantia* with ethyl acetate	600 uL	$12/cm^2$x $0.5(63.6cm^2)$= 381.
Controls: *S.aureus* *E.coli*	Nutrient agar only	600 uL	Excess growth observed. $26/cm^2$x $63.6cm^2$=1653. $20/cm^2$x $63.6cm^2$=1272.

These colonies were estimated with the assistance of a colony counter. The number was estimated for $1cm^2$ and then calculated for the entire plate. The plate radius was 45mm, therefore the area was 63.6 cm^2

Table 5.0. Results of the Well diffusion for plant extracts against *C.albicans.*

Zones of inhibition (mm^2)	**Extract**	**Volume of Extract**	**Observations**
0	*Momordica.charantia* with ethyl acetate	100-600 uL	No zones of inhibition visible, scattered colonies.
0	*Momordica.charantia* with dichloromethane	100-600 uL	No zones of inhibition visible, scattered colonies.
75x45 =	*Momordica.charantia* with ethanol	600 uL	Complete zones of inhibition.
20x30 = 600 30x50 = 1500 50x70 = 3500	Nystatin	200 uL 400 uL 600 uL	Zones of inhibition
Controls	Diffusion well with four solvents		Scattered colonies
Reference(Nystatin)		600 uL	Complete zones of inhibition

Streak plate:

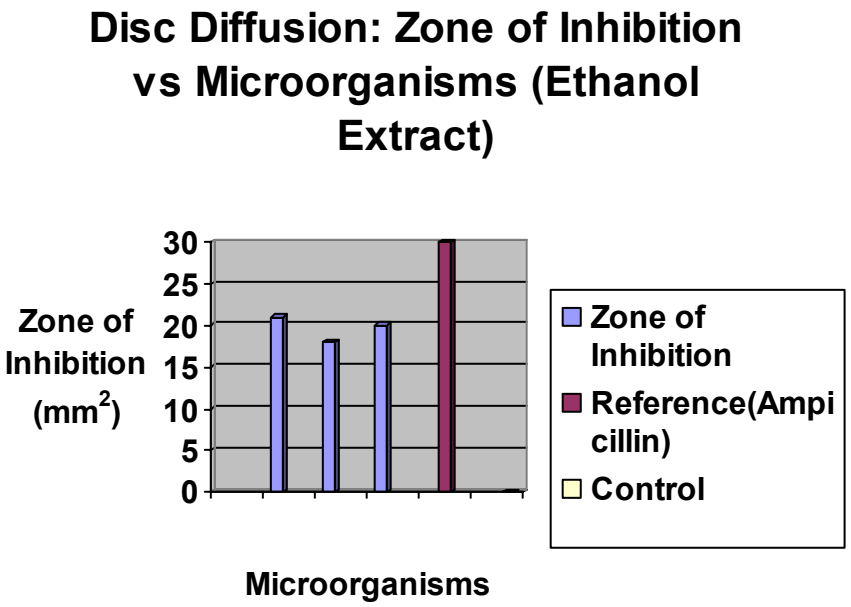
Table 6.0. showing the results of the streak plate method.

Bacterium	Solvent Extract	Volume of Extract	Observations
E.coli	*Momordica.charantia* with ethanol	600uL	Limited growth
E.coli	*Momordica.charantia* With ethyl acetate	200-600uL	Limited growth
S.aureus	*Momordica.charantia* with ethyl acetate	200-600uL	Limited growth
S.aureus	*Momordica.charantia* with ethanol	200-600uL	Limited growth
Controls:	The four solvents: hexane,dichloromethane, ethyl acetate and ethanol	200-600uL	Growth of microorganism
Reference (Ampicillin)		200-600uL	Inhibition

Table 7.0. showing the results of the TLC for all the extracts.

Solvents	**Plants**	**No of spots visible by UV**	**R_f value= d moved by sample / d moved by solvent**
Hexane	*Momordica Charantia*	1	0.67
Dichloromethane/hexane, 90: 10, v/v)	*Momordica Charantia*	5	0.05 0.08 0.18 0.40 0.50
Ethylacetate/dichloromethane, 90: 10, v/v)	*Momordica Charantia*	1	0.11 0.39 0.58 0.97
Ethanol/hexane, 90: 10, v/v)	*Momordica Charantia*	1	0.14

Graphs: Bar graphs are shown in Fig. 3.0 (a) and 4.0 (a) whereas a corresponding line graph for Fig. 3.0 (a) is shown in Fig. 5.0 (a).



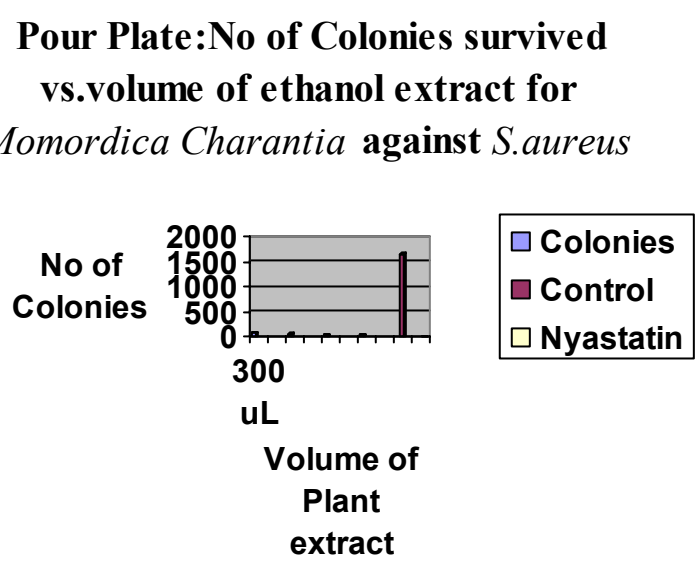
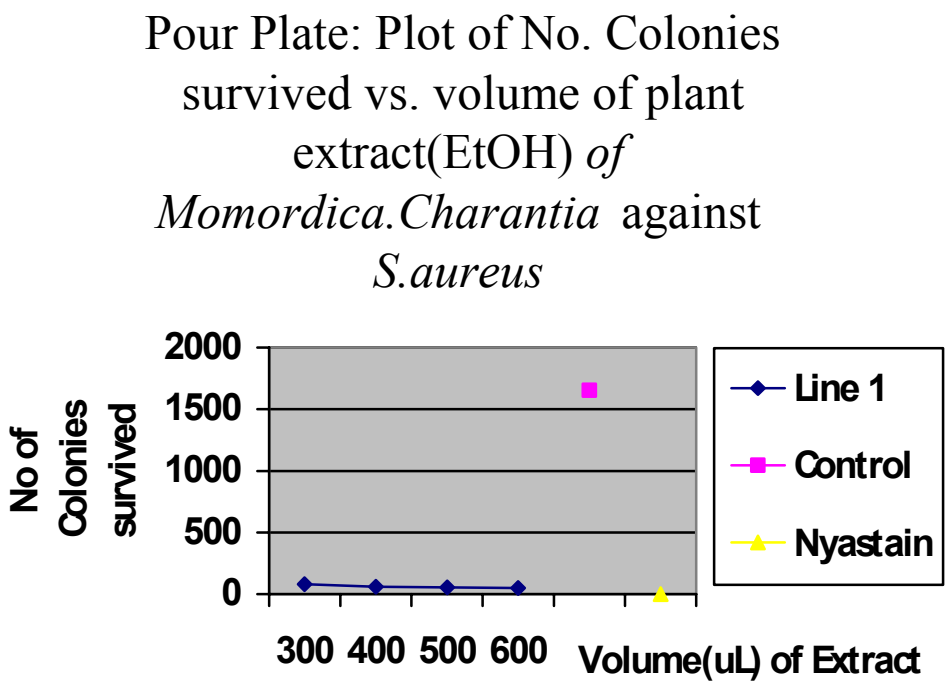


Fig. 4.0. (a)



(a)

Fig. 5.0. (a).

2.3.17. Discussion:

All four methods: Stokes disc diffusion sensitivity techniques, Pour plating, Well diffusion and Streak plate were successful in determining *Momordica Charantia's* antimicrobial activities. Antimicrobial activity follow the sequence: Ethanol extract > EtOAc extract > dichlormethane extract > hexane extract. Stokes disc diffusion indicates that the hexane, dichloromethane, ethylacetate extract of *Momordica Charantia* had neglible antimicrobial activity. Only a maximum zone of inhibition of 10 mm^2 was observed against *E.coli* for the EtOAc extract. However, the plant ethanol extract was effected against all three microorganisms studied. Significant zones of inhibition were observed. This range from 18 mm^2 to 21 mm^2. The former was obtained for *S.aureus* whereas the latter was obtained for *E. coli*.

The Well diffusion method was used primarily against *C. albicans*. It indicates that the EtOAc extract and dichloromethane extract of *Momordica Charantia* at volume of 100-600 uL induce no zones of inhibition. Instead scattered colonies were observed i.e microbial.This is consistent with the results obtained for the disc diffusion method. The ethanol extract of *Momordica Charantia* showed complete zones of inhibition. The zone of inhibition was observed to be: 75 x 45 mm^2. *Momordica Charantia's* hexane and dichloromethane extracts at 100-600ul showed zero zone of inhibition. The reference antibiotic: Nystatin showed zone of inhibition of 600 mm^2 to 3500 mm^2 as the volume of the extract increase from 200uL to 600uL respectively. For the control experiment, the well with the four solvents induced scattered colonies i.e negative inhibition. This indicates that the inhibition induced are truly due to the plant's active constituents rather than to the solvents used in the extraction process.

The Pour plate method indicate that the ethanolic extract of *Momordica Charantia* showed inhibition against the growth of bacteria, *S.aureus*. With increasing volume of extract from 300 uL to 600 uL, the number of colonies decreased from 80 to 50 against *S.aureus,* Table 4.0. In constrast, the EtOAc extract of *Momordica Charantia* at a volume of 600 uL induce the formation of 381 colonies i.e it was microbial. Control experiments for Pour plate indicate that the solvents induce growth of bacteria: *S.aureus and E.coli*, Table 4.0. However, the reference compound Ampicillin and nyastatin completely inhibit the

growth of microorganisms. For the pour plate method, the number of colonies were estimated using a colony counter.

For the Streak plate method, the ethanol extract of *Momordica Charantia* at a volume of 600 uL showed limited growth against *S.aureus and E.coli*. The EtOAc extract of *Momordica Charantia* at 200-600uL also showed limited growth against *E.Coli* and *S.aureus*. The control experiment indicated that all four solvents: hexane, dichloromethane, ethylacetate and ethanol showed growth of the two bacterial species whereas the reference compound Ampicillin showed complete inhibition at 200-600uL.

Fig. 3.0 (a) represent for the Disc diffusion method using the ethanol extract, a plot of the zone of inhibition vs. type of microorganism, Fig. 4.0 (a) represent for the pour plate method, a plot of the number of colonies survived vs. volume of *Momordica Charantia* ethanol extract against *S.aureus*. Fig. 4.0 (a) represent plots of the corresponding line graphs. For the disc diffusion technique, using the ethanol extract, the largest zone of inhibition was observed for *E.Coli*. As the volume of plant extract increased so too is the zone of inhibition for the well diffusion method. For the pour plate result, as the volume of the plant (*Momordica Charantia*) ethanolic extract increased, the number of colonies decreased

TLC analyses in various solvent system for each solvent type extract revealed the presence of spots that range from one to a maximum of five, Table 6.0. Each spot is presumbably due to a pure natural product or phytochemical. Each also has a specific R_f value. The larger the R_f value, the lower the polarity of natural product/phytochemicals The number of spots and R_f value for each spot is recorded in Table 5.0. For example for the dichloromethane extract of *Momordica Charantia*, five spots with R_f values of 0.05, 0.08, 0.18, 0.40 and 0.50 in dichloromethane/hexane, 90: 10, v/v) respectively were seen.

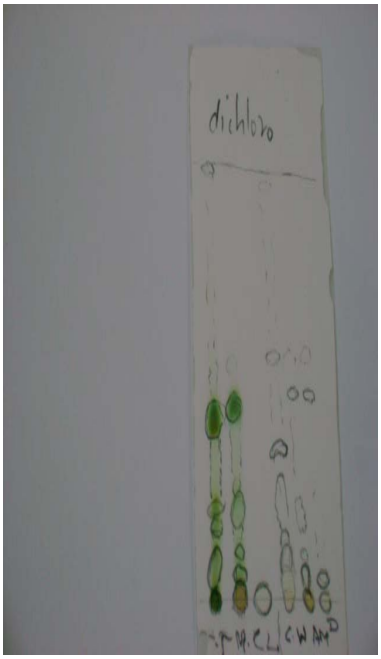


Fig. 6.0**. TLC analyses:** *Momordica Charantia* **etc.**

Conclusions:

It is clearly seen that *Momordica Charantia* has antimicrobial properties. However, antimicrobial activity is solvent dependent with the ethanol extract, the most potent and hexane the least. In general, the order of antimicrobial activity follow the sequence: Ethanol extract > EtOAc extract > dichlormethane extract > hexane extract. Thus, the ethanol extract of *Momordica Charantia* can be used as the active constituent of an antimicrobial cream. Future work such as isolation and purification of bioactive constituents should target the ethanol extract of *Momordica Charantia*. In Guyana's culture, *Momordica Charantia fruit* is edible and is used as "cooked carylla" eaten with rice. Also, an overnight aqueous extract of the plant leaves is used in the control of diabetes.

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