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Research Article

EVALUATION OF ANTIMICROBIAL STUDIES ON ETHANOLIC EXTRACTS AND POLY HERBAL FORMULATIONS OF *ABUTILON INDICUM, ARISTOLOCHEA BRACTEOLATE,* AND *ANDROGRAPHIS PANICULATA* BY USING FLOW CYTOMETER

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ABSTRACT

Abutilon indicum belonging to the family of malvaceae, It is a perennial shrub. It habitually grows as wild plant abundantly in wastelands and seashore to 1200 m high in India. *Aristolochia bracteolate* is a perennial, mostly prostrate but occasionally climbing or suberect, unpleasantly smelling herb. The plant is usually gathered from the wild and is used locally in traditional medicine. *Andrographis paniculata* is an annual herbaceous plant in the family *Acanthaceae*. The above mentioning the three plants have been traditionally used to treat infections and some diseases. In our study focused the preparation of ethanolic extract from above mentioning the three plant leaves. The various concentrations like 1%, 2.5%, and 5% poly herbal cream, ointment and gel was prepared and physically evaluated. The flow cytometric method analysed the two fungal strains *Trichophyton rubrum*, *Microsporum canis* and three bacterial strains *Staphylococcus aureus, Streptococcus pyogenes* and *Propionibacterium acnes*. Finally we reported on our plant extracts and polyherbal formulations having potential activity against these micro organisms.

Keywords: Abutilon indicum, Aristolochia bracteolate, Andrographis paniculata, Gel, cream.

INTRODUCTION

Dermatophytosis, additional normally identified as as ringworm, is a fungal infection of the skin. Ringworm is a misnomer. The infection isn't caused by a worm. It's caused by a fungus (Trichophyton rubrum, Microsporum canis). Ringworm infection can affect both humans and animals. The infection initially presents itself with red patches on affected areas of the skin and later spreads to other parts of the body. The infection may affect the skin of the scalp, feet, groin, beard, or other areas[1]. Acne vulgaris (or simply acne) is a long-term skin disease that occurs when hair follicles become clogged with dead skin cells and oil from the skin.[2] Acne is causative organisms of *Propionibacterium acnes*. Acne is characterized by areas of blackheads, whiteheads, pimples, and greasy skin, and may result in scarring.[3,4,5] The resulting appearance can lead to anxiety, reduced self-esteem and, in extreme cases, depression or thoughts of suicide.[6,7] Impetigo is a highly contagious skin condition. It usually occurs on the face, neck, and hands of young children and infants. Children who wear diapers also tend to get it around the diaper area. Impetigo occurs more rarely in adults, usually following another skin condition or an infection. Impetigo is caused by two bacteria — streptococcus pyogenes and staphylococcus aureus[8]. Recommended treatment often depends on which bacteria are causing your impetigo. Outlook for this condition is good and it usually goes away within two to three weeks. In the present research work was carried out the Antimicrobial activity of ethanolic extracts and poly herbal formulation of the mentioning the plants of *Abutilon indicum*, *Aristolochia bracteolate* and *andrographis paniculata*.

MATERIALS AND METHODS

Plant material

The plant of *Abutilon indicum, Aristolochea bracteolate,* and *Andrographis paniculata was* collected from Thirumalaisamudram 7km away from Thanjavur (Tamil Nadu) in the month of December 2010. The plants was identified by local people of that village and authenticated by Dr. N.Ravichandran, Asst. Professor, Drug Testing Laboratory, CARISM, SASTRA University Thanjavur, and the Voucher specimen is preserved in laboratory for future reference.

Chemicals

All the reagents used were of analytical grade obtained from S.D. fine chemicals, Ltd, and Hi Media, Mumbai.

Preparation of Topical Poly herbal Gel 1%, 2.5% and 5 %[9]

Ingredients	1% Gel	2.5% Gel	5% Gel
Abutilon indicum ethanolic Extract	0.3gm	0.75gm	1.5gm
Andrographis paniculata ethanolic extract	0.3gm	0.75gm	1.5gm
Aristolochea bracteolate ethanolic extract	0.3gm	0.75gm	1.5gm
Tragacanth	0.3gm	0.3gm	0.3gm
Xanthum gum	0.6gm	0.6gm	0.6gm
Methyl paraben	51mg	51mg	51mg
Prophyl paraben	90mg	90mg	90mg
EDTA	5mg	5mg	5mg
Potassium hydroxide	200mg	200mg	200mg
Glycerin	1.2ml	1.2ml	1.2ml

1% Gel Preparation

Each 0.3g of sample, 0.3 g of tragacanth dissolved in 10 ml of distilled water, 0.6 g of xanthun gum in a beaker dissolved in 10 ml of distilled water. Warm it over a water bath, Mixed both. Then 51 mg of methylparaben, 90mg of propylparaben and 5mg of EDTA dissolved in 5ml of water. Incubate at 6hrs (Room temp.).200mg of KOH dissolved in 2ml of water, add 1.2ml of glycerin. All the ingredients are mixed together.

2.5% Gel Preparation

Each 0.75g of sample, 0.3 g of tragacanth dissolved in 10 ml of distilled water, 0.6 g of xanthun gum in a beaker dissolved in 10 ml of distilled water.Warm it over a water bath, Mixed both. Then 51 mg of methylparaben, 90mg of propylparaben and 5mg of EDTA dissolved in 5ml of water. Incubate at 6hrs (Room temp.).200mg of KOH dissolved in 2ml of water, add 1.2ml of glycerin. All the ingredients are mixed together.

5% Gel Preparation

Each 1.5g of sample, 0.3 g of tragacanth dissolved in 10 ml of distilled water, 0.6 g of xanthun gum in a beaker dissolved in 10 ml of distilled water.Warm it over a water bath, Mixed both. Then 51 mg of methylparaben, 90mg of propylparaben and 5mg of EDTA dissolved in 5ml of water. Incubate at 6hrs (Room temp.).200mg of KOH dissolved in 2ml of water, add 1.2ml of glycerin. All the ingredients are mixed together.

Evaluation of Topical Poly herbal Gel 1%, 2.5% and 5 %

The evaluation topical poly herbal gel parameters like appearance, pH, Congealing temperature, acidity rancidity and consistency reported on the table No: 12.

Preparation of Topical Polyherbal Ointment 1%, 2.5% and 5 %[10]

Ingredients	1% ointment	2.5% ointment	5% ointment
Abutilon indicum ethanolic Extract	0.3gm	0.75gm	1.5gm
Andrographis paniculata ethanolic extract	0.3gm	0.75gm	1.5gm
Aristolochea bracteolate ethanolic extract	0.3gm	0.75gm	1.5gm
Glycerine	1.5gm	1.5gm	1.5gm
Petroleum jelly	25gm	25gm	25gm

1% Ointment

Each 0.3 g mixed with 1.5 g of glycerine then kept in water bath stirred well.25g of petroleum jelly in a beaker melt over a water bath. Both phases are mixed together.

2.5% Ointment

Each 0.75g mixed with 1.5 g of glycerine then kept in water bath stirred well.25g of petroleum jelly in a beaker melt over a water bath. Both phases are mixed together.

5% Ointment

Each 1.5g mixed with 1.5 g of glycerine then kept in water bath stirred well.25g of petroleum jelly in a beaker melt over a water bath. Both phases are mixed together.

Evaluation of Topical Poly herbal ointment 1%, 2.5% and 5 %

The evaluation of Topical poly herbal ointment parameters like appearance, pH, Congealing temperature, acidity, rancidity and consistency reported on the table No: 13

Preparation of topical poly herbal cream 1%, 2.5% and 5 %[11,12]

Ingredients	1% cream	2.5% cream	5% cream
Abutilon indicum ethanolic Extract	0.3gm	0.75gm	1.5gm
Andrographis paniculata ethanolic extract	0.3gm	0.75gm	1.5gm
Aristolochea bracteolate ethanolic extract	0.3gm	0.75gm	1.5gm
Methyl paraben	51mg	51mg	51mg
Prophylparaben	90mg	90mg	90mg
Borax	300mg	300mg	300mg
Liquid paraffin	18gm	18gm	18gm
Beeswax	9gm	9gm	9gm

1% Cream

Each 0.3g of sample, Then 51 mg of methylparaben, and 90mg of propylparaben dissolved in 3ml of water. 300mg of borax dissolved in 3ml of water. 18g of liquid paraffin mixed with 9g of melted bees wax .Mix both phases together.

2.5% Cream

Each 0.75g of sample, Then 51 mg of methylparaben, 90mg of propylparaben dissolved in 3ml of water. 300mg of borax dissolved in 3ml of water. 18g of liquid paraffin mixed with 9g of melted bees wax .Mix both phases together.

5% Cream

Each 1.5g of sample, Then 51 mg of methylparaben, 90mg of propylparaben dissolved in 3ml of water. 300mg of borax dissolved in 3ml of water. 18g of liquid paraffin mixed with 9g of melted bees wax .Mix both phases together.

Evaluation of Topical Poly herbal cream 1%, 2.5% and 5 %

The evaluation of Topical poly herbal cream parameters like appearance, pH, Congealing temperature, acidity rancidity and consistency reported on the table No: 14.

Antifungal activity[13]

Fungal culture

1. The freeze dried culture of the fungus was revived as per prescribed protocol
2. The freeze dried fungus culture was added to the sterile Potato Dextrose broth (5 ml) (PDB) under aseptic conditions.
3. 50ul of the suspension was inoculated on Potato dextrose agar (PDA) plates.
4. The inoculated PDA plates were incubated at 37°C for 5 days.
5. At the end of 5 days fungal spores were harvested from the fully grown fungal cultures. For this 2ml of sterile PDB was added to the culture plates and the plates were gently rinsed and the suspension removed from plates and collected in sterile 15 ml centrifuge tubes.
6. This suspension was further filtered using double layered sterile cotton gauze to remove mycelium.
7. The suspension was centrifuged for 5min at 120g
8. The supernatant was discarded; the pelleted spores were washed and resuspended in 5ml of sterile PDB.
9. The suspension was again centrifuged for 5minutes at 120g.
10. The supernatant was discarded and the pelleted spores were resuspended in sterile PDB to obtain an OD of 0.91 and used for the antifungal assays.

Stock preparation

Stock solutions of the formulations (20mg) (ointment, gel, cream) were made in 2ml of 70% ethanol. The solutions were sonicated for 15minutes and then centrifuged (5min, 800g). The clear supernatant from these solutions were collected and used in the assays.

Treatment

500µl from the stock was added to 5ml of spore suspension. This was incubated for 4hours at 37C and then analyzed in flow cytometer.

Flow Cytometeric Analysis

Flow Cytometeric analysis was done using BD FACS calibur. 500µl of the sample was stained with 10µl Propidium iodide staining solution (PI Staining Solution: 1mg/ml PI in PBS). The samples were incubated in the dark for 10mins and then analyzed.

FACS acquisition& analysis

The samples were run on low mode. Individual cells were detected by their forward-angle (FSC) and right-angle scatter (SSC) of incident 488-nm laser light. This light scatter data plot was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. The light scatter gated PI fluorescence of individual cells was acquired by using a 485-nm band-pass filter (FL2) and displayed in single parameter dot plots and histograms. By using the cytometer's onboard software (Cell Quest Pro), the fluorescence dot plots were integrated to determine the percentage of PI^-(living) and PI^+ (dead) fungal cells.

Antibacterial activity

Bacterial culture

1. The freeze dried culture of the bacteria was revived as per prescribed protocol
2. The freeze dried bacteria culture was added to the sterile Trypticase soy broth (5 ml) under aseptic conditions.
3. 50ul of the suspension was inoculated on blood agar plates.
4. The inoculated blood agar plates were incubated at 37°C for further uses.
5. The bacterial culture inoculated in the broth was incubated overnight at 37°C.
6. The growth of the bacteria was observed by its turbidity. The OD was also measured using UV-Vis spectrophotometer. The OD_{600} was found to be 0.92.

Stock preparation

Stock solutions of the formulations (20mg) (extracts, ointment, gel, cream) were made in 2ml of 70% ethanol. The solutions were sonicated for 15minutes and then centrifuged (5min, 800g). The clear supernatant from these solutions were collected and used in the assays.

Treatment
500µl from the stock was added to 5ml of bacterial suspension. This was incubated for 4hours at 37˚C and then analyzed in flow cytometer.

Flow Cytometeric Analysis
Flow Cytometeric analysis was done using BD FACScalibur. 500µl of the sample was stained with5µl Propidium iodidestaining solution(PI Staining Solution: 1mg/ml PI in water). The samples were incubated in the dark for 10mins and then analyzed.

FACS acquisition& analysis
The samples were run on low mode. Individual cells were detected by their forward-angle (FSC) and right-angle scatter (SSC) of incident 488-nm laser light. This light scatter data plot was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. The light scatter gated PI fluorescence of individual cells was acquired by using a 630-nm band-pass filter (FL3) and displayed in single parameter dot plots and histograms. By using the cytometer's onboard software (Cell Quest Pro), the fluorescence dot plots were integrated to determine the percentage of PI^-(living) and PI^+ (dead) bacterial cells.

Bacterial culture
1. The freeze dried culture of the bacteria was revived as per prescribed protocol
2. The freeze dried bacteria culture was added to the sterile Nutrient broth (5 ml)under aseptic conditions.
3. 50ul of the suspension was inoculated on nutrient agar plates.
4. The inoculated Nutrient agar plates were incubated at 37°C for further uses.
5. The bacterial culture inoculated in the broth was incubated overnight at 37°C.
6. The growth of the bacteria was observed by its turbidity. The OD was also measured using UV-Vis spectrophotometer. The OD_{600} was found to be 0.92.

Stock preparation
Stock solutions of the formulations (20mg) (extracts, ointment, gel, cream) were made in 2ml of 70% ethanol. The solutions were sonicated for 15minutes and then centrifuged (5min, 800g). The clear supernatant from these solutions were collected and used in the assays.

Treatment
500µl from the stock was added to 5ml of bacterial suspension. This was incubated for 4hours at 37˚C and then analyzed in flow cytometer.

Flow Cytometeric Analysis
Flow Cytometeric analysis was done using BD FACScalibur. 500µl of the sample was stained with10µl Propidium iodidestaining solution(PI Staining Solution: 1mg/ml PI in water). The samples were incubated in the dark for 10mins and then analyzed.

FACS acquisition& analysis
The samples were run on low mode. Individual cells were detected using FSC and SSC detector by exciting with a 488-nm laser light. This light scatter dot plot was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. The light scatter gated PI fluorescence of individual cells was acquired by using FL2 detector and displayed as dot plot. By using the cytometer's onboard software (Cell Quest Pro), the fluorescence dot plots were integrated to determine the percentage of PI^- (living) and PI^+ (dead) bacterial cells.

Bacterial culture[14, 15]
1. The freeze dried culture of the bacteria was revived as per prescribed protocol
2. The freeze dried bacteria culture was added to the sterile Trypticase soy broth(5 ml)under aseptic conditions.
3. 50ul of the suspension was inoculated on blood agar plates.
4. The inoculated blood agar plates were incubated at 37°C for further uses.
5. The bacterial culture inoculated in the broth was incubated overnight at 37°C.
6. The growth of the bacteria was observed by its turbidity. The OD was also measured using UV-Vis spectrophotometer. The OD_{600} was found to be 0.92.

Stock preparation
Stock solutions of the formulations (20mg) (extracts, ointment, gel, cream) were made in 2ml of 70% ethanol. The solutions were sonicated for 15minutes and then centrifuged (5min, 800g). The clear supernatant from these solutions were collected and used in the assays.

Treatment
500µl from the stock was added to 5ml of bacterial suspension. This was incubated for 4hours at 37˚C and then analyzed in flow cytometer.

Flow Cytometeric Analysis
Flow Cytometeric analysis was done using BD FACScalibur. 500µl of the sample was stained with5µl Propidium iodidestaining solution(PI Staining Solution: 1mg/ml PI in water). The samples were incubated in the dark for 10mins and then analyzed.

FACS acquisition& analysis
The samples were run on low mode. Individual cells were detected by their forward-angle (FSC) and right-angle scatter (SSC) of incident 488-nm laser light. This light scatter data plot was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. PI fluorescence of individual cells was acquired by using a FL3 detector (Em = 630-nm) and displayed in dot plots. Flow cytometer's onboard software (Cell Quest Pro), was used to analyze the fluorescence dot plots and to determine the percentage of PI^-(living) and PI^+ (dead) bacterial cells.

Antifungal activity
Fungal culture

1. The freeze dried culture of the fungus was revived as per prescribed protocol
2. The freeze dried fungus culture was added to the sterile Sabouraud Dextrose broth (5 ml) (SDB) under aseptic conditions.
3. 50ul of the suspension was inoculated on Sabouraud dextrose agar (SDA) plates.
4. The inoculated SDA plates were incubated at 37°C for 5 days.
5. At the end of 5 days fungal spores were harvested from the fully grown fungal cultures. For this 2ml of sterile SDB was added to the culture plates and the plates were gently rinsed and the suspension removed from plates and collected in sterile 15 ml centrifuge tubes.
6. This suspension was further filtered using double layered sterile cotton gauze to remove mycelium.
7. The suspension was centrifuged for 5min at 120g
8. The supernatant was discarded; the pelleted spores were washed and resuspended in 5ml of sterile SDB.
9. The suspension was again centrifuged for 5minutes at 120g.
10. The supernatant was discarded and the pelleted spores were resuspended in sterile SDBto obtain an OD of 0.8 and used for the antifungal assays.

Stock preparation
Stock solutions of the formulations (20mg) (ointment, gel, cream) were made in 2ml of 70% ethanol. The solutions were sonicated for 15minutes and then centrifuged (5min, 800g). The clear supernatant from these solutions were collected and used in the assays.

Treatment
500µl from the stock was added to 5ml of spore suspension. This was incubated for 4hours at 37C and then analyzed in flow cytometer.

Flow Cytometeric Analysis
Flow Cytometeric analysis was done using BD FACS calibur. 500µl of the sample was stained with 10µl Propidium iodide staining solution (PI Staining Solution: 1mg/ml PI in PBS). The samples were incubated in the dark for 10mins and then analyzed.

FACS acquisition& analysis
The samples were run on low mode. Individual cells were detected by their forward-angle (FSC) and right-angle scatter (SSC) of incident 488-nm laser light. This light scatter data plot was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. The light scatter gated

PI fluorescence of individual cells was acquired by using a 630-nm band-pass filter (FL3) and displayed in single parameter dot plots and histograms. By using the cytometer's onboard software (Cell Quest Pro), the fluorescence dot plots were integrated to determine the percentage of PI^-(living) and PI^+ (dead) fungal cells.

RESULTS

Table 12: Evaluation of Topical Poly herbal Gel 1%, 2.5% and 5%

S.No	Test parameters	1% Gel	2.5%Gel	5% Gel
1.	Appearance	Brown coloured gel with pleasant odour	Brown coloured gel with pleasant odour	Brown coloured gel with pleasant odour
2.	pH (1% Solution)	3.60	3.80	3.88
3.	Congealing temperature	40^0c	41^0c	43^0c
4.	Acidity	0.5ml of M/10 NaoH	0.6ml of M/10NaoH	0.8ml of M/10 NaoH
5.	Rancidity	No pink colour develops	No pink colour develops	No pink colour develops
6.	Consistency	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles

Table 13: Evaluation of Topical Poly herbal ointment 1%, 2.5% and 5%

S.No	Test parameters	1% Ointment	2.5%Ointment	5%Ointment
1.	Appearance	Greenish brown coloured ointment with pleasant odour	Greenish Brown coloured ointment with pleasant odour	Brown coloured ointment with pleasant odour
2.	pH (1% Solution)	3.68	3.76	3.82
3.	Congealing temperature	40^0c	42^0c	44^0c
4.	Acidity	1.0ml of M/10 NaoH	1.1ml of M/10NaoH	1.3ml of M/10 NaoH
5.	Rancidity	No pink colour develops	No pink colour develops	No pink colour develops
6.	Consistency	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles

Table 14: Evaluation of Topical Poly herbal Cream 1%, 2.5% and 5%

S.No	Test parameters	1% Cream	2.5% Cream	5% Cream
1.	Appearance	Cream coloured with pleasant odour	Light brownish cream coloured cream with pleasant odour	Brown coloured cream with pleasant odour
2.	pH (1% Solution)	3.62	3.64	3.69
3.	Congealing temperature	40^0c	42^0c	44^0c
4.	Acidity	0.9ml of M/10 NaoH	1.1ml of M/10NaoH	1.3ml of M/10 NaoH
5.	Rancidity	No pink colour develops	No pink colour develops	No pink colour develops
6.	Consistency	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles	Excellent smoothness and free from gritty particles

Antifungal activity

Effect of different formulations on PI staining of *Microsporum canis* fungal spores

Antifungal effects can be detected by flow cytometric analysis of PI-treated fungal spores. The effects of the fungal spores are displayed in Table 1. In Fig. 1A, the PI fluorescence for untreated ***Microsporum canis*** spores illustrates the auto fluorescence profile attained. Figures 1B to G display the PI fluorescence for the extracts and formulations treated with fungal spores. The cells that developed PI fluorescence intensity greater than that noted for the untreated control cells were defined as **PI^+**. It is evident from the dot plots that **T3 (**Abutilon indicum) **(**77.21**%)** and **T6 (**2.5% cream) **(**78.315**%)** has the highest antifungal activity among the extract and formulations group respectively. **T2 (**Andrographis paniculata) **extract** showed the highest viability of 74.415**%** while **T1 (**Aristolochea bracteolate) had much lower percentage of live cells (2.63**%).** Among the formulations of ointment, cream and gel, the **T5 (**2.5% ointment) formulation showed the highest viability **(**74.24**%)** followed by **T4 (**2.5% gel) with 62.58**%** viable cells.

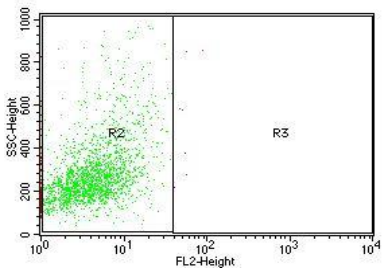

Figure 1A: control

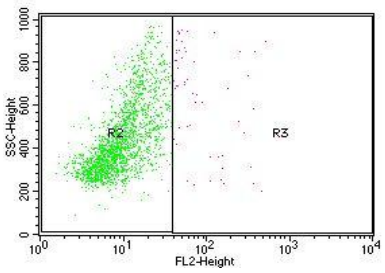

Figure 2B: T1

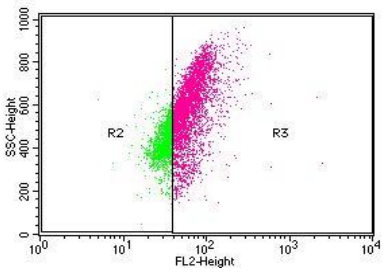

Figure 3C: T2

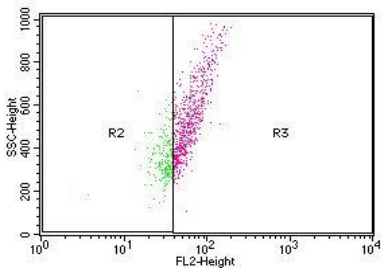

Figure 4D: T3

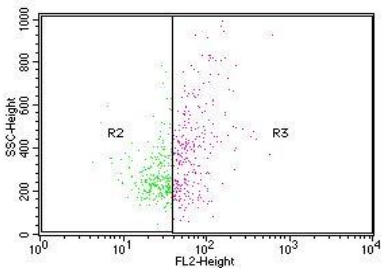

Figure 5E: T4

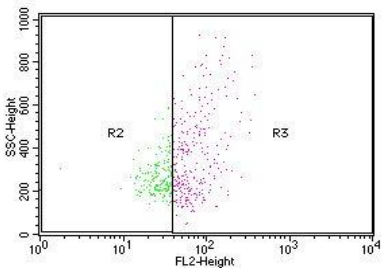

Figure 6F: T5

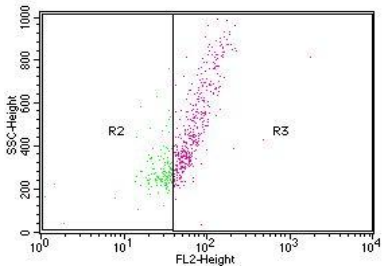

Figure 7G: T6

Fig. 1: Representative figures of flow cytometric dot plots of antifungal activity of the formulations on *Microsporum canis*

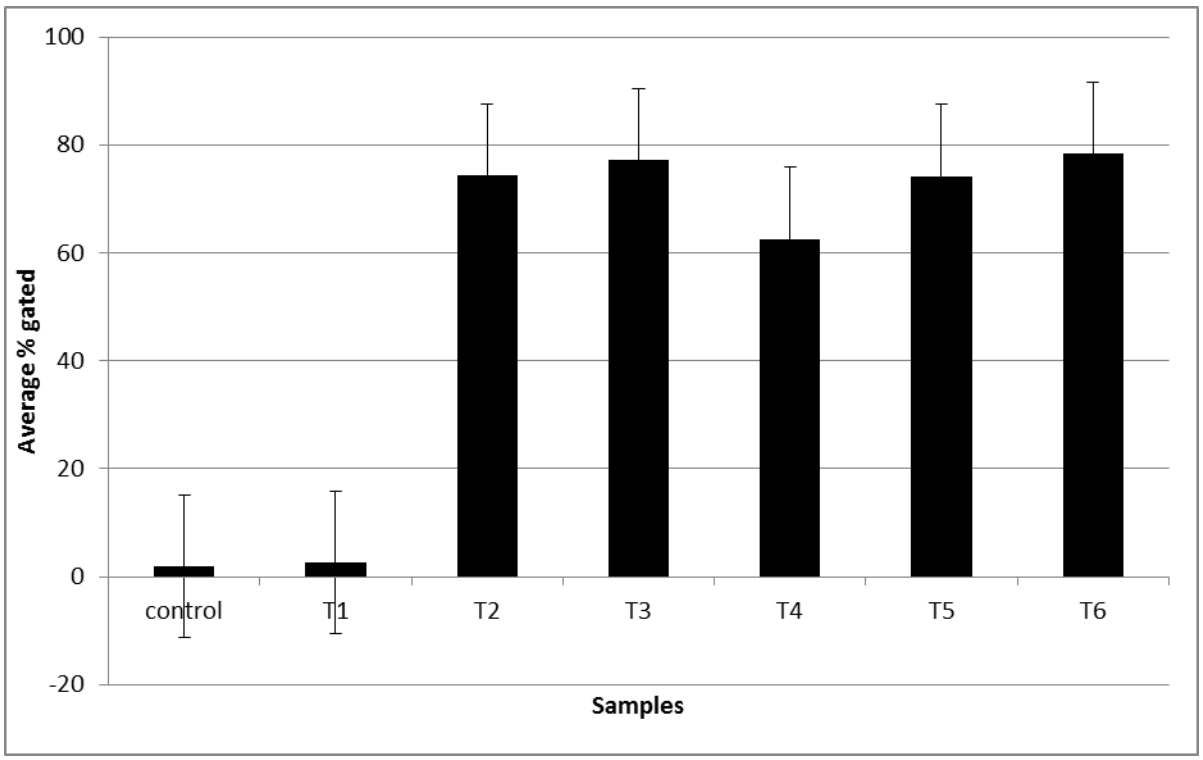

Chart 1: Gated percentage of PI Positive cells in the different formulations. Error bars represent the SD from the mean of at least duplicate readings. These data is representative of 2 independent experiments. *p* value was calculated with reference to no extract values and was <0.0001.

Table 1: Data Summary
Antibacterial activity

Samples	PI +ve gated	variance	std deviation
control	1.94	0.0098	0.098
T1	2.63	0.0968	0.311
T2	74.415	1.05125	1.025
T3	77.21	0.2312	0.480
T4	62.58	0.6272	0.791
T5	74.24	0.1058	0.325
T6	78.315	0.22445	0.473

Effect of different formulations on PI staining of *Propionibacterium acnes*

Antibacterial effects can be detected by flow cytometric analysis of PI-treated bacterial suspension. The effect of the bacteria is displayed in Table 1. In Fig. 1A, the PI fluorescence for untreated ***Propionibacterium acnes*** illustrates the autofluorescence profile attained. The dot plots were analyzed and it was found that **T2 (**Andrographis paniculata) had **52.32%** while **T5 (**2.5% ointment) had **45.13%**of dead cells among the extract and formulations group respectively. **T1(**Aristolochea bracteolate) **extract** had **30.07% of PI positive cells**while **T3(**Abutilon indicum) had **24.94%.** Among the formulations of ointment, cream and gel, the **T6 (**2.5% cream) formulation showed cell death of **35.48%** followed by **T4(**2.5% gel) with **27.98%**.

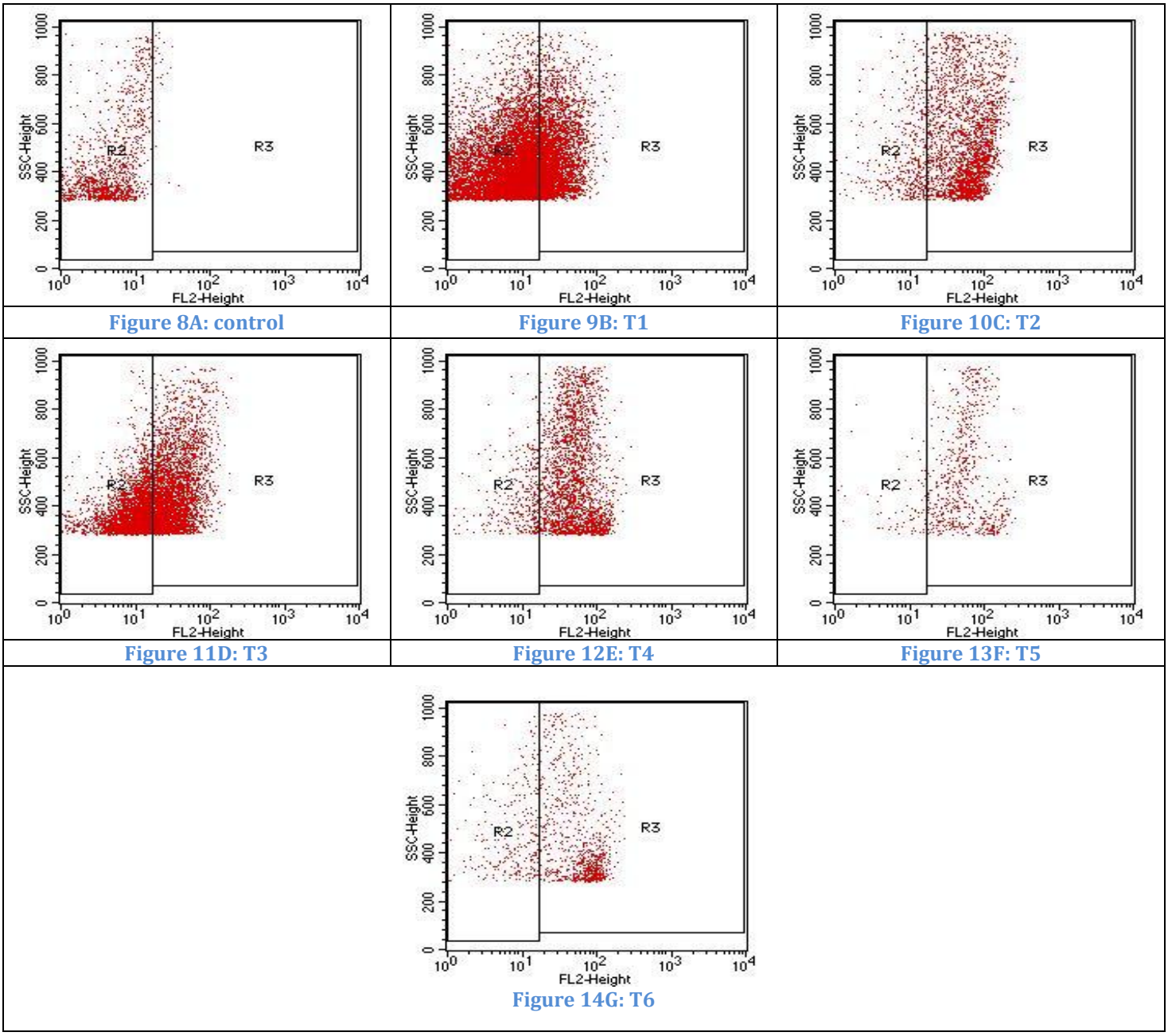

Figure 8A: control | Figure 9B: T1 | Figure 10C: T2

Figure 11D: T3 | Figure 12E: T4 | Figure 13F: T5

Figure 14G: T6

Fig. 1: Representative figures of flow cytometric dot plots of antibacterial activity of the formulations on *Propionibacterium acnes*

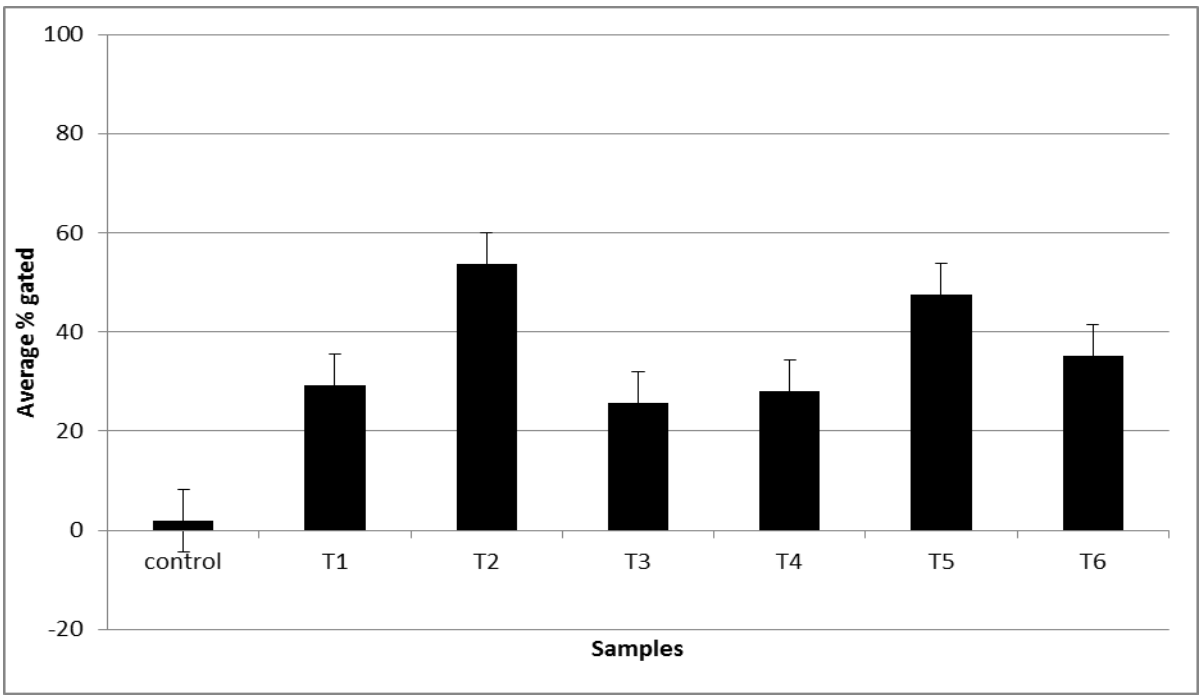

Chart 1: Gated percentage of PI Positive cells in the different formulations. Error bars represent the SD from the mean of at least duplicate readings. These data is representative of 2 independent experiments. *p* value was calculated with reference to no extract values and was <0.0001

Table 1: Data Summary

Data Summary							
	control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Live cells gated %							
PI $^{+ve}$ gated %	2.01	30.07	53.32	24.94	27.98	45.13	35.48
Mean	1.94	29.245	53.665	25.675	28.065	47.51	35.13
Variance	0.0098	1.3612	0.2380	1.0804	0.0144	2.8888	0.245
Std.Dev.	0.0989	1.1667	0.4879	1.0394	0.1202	0.537	0.4949
Std.Err.	0.137	6.309	3.574	5.266	0.636	3.704	2.933

Effect of different formulations on PI staining of Streptococcus aureus

Antibacterial effects can be detected by flow cytometric analysis of PI-stainedbacterial suspension. Among the individual plant extracts **T2 (**Andrographis paniculata) showed significantly higher percentage (**63.16%)** of cell death (Chart 1) compared to the control group which showed only **7.56 %** of cell death. The other two plant treatments **T1 (**Aristolochea bracteolate) and **T3 (**Abutilon indicum) did not show significant antibacterial activity against *Streptococcus aureus* (**6.85% and 13.35% respectively**) (Table 1).

However, among the plant formulations, all three formulations of ointment, cream and gel (T4 (2.5% gel), T5 (2.5% ointment) and T6 (2.5% cream) showed significantly higher activity of **73.51%**, **79.30%** and **70.7%** compared to the control (Table 1).

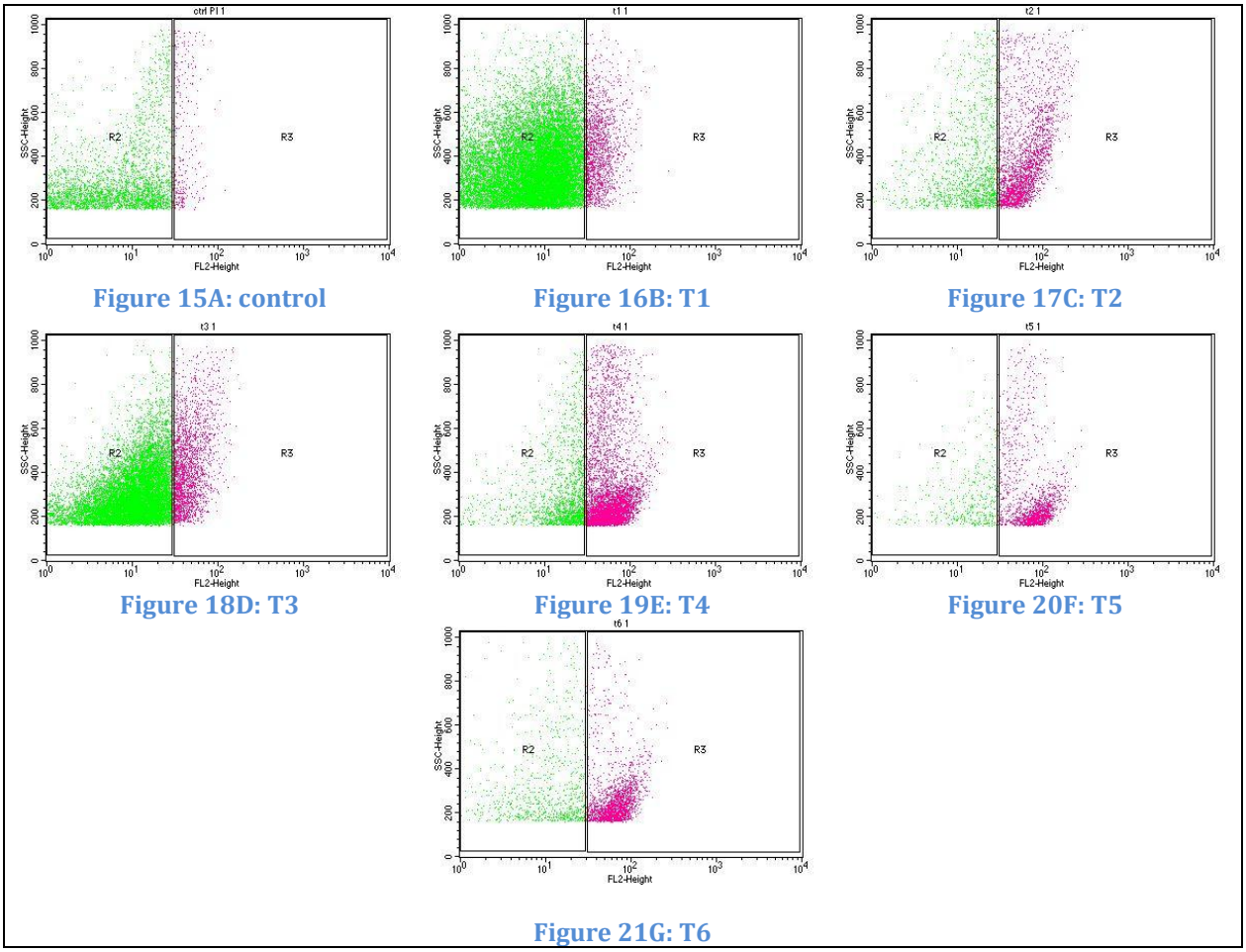

Figure 15A: control Figure 16B: T1 Figure 17C: T2

Figure 18D: T3 Figure 19E: T4 Figure 20F: T5

Figure 21G: T6

Fig. 1: Representative figures of flow cytometric dot plots of antibacterial activity of the formulations on *Streptococcus aureus*.

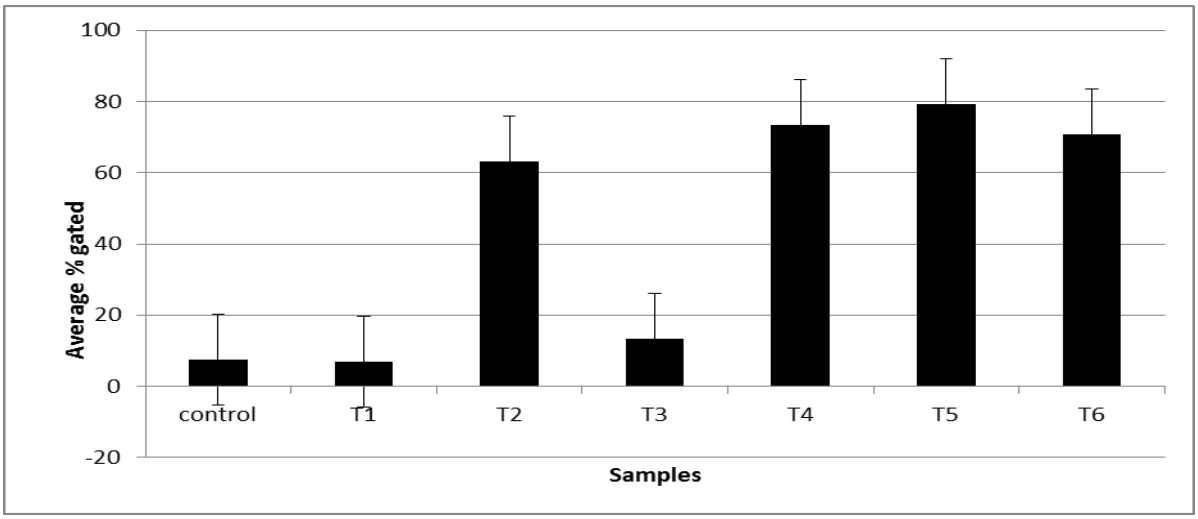

Chart 1: Gated percentage of PI Positive cells in the different formulations. Error bars represent the SD from the mean of at least duplicate readings. These data is representative of 2 independent experiments. *p* value was calculated with reference to no extract values and was <0.0001.

Table 1: Data Summary

Data Summary							
	control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Live cells gated %	91.38	92.39	32.25	85.62	24.71	19.42	28.08
PI $^{+ve}$ gated %	7.56	6.85	63.16	13.35	73.51	79.30	70.7
Mean	8.56	7.105	63.54	13.625	73.02	79.54	70.2
Variance	2	0.13005	0.2888	0.15125	0.4802	0.1152	0.5
Std.Dev.	1.414214	0.360624	0.537401	0.388909	0.692965	0.339411	0.707107
Std.Err.	1	0.255	0.38	0.275	0.49	0.24	0.5

Effect of different formulations on PI staining of *Streptococcus pyogenes*

Antibacterial effects can be detected by flow cytometric analysis of PI-treated bacterial suspension. The effect of the formulation and plant extract on bacteria is displayed in Table 1. In Fig. 1A, the PI fluorescence for untreated **Streptococcus pyogenes** illustrates the auto fluorescence profile attained. The dot plots were analyzed, **T2 (**Andrographis paniculata) with **54.01%** and **T5 (**2.5% ointment) with **49.13%**of PI +ve cells had the most antimicrobial activity among the extract and formulation groups respectively. In individual plant extracts, **T1 (**Aristolochea bracteolate) **extract** had **31.07%**and **T3 (**Abutilon indicum) had **26.32%**of PI positive cells while**.** Among the formulations of ointment, cream and gel, the **T6 (**2.5% cream) formulation showed cell death of **37.81%** followed by **T4 (**2.5% gel) with **26.41%**.

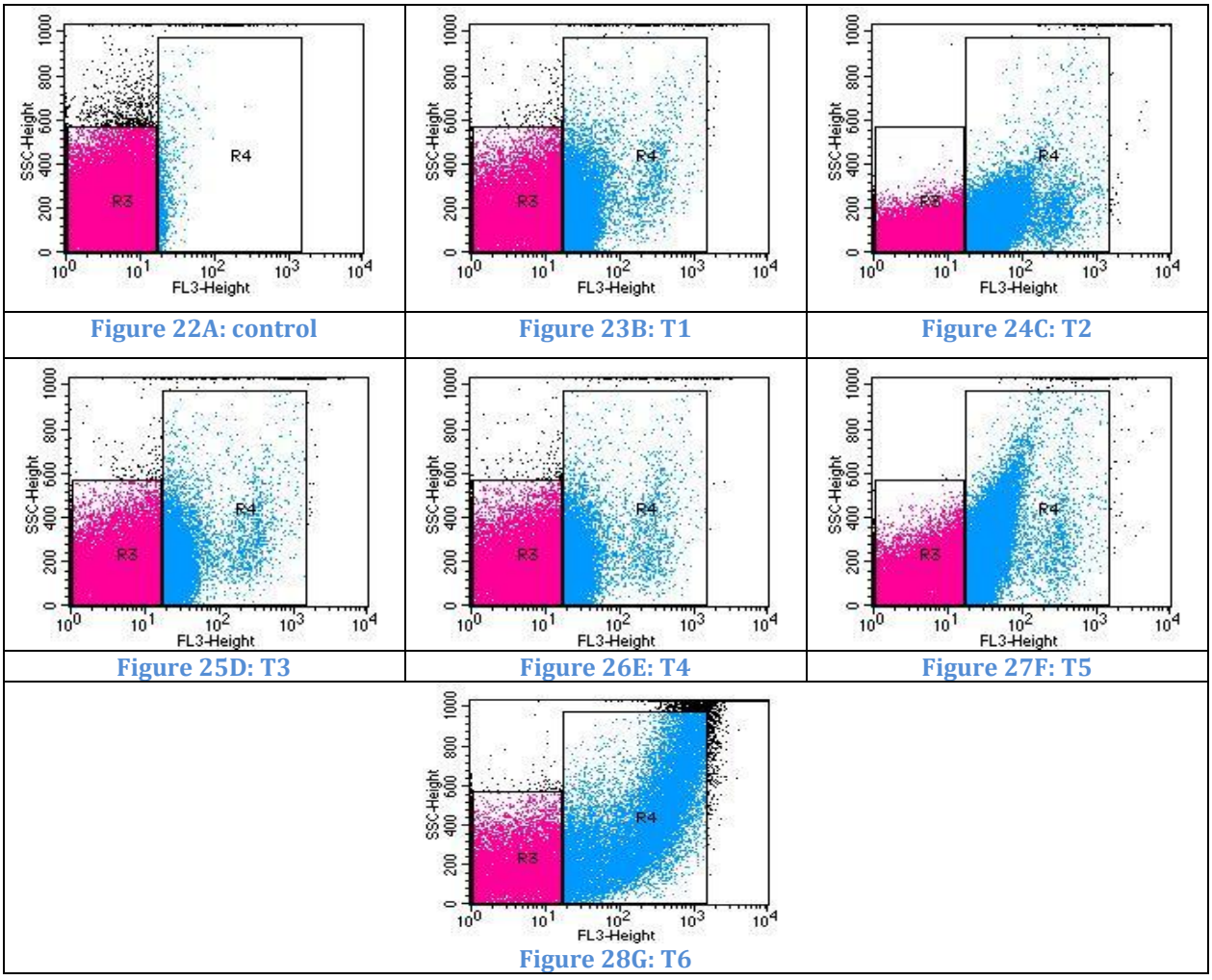

Figure 22A: control | Figure 23B: T1 | Figure 24C: T2

Figure 25D: T3 | Figure 26E: T4 | Figure 27F: T5

Figure 28G: T6

Fig. 1: Representative figures of flow cytometric dot plots of antibacterial activity of the formulations on *Streptococcus pyogenes*

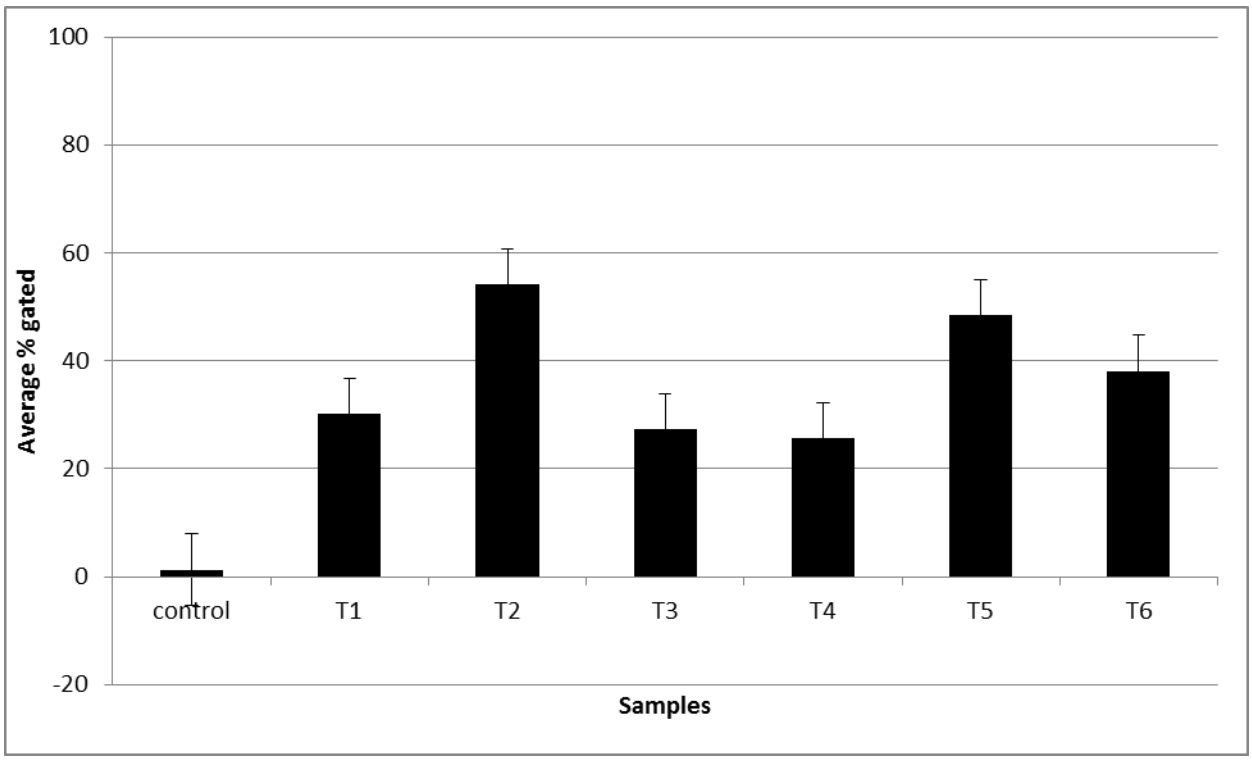

Chart 1: Gated percentage of PI Positive cells in the different formulations. Error bars represent the SD from the mean of at least duplicate readings. These data is representative of 2 independent experiments. *p* value was calculated with reference to no extract values and was <0.0001

Table 1: Data Summary

Data Summary							
	control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Live cells gated %		56.59	31.44	60.49	60.09	39.46	29.33
PI $^{+ve}$ gated %	1.32	31.07	54.01	26.32	26.41	49.13	37.81
Mean	1.305	30.245	54.885	27.23	25.675	48.51	38.135
Variance	0.00045	1.3612	1.531	1.656	1.080	0.768	0.211
Std.Dev.	0.0212	1.166	1.237	1.286	1.039	0.876	0.459
Std.Err.	0.024	6.416	9.167	6.715	5.266	6.106	2.838

Effect of different formulations on PI staining of Trichophyton rubrum fungal spores

Antifungal effects can be detected by flow cytometric analysis of PI-treated fungal spores. The effects of the fungal spores are displayed in Table 1. In Fig. 1A, the PI fluorescence for untreated *Trichophyton rubrum* spores illustrates the autofluorescence profile attained. Figures 1B to G display the PI fluorescence for the extracts and formulations treated with fungal spores. The cells that developed PI fluorescence intensity greater than that noted for the untreated control cells were defined as **PI^{+}**. It is evident from the dot plots that **T3 (**Abutilon indicum) **(76.485%)** and **T6 (**2.5% cream) **(91.33%)** has the highest antifungal activity among the extract and formulations group respectively. **T1(**Aristolochea bracteolate) **extract** showed the highest viability of **96.54%**while **T2 (**Andrographis paniculata) had much lower percentage of live cells (**26.465%).** Among the formulations of ointment, cream and gel, the **T5 (**2.5% ointment) formulation showed the highest viability **(22.57%)** followed by **T4 (**2.5% gel) with **46.235%** viable cells.

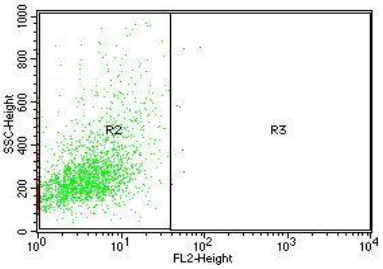

Figure 29A: control

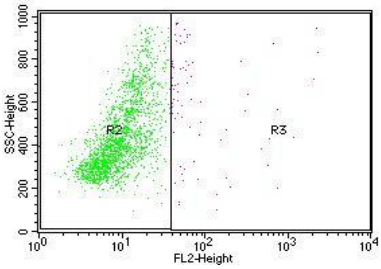

Figure 30B: T1

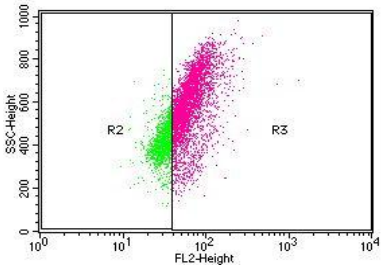

Figure 31C: T2

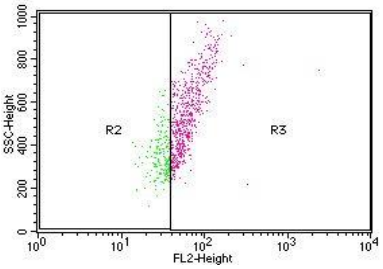

Figure 32D: T3

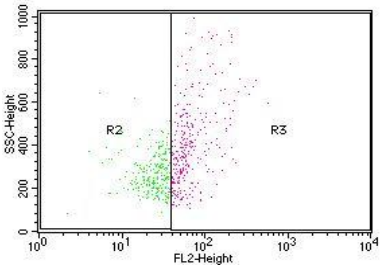

Figure 33E: T4

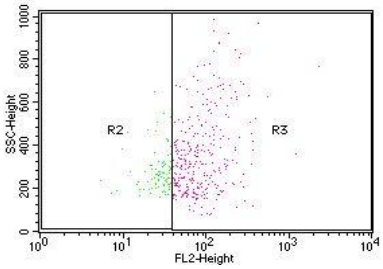

Figure 34F: T5

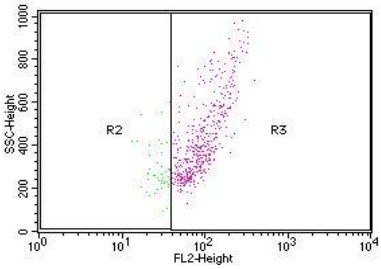

Figure 35G: T6

Fig. 1: Representative figures of flow cytometric dot plots of antifungal activity of the formulations on *Trichophyton rubrum*

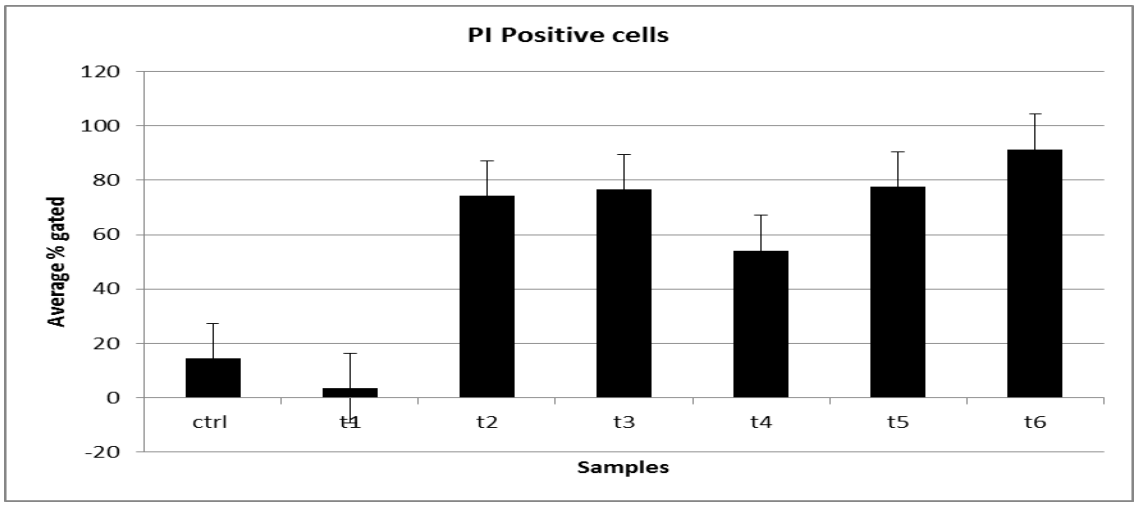

Chart 1: Gated percentage of PI Positive cells in the different formulations. Error bars represent the SD from the mean of at least duplicate readings. These data is representative of 2 independent experiments. *p* value was calculated with reference to no extract values and was <0.0001

Table 1: Data Summary

Data Summary							
	control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Live cells gated %	85.68	96.54	26.465	24.1	46.235	22.57	8.95
PI $^{+ve}$ gated %	14.5	3.485	74.22	76.485	54.145	77.56	91.33
Mean	14.5	3.485	74.22	76.485	54.145	77.56	91.33
Variance	0.3042	0.8064	0.5618	2.2684	2.0605	2.3762	1.3122
Std.Dev.	0.5515	0.898	0.7495	1.5061	1.4354	1.5415	1.1455
Std.Err.	0.39	0.635	0.53	1.065	1.015	1.09	0.81

CONCLUSION

Flow cytometric analysis revels that T3 (Abutilon indicum) is a highly potent and rapidly acting fungicidal extract against ***Microsporum canis*** when compared to T1(Aristolochea bracteolate) and T2(Andrographis paniculata). All the three formulations of ointment, cream and gel had a comparatively higher antifungal activity compared to the individual extracts. Among the formulations all the formulations (T4 (2.5% gel), T5 (2.5% ointment), T6 (2.5% cream)) showed significant activity against ***Microsporum canis*** with the formulation T6 showing the highest activity among the three.

Flow cytometric analysis reveals that T2 (Andrographis paniculata) is a highly potent and rapidly acting bactericidal extract against ***Propionibacterium acnes***when compared to T1(Aristolochea bracteolate) and T3(Abutilon indicum). All the three formulations of ointment, cream and gel had a comparatively higher antibacterial activity compared to the individual extracts. All the formulations (T4(2.5% gel) T5(2.5% ointment), T6(2.5% cream) showed significant activity against ***Propionibacterium acnes*** with the formulation T5 (2.5% ointment)showing the highest activity among the three.

Flow cytometric analysis reveals that among individual plant extracts T2 (*Andrographis paniculata*) is highly potent and rapidly acting bactericidal extract against *Streptococcus aureus* when compared to T1 (*Aristolochea bracteolate*) and T3 (*Abutilon indicum*). All the three formulations of ointment, cream and gel had a comparatively higher antibacterial activity compared to the individual extracts. All the formulations (T4 (2.5% gel), T5 (2.5% ointment), T6 (2.5% cream) showed significant activity against *S. aureus* with the formulation T5 (2.5% ointment) showing the highest activity among the three.

Flow cytometric analysis reveals that T2 (Andrographis paniculata) is a highly potent and rapidly acting bactericidal extract against ***Streptococcus pyogenes*** when compared to T1 (Aristolochea bracteolate) and T3 (Abutilon indicum). All the three formulations of ointment, cream and gel had a comparatively higher antibacterial activity compared to the individual extracts. Among the formulations all the formulations (T4 (2.5% gel), T5 (2.5% ointment), T6 (2.5% cream) showed significant activity against ***Streptococcus pyogenes*** with the formulation T5 (2.5% ointment) showing the highest activity among the three.

Flow cytometric analysis revels that T3 (*Abutilon indicum*) is a highly potent and rapidly acting fungicidal extract against *Trichophyton rubrum* when compared to T1 (*Aristolochea bracteolate*) and T2 (*Andrographis paniculata*). All the three formulations of ointment, cream and gel had a comparatively higher antifungal activity compared to the individual extracts. Among the formulations all the formulations (T4 (2.5% gel), T5 (2.5% ointment), T6 (2.5% cream) showed significant activity against *T. rubrum* with the formulation T6 (2.5% cream) showing the highest activity among the three.

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