JOURNAL OF CHEMICAL ENGINEERING AND INDUSTRIAL BIOTECHNOLOGY (JCEIB)
ISSN: 2600-7347 e-ISSN: 0126-8139
VOL. 9, ISSUE 1, 13 – 19
DOI: https://doi.org/10.15282/jceib.v9i1.9283

ORIGINAL ARTICLE

Effect of Custard Apple (*Annona squamosa L.*) Seeds Extracts on In-Vitro Activity of *Malassezia globosa*

N. Aliyu* and V. A. Duniya

Department of Chemistry/Biochemistry, Nuhu Bamalli Polytechnic, Zaria, Kaduna State, Nigeria.

ABSTRACT – Scalp is unique among skin areas in humans. It is characterized by a thick skin layer with high follocular density and sebaceaous glands. The presence of these glands along the dark and warm environment of the scalp makes it more prone to mycotic infections like dandruff. This research was aimed to ascertain the potential of custard apple seed phytoconstituents on dandruff causing *Malassezia globosa*. The alkaloid, flovonoid, saponin and tannin extracts were not effective on *M. globosa.* However, glycoside extract was more effective than the crude extract. Glycoside extract had inhibition zones of 35±0.3, 20±0.5, 14±0.2 and 12±1mm at 100, 50, 25 and 12.5mg/ml respectively while crude had zones of 15±0.4, 12±0.2, 10±0.1 and 0±0mm at 100, 50, 25 and 12.5mg/ml respectively. This study suggests the glycoside extract of custard apple seed could offer potential therapeutic benefits in the treatment of dandruff due to its fungicidal properties.

ARTICLE HISTORY
Received: 24 April 2023
Revised: 20 July 2023
Accepted: 24 July 2023

KEYWORDS
Custard apple
Inhibitory concentration
Flavonoids

INTRODUCTION

Scalp disorders are not among severe physical illness or morbidity, yet are of great social concern. Scalp and hair conditions have more of psychological impact in human societies. Scalp disorders include fungal and bacterial infestations that cause problems like alopecia, seborrheic dermatitis, ring worm, scalp psoriasis, scalp folliculitis, head lice and dandruff [1]. Malassezia spp. is an opportunistic dimorphic, Basidiomycetous fungi, associated with a variety of diseases including dandruff. The conditions that cause Malassezia related infections in humans are not fully understood but researchers have been able to determine the role of different factors including genetic and environmental factors, imbalance in skin normal biota, and immune suppression [2]. In the analysis of medicinal plants, extraction is very paramount to extract the desired chemical components from the plant materials. Phytochemicals are bioactive compounds found in plants that exert certain pharmacological effect, such as alkaloids, flavonoid, saponin, tannin, glycosides and so on. Phytochemicals may be non-polar to polar considering the suitability of the methods of extraction [3]. Basic steps, such as pre-washing, drying, and grinding to obtain a homogenous sample and to increase the contact of the sample surface with the solvent system. These steps ensure that potential active constituents (alkaloids, flavonoids, tannins, saponin and glycosides) are not lost, distorted or destroyed during the preparation of the extract from plant samples. These constituents are responsible for the effects on microorganisms or metabolic activity. If the plant was selected on the basis of traditional uses [4] as in the case of this study, then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. The selection of solvent system and extraction temperature largely depends on the specific nature of the bioactive compound being targeted [5].

Tannins are polymeric phenolic astringent compounds with numerous hydroxyl groups and quite diverse in chemical structure. Tannin are found in the bark of trees, wood, leaves, fruits, seeds, rots and plant galls. Certain tannin may help prevent disease and provide antioxidant and anti-inflammatory benefits. Flavonoids possesses a number of medicinal benefits including anticancer, antioxidant, anti-inflammatory and antiviral properties. They are a group of natural phenolic substances found in fruits, vegetables, grains, flowers, tea and wine. Saponins are heterosides synthesized by several plants reported to have a defensive role which was highlighted for the first time by Appelbaum in 1969. Saponins are used for industrial as well as for pharmacological purposes. Several saponosides are used by pharmaceutical industry for obtaining drugs or by cosmetics industry for their detergent property [6]. Glycosides are compound formed from a simple sugar and another compound by replacement of a hydroxyl group. It is deeply used to aid the scalp and hair split ends and contribute to soothing remedy for dehydration, calms inflammation, and reduces oiliness. Types of glycoside include flavonoid-glycoside, saponin-glycoside and so on.

Custard apple belongs to the family Annonaceae and has the botanical name *Annona squamosa L.* It has been known as sweetsop and sugar apple. It is a green, cone-shaped fruit with scaly skin and creamy sweet flesh [7]. The plants is also useful in destroying lice in the hair and having abortifacient, antimutagenic, scavenging, antimicrobial, antidiabetic, licicidal, hepatoprotective, antithyroid, antigenotoxic antiplasmodial and molluscicidal activity [8]. Custard apple seeds have been used as folk medicine worldwide. Khare et al. [9] reported the presence of some phytochemicals in the seeds. In traditional medicine, custard apple seeds were mainly used to treat various digestive disorders and as an insecticidal agent. Phytochemical and pharmacological research on custard apple seeds has shown acetogenin as the major bioactive

*CORRESPONDING AUTHOR | N. Aliyu | ✉ nafnaf4U@gmail.com

compound. Acetogenin is recognized as the most potent mitochondrial complex I inhibitor (effective in concentrations up to nanomolar) [10]. It was observed from past reports that the extracts of seeds of custard apple have good antibacterial properties [10], antimicrobial properties [11] and based on research [12], the aqueous extracts and organic extract solvent from the roots, leaves, fruits, and seeds of custard apple reported to be an insecticide, inhibiting the activity of insect feeding (antifeedant), and repellent to some kind of important pest of agriculture and pest in storage, hence the need to ascertain the effectivity of five bioactive components and compare the activity with a fraction consisting all components (crude) on dandruff causing microorganism alongside a control.

MATERIALS AND METHOD

Sample collection and Preparation

Custard apple (*Annona squamosa* L) fruits were harvested from the local garden in Kabama layout, Sabon gari, Zaria. The fruits, in full maturity were washed under running water, then sliced. The seeds were separated with a steel spoon. The seeds were collected and cleaned from the residue of fruit, then washed completely with tap water and air dried. The dried seeds were blended, sieved and stored in tight jar until used.

Custard apple seeds were crushed into a powder using an electronic blender, and then sieved through a 20mm mesh size sieve to obtain the powdered form. Maceration was carried out by weighing ten grams (10g) of the powdered sample and placing it into a stoppered container along with a solvent mixture. The mixture was allowed to stand for a period of 7 days with frequent agitation, ensuring the soluble matter dissolved. Subsequently, the mixture was filtered using filter paper to obtain fractions of each component.

Figure 1. Maceration of each phytochemical component.

SAMPLE FRACTIONATION

Alkaloid

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. 80% ethyl acetate, ethanol, acetone and water was added, and was allowed to stand for 7 days, heated at 40 ^{o}C for 30mins. Then it was allowed to cool and filtered. The extract was dissolved in 20ml water, partitioned by mixing 20ml diethyl ether in separating funnel to remove pigments and lipids. The aqueous layer was placed in another separating funnel and made alkaline by adding dilute ammonia solution. Chloroform (50ml) was added, and was shook gently to allow separation. The chloroform layer, which contains alkaloids, was carefully poured into a beaker and then concentrated to dryness [13].

Tannin

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. Then 30% ethanol, methanol, acetone and water was added, and was allowed to stand for 7 days, heated at 40 ^{o}C for 30mins. Then it was allowed to cool and filtered. The extract was solubilized in 50ml of water/ethanol (95:5v/v) and extracted three times with chloroform (50ml) to remove lipophilic material. Then the aqueous phase was extracted three times with ethyl acetate (50ml) to obtain two distinctive fractions. The organic fraction was concentrated to dryness before used [14].

Saponin

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. Then 30% ethanol, methanol, acetone and water was added, and was allowed to stand for 7 days, heated at 40 ^{o}C for 30mins. Then it was allowed to cool and filtered. The extract was then defatted with 20ml petroleum ether. Next, the extract was dissolved in water and shaken with n-butanol. N-butanol aliquots are combined and the liquids removed to give saponin extract. Diethyl ether was used to precipitate [15].

Glycosides

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. Then 70% ethanol, water, ethyl acetate and acetone was added, and was allowed to stand for 7 days, heated at 40 ^{o}C for 30mins. Then it was allowed to

cool and filtered. 2.5ml solution of 10% sulphuric acid was added into the 25ml extract and was stirred and kept in hot water bath for 5min after which it was filtered. The filtrate was allowed to cool before 1ml 10% ammonia was added and shaken. Filtrate changed to pinkish red, indicative of glycoside [16] which was concentrated by heating on water bath.

Flavonoids

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. Then 70% ethanol, water, ethyl acetate and acetone was added, ans was allowed to stand for 7 days, heated at 40 ºC for 30mins and allowed to cool then filtered. Distilled water (400ml) was added to 25g extract, and was partitioned with 150ml of diethyl ether three times. Then the aqueous fraction was partitioned with 150ml ethyl ether three times. The aqueous fraction was further partitioned with N-butanol (150ml) saturated with water three times. The butanol fraction was partitioned with 50ml of 1% potassium hydroxide three times. The potassium hydroxide fraction was acidified with dilute hydrochloric acid. The N-butanol fraction contain flavonoids [17].

Crude

Ten grams of custard apple seed powder was weighed into stoppered sample bottle. Then 70% ethanol, water, ethyl acetate and acetone was added, allowed to stand for 7 days, heated at 40 ºC for 30mins and allowed to cool then filtered. This was termed 'crude' because no fractionation was carried out. Solvents with different polarities were used to extract both polar and non-polar components, and hence it contains all the bioactive components.

Figure 2. Sample fractionation

Figure 3. Fractionated component

Cultivation of Malassezia globosa

Scalp scrapings of dandruff was grown on potato dextrose agar and incubated for 3 days at room temperature.

Direct microscopy of fungi

Fungal culture was taken on slide containing a drop of 10% potassium hydroxide with methylene blue and covered with cover slip. The slide was heated over the flame to remove air bubbles and observed under 10X and 45X magnification objective lenses of microscope. Direct microscopy show the typical mixture of organisms present. The presence of globose blastoconidia and pseudo- mycelia coupled with mycelia in the form of 'sphagetti' indicate the most identifiable difference of *M. globosa* from the other species of *Malassezia* [18].

Antifungal activity

Antifungal activity of custard apple seed extracts was tested against dandruff causing microorganism (*Malassezia globosa*) using Muller Hington agar (MHA) by well diffusion method. Fungal lawn of 0.1 ml each of fungal culture was made on separate solidified MHA plates. A well was made in the centre with the help of sterilized borer, 0.5 ml each of different concentrations (12.5, 25, 50 and 100mg/ml) of the extracts were poured in the wells of separate MHA plates. A positive control with ciprofloxacin drug was prepared. All the fungal culture plates were incubated at 37 ^{o}C for 2-4 days. After incubation, the plates were observed for zones of inhibition [18] which diameter of zone were measured with a sterilized meter rule (mm).

Minimum Inhibitory Concentration (MIC) was determined as described by Krasteva et al. [19] with slight modification. The fungal inoculum was diluted to obtain a suspension corresponding to a cell concentration of 5×10^6 CFU/ml. various concentration/dilutions of the extracts were prepared and 2ml of each extracts was added to diluted inoculum in tubes making a final concentration of 100, 50, 25 and 12.5mg/ml. The cultures were incubated at 32 ^{o}C for 48 h. the growth of cells was monitored and compared with McFarland standard, the more turbid the tube the more the fungal growth, the less turbid the tube the less the fungal growth and a clear tube indicates no growth (no activity). MIC was defined as the lowest concentration that inhibited visible fungal growth. The MFC value is defined as the dilution that inhibits growth after incubation for 5-7 days.

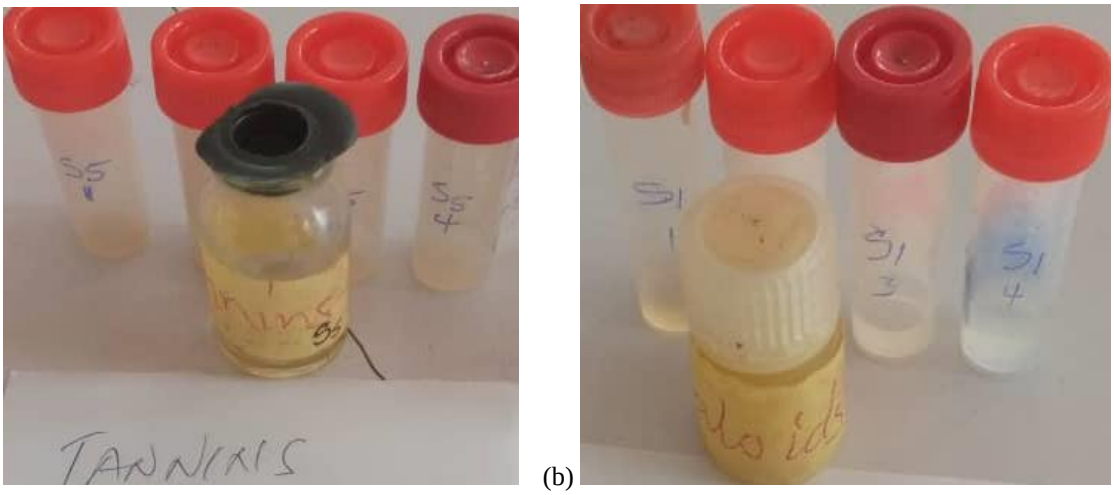


Figure 4. Varying concentrations of extracts of used for MIC and MFC: (a) Tannin (b) Alkaloids.

Data Analysis

The average of duplicate independent readings for the organism was recorded. Data were represented as mean ± standard deviation.

EXPERIMENTAL RESULTS

The result of morphology and view under light microscopy as in Figure 5 show hyphae and some spores which correspond to sphagetti and meatball appearance of mycelia and microconidia of *M. globosa.* This is similar to the report by Isa et al. [20] who isolated and identified *Malassezia globosa* associated with dandruff.

The inhibition zone as shown in Figure 6 was an area around the paper disc that is not overgrown with *M. globosa*. The diameter of the inhibition zone formed shows the effectiveness of seed extract of custard apple. The wider the inhibition zone, the more effective the concentration of seed extract of custard apple.

Inhibitory studies showed that alkaloid, flavonoid, tannin and saponin extracts were not effective on *M. globosa* hence the 0mm zone of inhibition. This finding is similar with report of Ishaku et al. [21] who recorded no zone of inhibition of *Lawsonia inermis* leaves against *Malassezia sp*. However, glycoside extract was effective on *M. globosa* with zones of inhibition 35, 20, 14, 12mm at 100, 50, 25, 12.5mg/ml respectively. This may be due to the fact that glycosides are steroids having the ability to exert specific powerful action by disrupting structure and function of cell wall/membrane, mitochondria, biofilm, and mycotoxin synthesis, hence a very small amount can exert beneficial simulation. This study is different from Murarkar et al. [18], who reported 0.5ml each of lemon juice, neem leaves, onion juice, yoghurt and marketed shampoo with inhibition zones of 18, 13, 20, 18 and 15mm against *M. globosa*. This study is in accordance with Wang et al. [22], who reported 0mm inhibition zone of 32 and 64mg/ml ketoconazole, itraconazole, amphotericin B, and fluconazole against *M. globosa*. The crude extract also show inhibition zones of 15, 12, 10 and 0mm at 100, 50, 25 and 12.5mg/ml respectively. Decreased activity when compared with glycoside extract may be due to combined effect/interference from other inactive phytochemical components as shown in Table 1. The control (Ciprofloxacin) had inhibition zone of 35mm, whose effect can only be compared with 100mg/ml of glycosides extract.

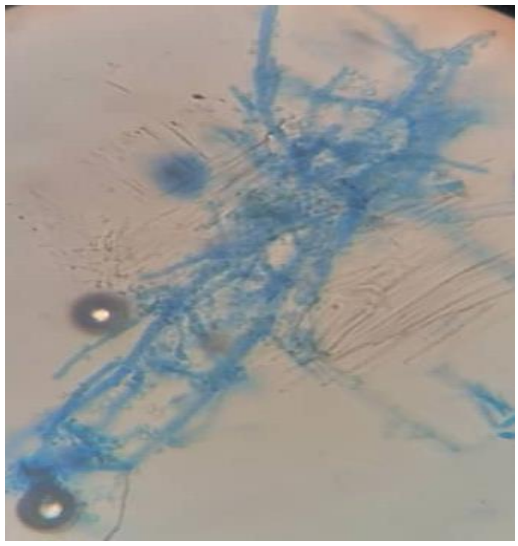
(a)  (b) 

Figure 5. (a) Growth of *M. globosa* in a plate. (b) Microscopic view of mycelia of *M. globosa*.

Figure 6. Zone of inhibitions of different concentration of crude extract.

Table 1. Sensitivity test (Inhibitory activity) of custard apple seed extracts on *M. globosa*.

Components	**Zone of inhibition (mm) at varying concentration of the extracts (mg/ml)**				
	100	50	25	12.5	Control (Ciprofloxacin)
Alkaloid	0±0.00	0±0.00	0±0.00	0±0.00	35±0.00
Flavonoid	0±0.00	0±0.00	0±0.00	0±0.00	35±0.00
Saponin	0±0.00	0±0.00	0±0.00	0±0.00	35±0.00
Tannin	0±0.00	0±0.00	0±0.00	0±0.00	35±0.00
Glycoside	35±0.30	20±0.50	14±0.20	12±1.00	35±0.00
Crude	15±0.20	12±0.20	10±0.10	0±0.00	35±0.00

Table 2 show the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extracts against *M. globosa*. Alkaloids, flavonoid, saponin and tannin were not effective (no activity). The tube mixtures remain turbid for both MIC and MFC. The MIC for glycoside and crude extracts are 25 and 50mg/ml, while the MFC

are 50 and 100mg/ml respectively. The tubes were clear which signifies activity/absence of *M. globosa* growth. The effect of glycoside extract may be attributed to the fact that its structure can bound/synergize with other compound to exert a desirable effect. This study is in accordance with Afzal et al. [23] who reported antifungal properties of glycosides isolated from *Albizia kalkora*. This study is in contrast with Vu et al. [24] who reported MIC of 2.5µl/ml of *M. arvensis* essential oil on *Malessezia* genus fungi. This study is also different from Sibi et al. [25] who reported herbal ingredients like tea tree oil, rosemary oil, coleus oil, clove oil, pepper extract, neem extract, and basil extract also recorded anti-Malassezia activity with lower MIC values.

Table 2. Inhibitory concentration of custard apple seed extracts on *M. globosa.*

	Concentration of extract (mg/ml) for:	
	MIC	MFC
Alkaloid	-	-
Flavonoid	-	-
Saponin	-	-
Tannin	-	-
Glycoside	25	50
Crude	50	100

KEY: - means no activity

CONCLUSION

This research indicate the significance of custard apple seed which contains phytochemicals. The glycoside and crude extracts inhibited the growth of *Malassezia globosa* by 35 and 15mm respectively. However, it is paramount to emphasize the importance of the use of minimum fungicidal concentration of 50mg/ml of glycoside extract in cosmoceuticals to treat *Malassezia* fungal infections. Furthermore, sensitivity test could be carried out on other *Malassezia* strain so as to ascertain the extracts' effect and investigation to identify the active agent(s) responsible for the fungicidal effect.

ACKNOWLEDGEMENT

The authors would like to thank the management of Nuhu Bamalli Polytechnic, Zaria, Kaduna State under Tertiary Education Trust Fund, Institution based Research for funding this study.

REFERENCES

[1] M. Narshana, P. Ravikumar, "An overview of dandruff and novel formulations as a treatment strategy", *International Journal of Pharmaceutical Sciences and Research.* ISSN 0975-8232, 2019.

[2] G. Ravichandran, V. S. Bharadwaj, S. Kolhapure. Evaluation of the clinical efficacy and safety of "Anti-Dandruff Shampoo" in the treatment of dandruff. *The Antiseptic*, 201:5-8, 2004.

[3] S. Sasidharan1, Y. Chen, D. Saravanan, K.M. Sundram, L. Yoga Latha. Extraction, isolation and characterization of bioactive compounds from plants' extracts, *Afr J Tradit Complement Altern Med.* 8(1):1-10 1, 2011.

[4] D. S. Fabricant and N. R. Farnsworth. The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect*. 109, 69–75, 2001.

[5] P. Cosa, A. J. Vlietinck, D. V. Berghe, L. Maes. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *J. Ethnopharmacol.* 106: 290–302, 2006.

[6] P. N. Bajad, A. B. Pardeshi and V. P. Pagore. Extraction, isolation and quantification of saponin from Dodonaea viscosa JACQ. *The Pharma Innovation Journal*, 8(5): 41-44, 2019.

[7] K. V. Yathish, B. R. Omkaresh, R. Suresh, *International Journal of Engineering Research and Technology,* vol 2: 31-36, 2013.

[8] A. Bhattacharya, Debnath, S. K., Debnath M. D., Kar D. R. Phytochemical and pharmacological evaluation of the seeds of Annona squamosa Linn. *Int. J Pharm. Pharm. Sci*. 4:92-94, 2012.

[9] R. K. Khare, G. Das, S. Kumar, V. Gautam, S. Nath, R. Verma, V. Poonia, S. Sachan, M. N. Reddy and N. Yadav. Phytochemical analysis of custard apple (Annona squamosa) and Karanja (Pangamia pinnata) seed extracts. *The Pharma Innovation Journal*, SP-11(1): 480-484, 2022.

[10] N. P. Ristiati, N. L. P. M Widiyanti, S. Mulyadiharja, I. P. A. Putra, "Effectivity of custard apple's (Annona squamosa) seed extract in various concentrations on the growth of *Escherichia coli*", *IOP Conf. Series: Journal of Physics: Conf. Series* 1116 052054. doi:10.1088/1742-6596/1116/5/052054, 2018.

[11] H. Dalia, Eshra, Attia R. Shehata, Abdel-Nabey A. Ahmed, Jehan I. Saber. Physicochemical Properties of the Seed Kernels and the Oil of Custard Apple (Annona squamosa L.). International Journal of Food Science and Biotechnology. Vol. 4, No. 4, 2019, pp. 87-93.

[12] A. Febrianni, *Prosiding Seminar Nasional dan Lokakarya Forum Komunikasi Perguruan Tinggi Pertanian Indonesia 2-4 September* (Bogor: Fakultas Pertanian Institut Pertanian Bogor) 2011.

[13] G. E. Trease, W. C. Evans, "Pharmacognosy". 12th edn. Bailliere Tindall and Co Publisher, London, 176-180, 1989.

[14] M. A. Wen, W. T. Pierre, J. Michael, L. Hua, L. T. Pierre, "Isolation of condensed tannins in individual size from grape seeds and their impact on astringency perception". *BIO Web of Conferences*, EDP Sciences 7, p5, 2020.

[15] H. Usman, U. K. Abubakar, O. I. Olajide, M. E. Ali and A. A. Ibrahim, "Phytoconstituent evaluation and antimicrobial efficacy of the crude flavonoid and saponin rootbark extract of *Terminalia avicentrinoides* and *Ficus polita*", *Journal of Herbmed Pharmacology* 7(2) 106-111, 2018.

[16] A. Sakalpanich, W. Gritsanapan, "Extraction method for high concentration of anthraquinone from K Cassia fistula pods". *J Health Resources* 22(4): 167-172, 2008.

[17] K. Murarkar, S. Rathi, A. Chandak, "Antimicrobial activity of natural herbal products against dandruff causing fungus and bacteria", World Journal of Pharmaceutical Research Volume 8, Issue 1, 1460-1467, 2018.

[18] K. Murarkar, R. Surbhi and A. Chandak. Antimicrobial activity of natural herbal products against dandruff causing fungus and bacteria. World Journal of Pharmaceutical Research Volume 8, Issue 1, 1460-1467.

[19] D. Krasteva, Y. Ivanov, Z. Chengolova and T. Godjevargova, Antimicrobial potential, antioxidant activity and phenolic content of Grape seeds extracts from four grape varieties. *Microorganisms*, 11. 395, 2023.

[20] S. Isa, H. Sa'ad, M. F. Umar, M. M. Muhd, "Isolation and identification of *Malassezia globosa*, Associated with Dandruff among Female Students of Gombe State University", *Greener Journal of Microbiology and Antimicrobials* ISSN: 2354-2284 Vol. 1 (1), pp. 001-006, 2013.

[21] M. J. Ishaku, R. G. Egah, L.Y. Adogo and A. Z. Koggie, Isolation and antifungal effects of plants extracts on Malassezia species isolated from scalps of primary school pupils and Bingham University students, *Annual Research & Review in Biology*, 36(1): 36-43, 2021.

[22] K. Wang, L. Cheng, W. Li, H. Jiang, X. Zhang, S. Liu, Y. Huang, M. Qiang, T. Dong, Y. Li, J. Wang, S. Feng and H. Li, "Susceptibilities of *Malassezia* strains from *Pityriasis versicolor, Malassezia folliculitis* and seborrheic dermatitis to antifungal drugs, *Helyon 6*, e04203, 2020.

[23] M. Afzal, E. Ahmed, A. Sharif, A. Javaid, "Antifungal potential of two new triterpenoidal glycosides from the Albizia kalkora". Available http://www.researchgate.net/publication/366633221, 2022. [Accessed 5 May, 2023].

[24] T. X. Vu, T. B. Tran, C. Q. Hoang, H. T. Nguyen, M. X. B. Phan, A. N. Dao, M. T. Dinh, K. Soytong and H. Q. Nguyen. "Chemical compositions and Anti-*malassezia* properties of Vietnamese *Mentha arvensis* and *Piper bettle* essential oils, *International Journal of Agricultural Technology* Vol. 17(4):1619-1630 ISSN 2630-0192, 2021.

[25] D. Sibi, C. D. Silvanose and V. G. Jibin, Role of Malassezia furfur and M. globosa in Dandruff and Seborrheic Dermatitis. *J Clin Investigat Dermatol.* 11(1): 2, 2023.