ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
**Dutse Journal of Pure and Applied Sciences (DUJOPAS),** *Vol. 6 No. 1 March 2020*

# Antifungal Evaluation of *Cucurbita Pepo* Seed extracts against some Dermatophytes

***[1]Halima Isah, [2]Muhammad Yusha'u**
[1]Government Science and Technical College
Hadejia, Jigawa State, Nigeria

[2]Department of Microbiology
Bayero University, Kano, Nigeria
Email: halimaisah84@gmail.com

## Abstract
*A research on antifungal activity of Cucurbita pepo seed extracts against some dermatophytes was conducted. Extraction of C. pepo was achieved using soxhlet apparatus with methanol, dichloromethane and petroleum ether as solvents. The extracts were subjected to phytochemical screening for qualitative detection of plant secondary metabolites using standard procedures. Antifungal activity of the extracts against human isolates of Microsporum canis,Trichophyton terrestre, Trichophyton mentagrophytes and Trichophyton verrucosum, was determined using agar well diffusion method. Phytochemical screening revealed the presence of saponins, alkaloids, flavonoids, tannins, steroids and glycosides in all the extracts. The results of antifungal activity showed that all the extracts were active against the isolates tested. The methanolic extract has the highest activity followed by dichloromethane extract, while petroleum ether extract produced lowest zone of inhibition. The zone of inhibition ranges from 10mm to 30mm at concentration of 6.25 to 50mg/ml, for methanolic extract inhibition ranges from 10mm to 30mm, 10mm to 28mm for dichloromethane and 10 to 24mm for petroleum ether with M. canis exhibiting the highest* zone of inhibition(30mm). The result obtained shows that *C pepo* possess antifungal property.

**Keywords:** *Cucurbita pepo*, Antifungal activity, *Microsporum canis, Trichophyton terrestre, Trichophytonmentagrophytes and Trichophyton Verrucosum*.

## INTRODUCTION
Plants are important sources of medicines since the beginning of human civilization. In spite of tremendous developments in the field of allopathy during the 20th century, plants still continue to be the major source of drugs in modern as well as traditional medicine throughout the world. Approximately one third of all pharmaceuticals are of plant origin with over 60% of all pharmaceuticals being plant based (Sulaiman and Suresh, 2002).

Presently trend is more towards everything "natural", medicinal plants are value added for the content and chemical composition of their active components. In a wider context, there is a growing demand for plant-based medicines, health products, pharmaceuticals, food supplements, cosmetics etc. (Vennaposa *et al*., 2013).

Plants are important source for the discovery of new products of medicinal value for drug development and their secondary metabolites are unique sources for pharmaceuticals, food

**Author for Correspondence*

additives, flavors and other industrial values. Commercial importance of these secondary metabolites has resulted in a great interest in its production and in exploring possibilities of enhancing its production by means of tissue culture technology in the recent years. Plants cell culture technologies were introduced at the end of 1960's as a possible tool for both studying and producing plant secondary metabolite (Tiwara and Rana, 2015).

There is increased prevalence of antibiotics and antifungal resistance among organisms emerging from the extensive use of antimicrobes which renders current amtimicrobial agents insufficient to control atleast some infection (Riffel *et al*., 2002).

Superficial dermatophytoses affecting skin, hair and nail are among the most common public health problem in hot and humid climate of tropical countries(Pramod, 2015). Cutaneous mycoses are mostly caused by keratinophilic filamentous fungi called dermatophytes and are classified into three genera: *Trichophyton, Microsporum* and *Epidermophyton*(Pramod, 2015). So far, about 40 species of dermatophytes have been identified as human pathogens. Although infections caused by dermatophytes are generally limited to the surface regions of the skin, these fungi can behave in a manner invasive, causing deeper and disseminated infection, especially in immune compromised patients(Rodwell *et al. 2008).* World Health Organization estimates dermatophytes affect about 25% of the world population. Mycoses may have significant negative social, psychological, and occupational health effects and can compromise the quality of life significantly. Early recognition and treatment is essential to reduce morbidity and possibility of transmission. Treatment of dermatophytosis is generally long and costly. Dermatophytosis are often associated with relapses following the interruption of antifungal therapy. Recently, clinical failure has been observed in patients treated with antifungals and drug resistance has become an important problem(Pramod,2015). Although the prevalence of drug resistance in fungi is below that observed in bacteria, mycologists now believe that selective pressure will, over time, lead to more widespread resistance. Dermatophytoses are frequently associated with relapses following the interruption of antifungal therapy(Stephenson, 1997).Clinical resistance to antifungal agents was rare until the late 1990s, with only isolated cases in patients with chronic mucocutaneous candidiasis (Marichal and Vanden,1995).

*Cucurbita pepo* (pumpkin) belongs to the family *Cucurbitaceae* which includes cucumbers, melons, squash and gourds. The word 'pumpkin' comes from a Greek word "pepon" meaning large melon. While the English name is pumpkin or pompion, a term which dated as far back as 1547BC. The Cucurbitaceae include the genera of Citrullus e.g. *C. lanatus* (watermelon), Cucumis e.g. *C. melo* (Cucumber) (Bradley, 1992).

Ethnopharmacological studies show that *Cucurbita pepo* is used in many countries for treating numerous diseases, e.g., as an anti-inflammatory, antiviral, analgesic urinary disorders, anti-ulcer, antidiabetic and antioxidant (Smith, 1997; Wang *et al.,* 2007). Traditional medicine, particularly Ayurvedic systems (Ding *et al.,* 2002), and Chinese (Chaturvedi, 2012) have used different parts of the plant including flesh of the fruits and seeds (Caili *et al.,* 2006). In 2012 Chaturvedi, report that pumpkin exhibiting important physiological properties as wound healing, tumor growth inhibition, hypoglycemic effects and immunomodulating. The seeds are used as a vermifuge, treat problems of the urinary system, hypertension, prevents the formation of kidney stones, alleviate prostate diseases, and enhanced the erysipelas skin infection (Dhiman *et al.,* 2012; Ginley, 2011).

## MATERIAL AND METHODS

### Collection, Identification and handling of plant materials

The plant used for this research was *Cucurbita pepo (*seed)which was obtained from International Institute for Tropical agricultural research (IITA) at Tarauni LGA, Kano state.The pumpkin was properly washed and sliced using knife, the seeds were removed .The plant part was identified and confirmed with voucher specimens at the Department of Plant Biology, Bayero University Kano. Seed were air dried by spreading on sacks in an aerated room. This was then grounded in to powdered form using pestle and mortar in the laboratory, then sieved and packed in a clean, sterile container as described by Mukhtar and Tukur (1999).

### Extraction procedure

One thousand (1000) ml of solvents (methanol, dichloromethane and pet-ether) was added in to a round bottom flask on to which thimble containing hundred grams (100g) of powdered plant material (i.e. one after the other). The solvents were heated using heating mantle and begin to evaporate, moving through the apparatus to the condenser. The condensate then dropped in to the reservoir containing the thimble, once the level of the solvent reaches the siphon it pours back in to the flask and the cycle repeats. The process was run exhaustively. The solvents were evaporated using a rotary evaporator leaving the yield of extracted material in the flask. All extracts were kept at $4^{o}C$ until use as demonstrated by Fatope and Hamisu, (1997).

### Determination of some physical properties of the plant extract

The colour was assessed immediately after the removal of the solvent by evaporation process. Texture was assessed manually with the help of glass rod and feeling of the particulate nature of the resultant fraction in between globe fingers as described by Adoum *et al.;*(1997)

### Phytochemical screening

### Test for saponins

This was carried out as demonstrated by Sofowora (1993), plant extract (1ml) was transferred into a test-tube. Distilled water will be added to the test-tube and shaken vigorously. Persistent froth that last for about 15 minutes will indicate the presence of saponins.

### Test for tannins

This was done by measuring two milliliter of each of the extracts and diluted with distilled water in separate test tube and 2-3 drops of 5% ferric chloride ($FeCl_3$) was added. A green-black or blue coloration will indicate the presence of tannin as described by Ciulci, (1994).

### Test for flavonoids

To 4ml of each of the fractions a piece of magnesium ribbon was added followed by conc. HCl drop wise. A colour ranging from orange to red magenta indicates flavonoids (Sofowora, 1993).

### Test for alkaloids

This was carried out according to the method reported by Ciulci (1994). To 1.0ml of each extract in separate test tube, 2-3 drop of Meyer's reagent was added. A white precipitate indicates the presence of alkaloids.

**Test for steroids**
Two (2) mls of each extract was transferred in to a test tube containing chloroform (2ml), conc. $H_2S0_4$was subsequently added to form a lower layer. A reddish brown ring at the interface of the two liquids and a violet colour in the supernatant layer indicate the presence of the steroids (Sofowora, 1993).

**Test for glycosides**
Ten ml of 50% of $H_2S0_4$ was added to 1mI of the extract in a test tube, the mixture was heated for 15 minutes. Ten milliliters of Fehling solution was added and the mixture boiled. Appearance of brick red precipitate indicates the presence of glycosides (Sofowora, 1993).

**Test for terpenoids**
Two (2) ml of chloroform was added to 0.5ml of the extract, 3ml of conc. $H_2S\ 0_4$ was added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids (Ciulci, 1994).

**Test for anthraquinone:-**
Zero point five (0.5) ml of the extract was taken in to a dried test-tube and 5ml of chloroform was added and shacked for 5min. The extract was filtered and a drops of ammonia solution was added and shacked vigorously. A pink violet or red color in the ammoniacal layer (lower layer) indicates positive results (Ciulci, 1994).

**TEST ORGANISM USED**
The test micro-organisms used for the antifungal activity screening are *Trichophyton verrucosum*, *Trichophyton mentagrophytes*, *Trichophyton and Microsporum canis*. Samples were isolated from infected individuals at "Tsangayar Almajirai' at Daurawa Tarauni LGA Kano State, cultured and identified as described by (David, 1989;Ellis *et al.,* 2007;Polari *et al.,* 2015).

**Preparation of the different concentration of the extract**
Different concentrations of the extracts were prepared by serial doubling dilution in dimethyl sulfoxide (DMSO) from an initial stock solution to make the require concentration of 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml respectively as reported by Vashka (2009).

**Inoculum Standardization**
The inocula were prepared as described by Guarro *et al.,* (1998) Strains were sub cultured on SDA at $25^0$C for 3-5 days. The inoculum was prepared by flooding the surfaces of the subculture plates with 1% tween 80 and scraping the sporulated aerial mycelium with a loop. The suspensions obtained were then filtered through sterile gauze to remove the majority of hyphae and adjusted with a spectrophotometer at 530nm to have $5\times10^5$CFU/ml.

**Antifungal Assay**
A sterile Sabouraud's Dextrose Agar media incorporated with chloromphenicol and cyclohexamide was poured in to Petri plates. As soon as the agar solidified, five wells were made in to the plates with the help of a borer sterilized with alcohol and flame. A sterile swab was used to evenly distribute the standardized inoculums over the surface of the sterile sabouraud's dextrose agar; the plates were allowed to stand for 15minutes before use in the test. The extracts concentrations (0.1ml) were introduce in to the wells. The antibiotic (ketaconazole) was introduced in to the central well to serve as control. The plates were labeled and left to stand for about 30 minutes before 72hrs incubation at $25^o$C. The activity was determined by measuring the diameter of zone of inhibition (zones of clearance

produced around the or well). The diameter of inhibition zones was measured in mm and the results were recorded. The assay was done in triplicate (Jagesser, 2008).

## RESULTS

Physical characteristics of the plant extracted are presented in Table 1. From the data presented, the initial weight of the plant powder was 200g, which yielded (40%, 40% and 20% with methanol, dichloromethane and petroleum ether extracts respectively). Phytochemical screening of the plant extract showed that saponins, alkaloids, tannins, steroids, glycosides and flavonoids were present in all the extracts. However, only terpenoids and anthraquinone were absent (Table 2).

The result of sensitivity test showed that all the extract of *Cucurbita pepo* seeds were active against the tested isolates. The methanolic extract has the highest activity followed by dichloromethane extract, while petroleum ether extract produces lowest zone of inhibition. The zone of inhibition ranges from 10mm to 28mm at concentration of 6.25 to 50mg/ml, for methanolic ranges from 10mm to 30mm, 10mm to 28mm for dichloromethane and 10 to 24mm for petroleum ether. The methanolic extract of *C. pepo*seed inhibited the growth of all the tested organisms with exception of *Trichophyton terrestre*, which was resistant at all the concentrations used. It produces a high zone of inhibition against *M. canis* with 30mm zone of inhibition, which is similar to that produce by the ketaconazole(standard antifungal) (Table 3). The dichloromethane seed extracts produces high zone of inhibition on *T. mentagrophytes* with 28mm diameter zone of inhibition than the standard antifungal (ketoconazole) (Table 3), The petroleum ether extract exhibited activity against all the tested isolates with the exception of *M. canis and T. verrucosum,* which are resistant at all the concentrations used.

## DISCUSSION

In this study methanol and dichlromethane solvent produces a higher amount of extracts, while the petroleum ether yielded the least amount of extracts. Phytochemical screening of the plant extract showed that saponins, alkaloids, tannins, steroids, glycosides and flavonoids were present in all the extracts, which corresponds with the work of Chonoko *et a*l., (2011) and Perez, (2016), where saponins, tannins, flavonoids, alkaloids and steroids were present. Also Bourgard *et al.,* (1994) reported that the significant antifungal activity exhibited by the plant materials may be linked to the presence of steroids flavonoids, tannins, alkaloids and saponins which were reported to possess antimicrobial activity. However, anthraquinone and terpenone were absent, Table 2.

The presence of phenolic compounds predominantly in the seed and pulp of *Cucurbita pepo* such as alkaloids, saponins and steroids which have been found to be used asanti-inflammatory and anti- oxidant agents (David, 1989). Waterman (1992) also reported that the main class of phenolic predominant in both the fruit and leaf extract were alkaloids and flavonoids which are found useful in medicine as antimicrobial, anti-inflammatory and anti-oxidant agents.

The result of sensitivity test showed that all the extract of *Cucurbita pepo* seeds were active against the tested isolates. The methanolic extract has the highest activity(10mm-30mm) followed by dichloromethane extract (10mm-28mm), while petroleum ether extract produces lowest zone of inhibition (10mm-24mm). The zone of inhibition ranges from 10mm to 28mm at concentration of 6.25 to 50mg/ml. respectively. The methanolic extract of *C.pepo* seed inhibited the growth of all the tested organisms with exception of *Trichophyton terrestre*,

which was resistant at all the concentrations used. It produces a high zone of inhibition against *M. canis* with 30mm zone of inhibition, which is similar to that produce by the ketoconazole (standard antifungal) (Table 3).This result is in agreement with the work of Perez (2016), who reported the methanolic extract of fruit of *C. pepo* were evaluated for antimicrobial activity against bacterial strains *Bacillus cereus, Bacillus subtilis, Escherichia coli, Enterobacter aerogenes, Enterobacter agglomerans, Salmonella enteritidis, Salmonella cholerasius, Staphylococcus aureus, Pseudomonas aeruginosa, Candidaalbicans, Penicillium chrysogenum, Enterobacter faecalis, Klebsiellapneumonia, B. Bisphericus, B. thruengenesis* and *Cryptococcus meningotidis*, in which the extract showed moderate to high activity against all the investigated microbial strains. The dichloromethane seed extracts produces high zone of inhibition on *T. mentagrophytes* with 28mm diameter zone of inhibition than the standard antifungal (ketoconazole) (Table 3), this correspond with the findings of Noumedem *et al., (2013)* where *C. pepo* seed extract displayed the largest spectra of activity against *Providencia stuartii, Pseudomonas aeruginosa, Kpneumoniae, Escherichia coli, Enterobacter aerogenes* and *Enterobacter cloacae* and the extract from *C. pepo* was more active than chloramphenicolused as positive control on at least one of the tested MDR bacteria. The petroleum ether extract exhibited activity against all the tested isolates with the exception of *M. canis and T. verrucosum,* which are resistant at all the concentrations used.

**Table 1: Physical characteristics of the plant extracts**

| Extracts | % Yield | Color | Boiling point | Texture |
|---|---|---|---|---|
| CSM | 40 | Golden brown | 65$^{o}$C | Sticky and oily |
| CSD | 40 | Yellow | 40$^{o}$C | Oily |
| CSP | 20 | Yellow | 40$^{o}$C | Oily |

Key: CSM=*C pepo* seed methanolic extract, CSD=*C pepo* seed dichloromethane extract, CSP=*C pepo* seed petroleum ether extract.

**Table 2: Phytochemical component of *C pepo* seed extracts**

| Phytochemical component | Extracts | | |
|---|---|---|---|
| | **CSM** | **CSD** | **CSP** |
| **Alkaloid** | + | + | **+** |
| **Saponin** | + | + | **+** |
| **Tannin** | + | + | **+** |
| **Flavonoid** | + | + | **+** |
| **Steroid** | + | + | **+** |
| **Glycoside** | + | + | **+** |
| **Terpenoids** | - | - | **-** |
| **Anthraquinone** | - | - | **-** |

Key: CSM=*C pepo* seed methanolic extract, CSD=*C pepo* seed dichloromethane extract, CSP=*C pepo* seed petroleum ether extract, +=Present, - =Absent.

**Table 3: Activity of *C. pepo* seed oil extracts on the tested organism**

| | Zone of Inhibition (mm) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Organisms** | **CSM** | | | | **CSD** | | | | **CSP** | | | | **Control** |
| | **50** | **25** | **12.5** | **6.25** | **50** | **25** | **12.5** | **6.25** | **50** | **25** | **12.5** | **6.25** | **25** |
| *Microsporum canis* | 30 | 30 | 20 | 26 | 20 | 20 | 15 | 10 | 0 | 0 | 0 | 0 | 30 |
| *Trichophyton terrestre* | 10 | 0 | 0 | 0 | 20 | 15 | 0 | 0 | 15 | 15 | 0 | 0 | 20 |
| *Trichophyton mentagrophyte* | 24 | 20 | 8 | 8 | 28 | 26 | 16 | 12 | 18 | 16 | 16 | 11 | 27 |
| *Trichophyton verrucosum* | 16 | 10 | 10 | 0 | 20 | 16 | 15 | 0 | 20 | 0 | 0 | 0 | 40 |

Key: CSM=*C pepo* seed methanolic extract, CSD=*C pepo* seed dichloromethane extract, CSP=*C pepo* seed petroleum ether extract

## CONCLUSION

The result of this study showed that *Cucurbita pepo* seeds extract have exhibited antifungal activity against *T. mentagrophyte, T. terrestre, T, verrucosum and M. canis* due to the presence of some secondary metabolites

## RECOMMENDATION

Based on the findings from this study it is recommended that further research should be carried out to identify the toxicity of the plant as well as the bioactive compounds present in the plant and the extracts should also be tested against a wide range of pathogenic miroorganisms.

## REFERENCES

Adoum, O.A, Dabo N.T. and Fatope M.O., (1997) Bio-activities of some savannah plants in brine Shrimp lethality test an *invitro* antimicrobial assay. *International journal of pharmacognos*y 35 (5):334.

Bradley, P. R. (1992). British Herbal Compendium. British Herbal Medicine Association, Bournemouth, Dorset, UK. Vol 1, pp. 112–4.

Bourgard, F., Putaraud, A. and Guckert, A. (1994).Extraction of Coumanins from plant Material *(Leguminocea). Phytochemical Analysis* Pp. 127-132

Caili, F. Huan, S. and Quanhong, L. (2006). A review on pharmacological activities and utili Zation technologies of pumpkin.*Plantfoods Hom Nutri* 61:73-80

Chaturvedi, P. (2012). Antidiabetic potentials of Momordica charantia: multiple mechanisms behind the effects. *J Med Food* 15: 101-107.

Chonoko, U. G. and Rufai, A. B. (2011) Phytochemical screening and antibacterial activity of *Cucurbita pepo* (pumpkin) against S*taphylococcus aureus* and S*almonella typhi Bayero Journal of Pure and Applied Sciences*, 4(1): 145 – 147.

Ciulci, I. (1994). Methodology for the analysis of vegetable drugs chemical industries branch, Division of industrial operations. Unido, Romania: 24, 26 and 67.

David, G.W. (1989). Antimicrobial chemotherapy oxford mechanical publication pp.91-99.

Dhiman, K, Gupta, A, Sharma, D.K, Gill, N.S. and Goyal, A. (2012) A review on the Medicinaly important plants of the family Cucurbitaceae. *Asian J Clin Nutr* 4:3

Ding, Y.L, Deng, X.M, Wang, X.L, Wang, I.Q and Zhang, Y.P. (2002). Study on Newcastle Disease virus effect of five traditional Chinese herbs. *J Trad Chin Vet Med* 21:3-4

Ellis, D., Stephen, D., Helen, A., Rosemary, H., and Robyn, B. (2007): Description of Medical Fungi 2ndedition.Published by Authors, Australia, North Adelaide, P84-170.

Fatope, M.O. and Hamisu, I. (1993).Screening of higher plants reported as pesticides using Shrimp lethality assay. *International journal of pharmacognosy* 31:250-260.

Ginley, M. C, (2011) *Cucurbita pepo*. The encyclopedia of earth. Edible medicinal and non-medicinal plants. Volume 2. Fruits. Springer Netherland.

Guaro, J. Isabil, P., Carmen A., Carles, L. and Fernndez-Ballart, J. (1998). Inoculums preparation for invitro susceptibility testing of filamentous fungi. *Journal of antimicrobialchemotherapy* 42 P 385-387.

Jagesser R.C., Mars, A., and Gones, G.(2008).Selective anti-microbial properties of leaf extract against various micro-organisms using Disc diffusion and Agar well diffusion method *Journal of nature and science* Vol 6 (2):24-38,

Mukhtar, MD and Tukur A (1999). *Invitro* screening for activity of *Pistiastratiotes* extract NISEB journal 1 (1):51-60.

Marichal P, Vanden Bossche H. Mechanisms of resistance to azole antifungals. Acta Biochemica Polonica 1995; 42:509-16.

Noumedem, J.A.K., Mihasan, M., Lacmata, S.T., Stefan, M., and Kuiate, J.R., (2013) Antibacterial activities of the methanol extracts of ten Cameroonian vegetables against Gram-negative multidrug-resistant bacteria. *BMC Complement Altern Med* 13: 26-29.

Perez, G. R.M. (2016). Review of *Cucurbita pepo* its Phytochemistry and Pharmacology. *Med-chem* 6:012-021.

Polari, L. V., Tyothi, P. I., and Sai, L. K. (2015) clinicomycological study of Dermatophytosis in south India. *J. of Laboratory physicians* 7(2):84-89.

Pramod, K. N. (2015):Antifungal drugs and resistance:Department of Dermatology, STD and Leprosy, H. J. N.M. Medical College, Raipur-492001, (GG) India.

Riffel, A. L.F., Medina, V., Steffens, R. C., Santos, D.B and Bizani, A.B, (2002). Invitro antimicrobial activity of a new series of 1, 4-naphthaquinones .Braz. *J Med. Biol. Res*.35:811-818.

Rodwell GE, CL Boyles, Towersey L, Aly R. The prevalence of dermatophytes infectionin patient infected with human immunodeficiency virus. Int J Dermatol 2008;47:339-43.

Smith, B.D. (1997). The initial domestication of Cucurbita pepo in the Americas10, 000 years ago. *Sci* 276: 932-934.

Sofowora, E.A. (1993). Medical plant and Traditional Medicine in Africa (2nd edition), spectrum Books limited Pp44-58,210-212.

Stephen J, Investigators seeking new ways to stem rising tide of resistant fungi. Jour Amer Med Assoa. 1997; 277:5-6.

Sulaiman V, Suresh S. K. (2002): Traditional Medicinal plants. *Pharmatimes* 43 (5). 18.

Tiwari, R., and Rana, C.S.(2015), Plant Secondary Metabolites: a review. *International Journal of Engineering Research and General Science* Volume 3, Issue 5.

Vashka Hurinanthan. (2009). Immune modulatory effect of *Dichrostachys cinerea, Carpobrotus dimidiatus, Capparistomentosa and Leonotisleo*nurus. Durban Uni of Tech South Africa.

Vennaposa, S., Devareddy, S. K., Sumathi, N., and Senthil, K. (2013) .Phytochemical and Antimicrobial Evaluations of *Dichrostachys cinerea .International Research J. of Phar*.4 (1).

Wang, X.L., Liu, J., Chen, Z.H.B., Gao F, Liu,J. X., and Wang, X.L. (2007).PreliminarystudyOn Pharmacologically effect of *Cucurbita pepo* Dayanggua. *J TradChin Vet Med* 20: 6-9.

Waterman, P. H. (1992). Searching for Bioactive compounds various Strategies .*Journal of National products* 53(1) 13-22.