Original Research Article

Antioxidant Activities of Different Solvent Extracts of *MoringaOleiferaSeeds* Using DPPH Assay

Comment [m1]: No italics

Abstract

Moringaoleifera (Moringaceae) which grows in the tropical and subtropical regions of the world, has long unani system of medicine with ayurvedic history. The seeds are known to possess high medicinal properties. This study aimed to determine the antioxidant activity of *M.oleifera* seeds, extracted with different solvents (methanol, ethyl acetate and n-hexane) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. This research was carried out using different concentrationsof methanol, ethyl acetate, and n-hexane extracts, (5, 10, 15, 20, 25, 30 mg/mL).Vitamin C was also used as standard antioxidant control. The percentage of inhibition and IC_{50} were measured. The results showed that the DPPH free radicals were scavenged by all seed extracts in a concentration dependent manner.Moreover, the IC_{50} values for DPPH radicals with methanol, ethyl acetate and n-hexane extract of the *M.oleifera* seeds were found to be 30.77, 44.77 and 45.04 mg/mL, respectively. Interestingly, the IC_{50} value of all the extracts revealed very active antioxidant activity but lower than the standard vitamin C. This is due to their IC_{50} values < 50 mg/mL. The order of activity for all the assay was methanol > ethyl acetate > n-hexane. The results suggested that methanol extract of *M.oleifera* seeds has the most active antioxidant activity.

Comment [m2]: Make Italic

Comment [m3]: Rewrite the proper sentence.

Keywords: *Moringaoleifera* seeds, antioxidant activity, DPPH assay

1. INTRODUCTION

Moringaoleifera, called the "miracle tree,"mainly because every part of the Moringa tree is beneficial for humans and animals, originates from the sub-Himalayan region, Asia, Africa, and Arabia, and is becoming increasingly popular in Western countries.MO has been studied for its health properties, attributed to the numerous bioactive components, including vitamins, phenolic acids, flavonoids, isothiocyanates, tannins and saponins, which are present in significant amounts in various components of the plant [1].The seeds with its high nutritive value confer on the plant significant medicinal properties that have been hyped for their therapeutic properties in the treatment of various ailments.

M. oleifera seeds have been found to be good antioxidants, able to reduce oxidative damage associated with aging and cancer [2].In addition, it is used as potential antioxidant, anticancer, anti-inflammatory, antidiabetic and antimicrobial agent [3].The ability of *M. oleifera* seeds to counter the synthesis of reactive oxygen species has been attributed to existing antioxidants like lectins, monopalmitic acid, myricetin, oleic acid, tocopherols, tri-oleic triglycerides, amongst others [4,5,6].The concentration of its bioactive compounds varies depending on the growing region of the plant, since the different environmental conditions modify its synthesis [7].The extraction solvent and drying process used can also have a significant impact on the biological activities and metabolite contents of plant materials because distinct bioactive constituents with different chemical properties and polarities may have varying solubility in different solvents [8,9].

Moringa seeds have been described by [3,10] to contain phenolic compounds as ferulic acid, gallic acid, caffeic acid, cinnamic acid, protocatechuic acid, phytosterol, quercetin, chlorogenic acid epicatechin, catechin, vanillin, and rhamnoglucoside.Generally, antioxidants are compounds that can scavenge free radicals by interrupting radical chain reactions, or even prevent the reactive oxidants from being formed in the first place.

Exogenous antioxidants derived from natural sources such as plants (flavonoids, polyphenols,carotenoids, vitamins) or minerals (selenium, zinc, manganese) can remove free radicals, inhibiting the adverse effects caused by reactive oxygen species (ROS) in the form of oxidation of lipids, protein, and nucleotides. In this way, they prevent damage done to the cells by ROS. Considering the beneficial role of the free radical-scavenging bioactive substances in human health protection, they are still being searched and constantly investigated. The reasons for these are increasing understanding of the harmful nature of synthetic antioxidant such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) and the increasing resistance posed by microorganisms to synthetic antibiotics [11].

Several methods have been used to measure free radical scavenging capacities of plant. DPPH method received more attention due to its fast, reliable results, relatively simple, stable and the DPPH was available commercially in high purity [12].The DPPH radical scavenging activity has been widely used as a model system to investigate the scavenging activity of natural compounds [13,14].

The present study aimed to determine the antioxidant activity of *M.oleifera seeds*, extracted with different solvents; (methanol, ethyl acetate, n-hexane) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

2.MATERIAL AND METHOD

2.1. Plant Material and Extraction

Fresh seeds of *M.oleifera* were procured from "Yelwan-Tudun" market, Bauchi city in northeast Nigeria. The seeds were de-husked manually with the aid of mortar and pestle; the clean kernels were air dried for three weeks. Subsequently, samples were milled into fine powder with the aid of a manual grinding machine and then subjected to cold extraction process using different solvents such as methanol as polar solvent, ethyl acetate as semi polar solvent, and n-hexane as nonpolar solvents for 72 hr in an enclosed glass jar and filtered. The procedure with each solvent was repeated, evaporated to dryness and stored in a refrigerator (4 ^{0}C) prior to analysis.

2.2. Preparation of different crude extracts and Vitamin C standard solution

10 mg crude extract each of methanol, ethyl acetate, n-hexane and vitamin C were dissolved in 50 mL of methanol in 100 mL volumetric flask and made to mark to obtain 100 mg/mL stock solution.Each extracts were prepared into several concentrations of 5, 10, 15, 20, 25 and 30 mg/mL by taking aliquots of 2.5, 5, 7.5, 10, 12.5 and 15 mL from the stock solution of each extracts and bringing to 50 ml with methanol for being used subsequently in antioxidant activity assay.The extracts and standard were prepared based on the method [15,16] with slight modification.

2.3. Preparation of 0.2 mMDPPH Methanolic Solution

Weight of 39.4 mg of DPPH was added to 500 mL of methanol in a 1000 mL volumetric flask and allowed to dissolve, after which was made to mark to give 0.1 mM DPPH solution. This was replicated two times to give 0.2 mM DPPH solution.

2.4. Radical /ROS Scavenging Activity by DPPH Assay

The free radical scavenging activity or antioxidant activity of each extracts was determined using the stable radical DPPH (2,2-diphenyl-1- picrylhydrazyl) assay according to the standardized protocol with some modifications [17,18].When DPPH reacts with an antioxidant compound which can donate hydrogen, it is reduced to non- radical DPPH-H (2,2-diphenyl-1-picrylhydrazine), (DPPH + RH → $DPPH_2$ +R). The result of reduction DPPH radicals causes discoloration from purple colour to yellow pale colour which indicates the scavenging activity.Different concentrations (5, 10, 15, 20, 25 & 30 mg/mL) of each test samples and standard (vitamin C) were prepared. From each solution, 1 mL of each solution was taken in different test tubes.1 mL of the 0.2 mM DPPH solution was added to each tube containing 1 mL solution. The tube was shaken vigorously and incubated in the dark for 30 min. The absorbance of each solution was taken on a UV-Visible spectrophotometer (Shimadzu made) at 517 nm against an equal amount of DPPH and methanol as a control. The experiment for each extract was performed in triplicate and the percentage inhibition described as antioxidant activity was calculated based on the equation:

$$AA\,\% = 100 - \left(\frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} X\ 100 \right)$$

Where, $Abs_{control}$ = Absorbance value of control, and

Abs_{sample} = Absorbance value of tested sample or reference standard.

The percentage inhibition was plotted with the concentration (mg/mL) to obtain the linear regression equation y = bx + a. The IC_{50} value is obtained by replacing y with 50 and calculating the value for x. IC_{50} (Half maximal Inhibitory Concentration) is the concentration of the sample that can scavenge 50% of DPPH free radical in DPPH free radical scavenging method. The IC_{50}value is inversely proportional to the free radical scavenging activity or antioxidant property of the sample.The IC_{50} values of the samples were compared and the value closest to that of vitamin C is considered to have the best antioxidant property [19].

2.5. Statistical Analysis

The data obtained in this study were provided in terms of mean and standard deviation (mean±standard deviation). One-way analysis of variance (ANOVA) was carried out to compare the data. Further, to determine the statistically significant differences, Post-Hoc Tukey's test was performed at ($P < 0.05$). For representing the result of the concentration variation (mg/mL) on inhibition of DPPH free radical scavenging (%), linear regression on the antioxidant test has been utilized to calculate the IC_{50} value and Microsoft Excel 2010 was employed for the statistical and graphical evaluations.

3. RESULT AND DISCUSSION

3.1. Extractive values of different extracts

In this research, the crude extracts were prepared by cold extraction using methanol (polar), ethyl acetate (medium-polar) and n-Hexane (non-polar) respectively. This is because extraction procedures and solvent types influence the solubility of phytochemical components from particular plants material based on their polarity potential [20]. The total yield of different extracts was significantly affected by different solvents used for the extraction. The results of the yield of the different extracts are shown in Table **1.**

Table 1: Total yield of different solvent extracts of *M.Oleifera* seeds

Solvent extracts	Initial amount of *M.oleifera* seed used (g)	Yield of the extract (g ± SD)
Methanol	100	8.15 ± 0.52^{c}
Ethyl acetate	100	18.44 ± 0.03^{a}
n-Hexane	100	17.25 ± 0.84^{b}

*Each value is expressed as mean ± standard deviation (SD) (n = 4).Values in the column followed by a different letter superscript are significantly different as determined by the Turkey's test. ($P < 0.05$).

3.2. Determination of Antioxidant Activity by DPPHAssay

The Antioxidant Activity test of the seed extracts was performed by the free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) assay because it offers the first approach and most accepted model to test the ability of natural compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity [21,22,23].The radical scavenging activity of different extracts and vitamin C (taken as standard) was evaluated using the DPPH assay.Six varying concentrations (5, 10, 15, 20, 25 and 30 mg/mL) of different solvent extracts of *M. oleifera*demonstrated different percentage of inhibition.

However, it is noted that the efficiency of the antioxidant increased with the concentration of each tested extracts. The three extracts, at their highest concentration (30 mg/mL) of the three extracts showed the best antioxidant activity, where among them, the methanol extract was the highest (49.71%), followed by ethyl acetate and n-hexane extract (39.93 and 34.92%), respectively (Table **2)**.The radical scavenging activities of those three extracts, at their highest concentration (30 mg/mL), were lower than the antioxidant capacity of vitamin C (54.65%) at the same concentration.

Table 2. Absorbance and free radical scavenging of different solvent extracts of*Moringaoleifera*seedswith standard vitamin Cat different concentrations (DPPH scavenging assay method).

Plant part	Extract	Concentration (mg/mL)	Absorbance	% inhibition of DPPH free radical scavenging

M.Oleifera Seeds	Methanol	30	1.368	49.71
		25	1.213	44.08
		20	0.961	34.92
		15	0.820	29.80
		10	0.720	26.16
		5	0.560	20.35
	Ethyl acetate	30	1.099	39.93
		25	0.811	29.47
		20	0.751	27.29
		15	0.685	24.89
		10	0.563	20.46
		5	0.422	15.33
	n-Hexane	30	0.9561	34.92
		25	0.820	29.80
		20	0.677	24.60
		15	0.510	18.53
		10	0.410	14.90
		5	0.269	9.770
	Standard Vitamin C	30	1.504	54.65
		25	1.368	49.71
		20	1.235	44.88
		15	1.099	39.93
		10	0.951	34.56
		5	0.819	29.79

Absorbance value of control = 2.752

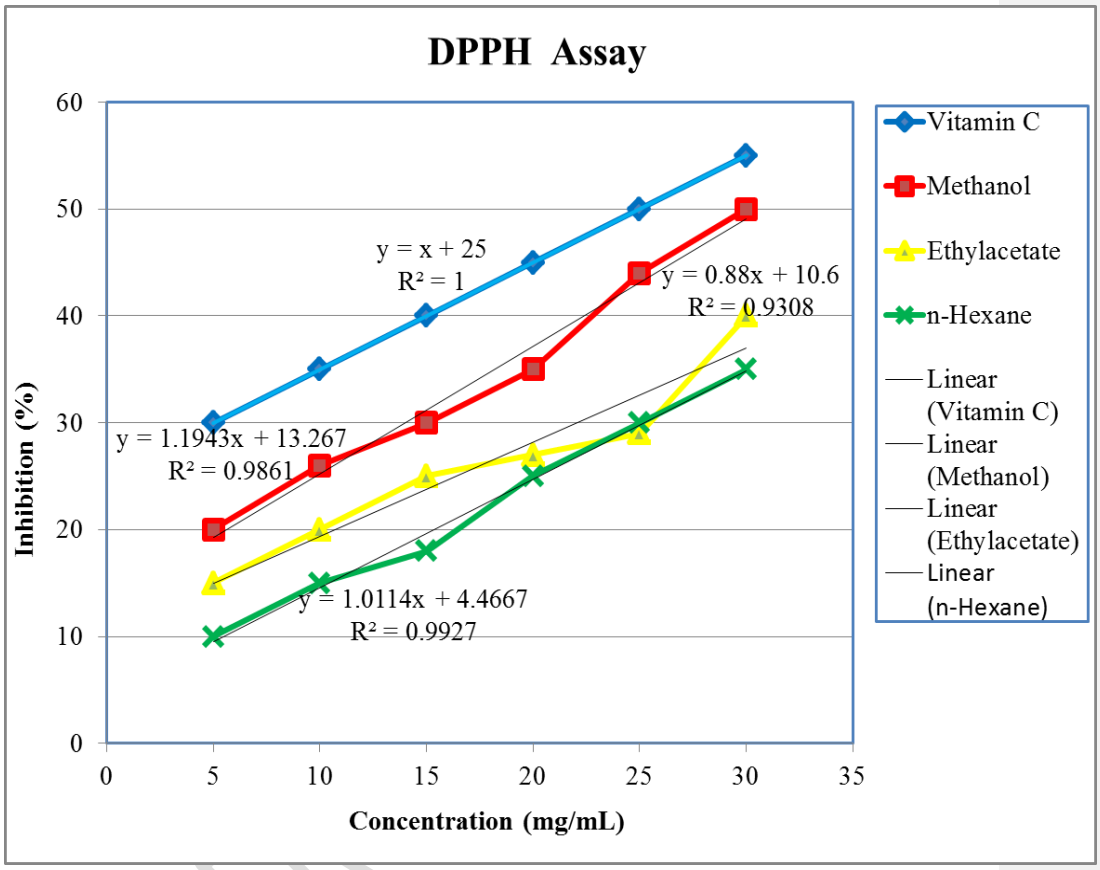

FIGURE 1: Graph of % inhibition of DPPH free radical scavenging against different concentrations in mg/mL of differentsolvent extracts of *M.oleifera* seed with standard vitamin C.

3.3. Determination of IC_{50} Values (DPPH Radical Scavenging Activity)

The IC_{50} values in mg/mL expresses the half maximal inhibitory concentration of the sample required to reduce by 50% the amount of free radical DPPH.IC_{50} value of each extracts and vitamin C (taken as standard) was calculated from the plot between different concentration and % inhibitionof DPPH free radical scavenging (Figure **1**).

A lower IC_{50} value corresponds to a higher antioxidant activity [24]. The observed IC_{50} value showed that methanol extract exhibited highest antioxidant activity (30.77 mg/mL) followed by ethyl acetate extract (44.77 mg/mL) and n-hexane extract (45.04 mg/mL), respectively where as standard vitamin C has IC_{50} value of 25 mg/mL (Figure **2**).

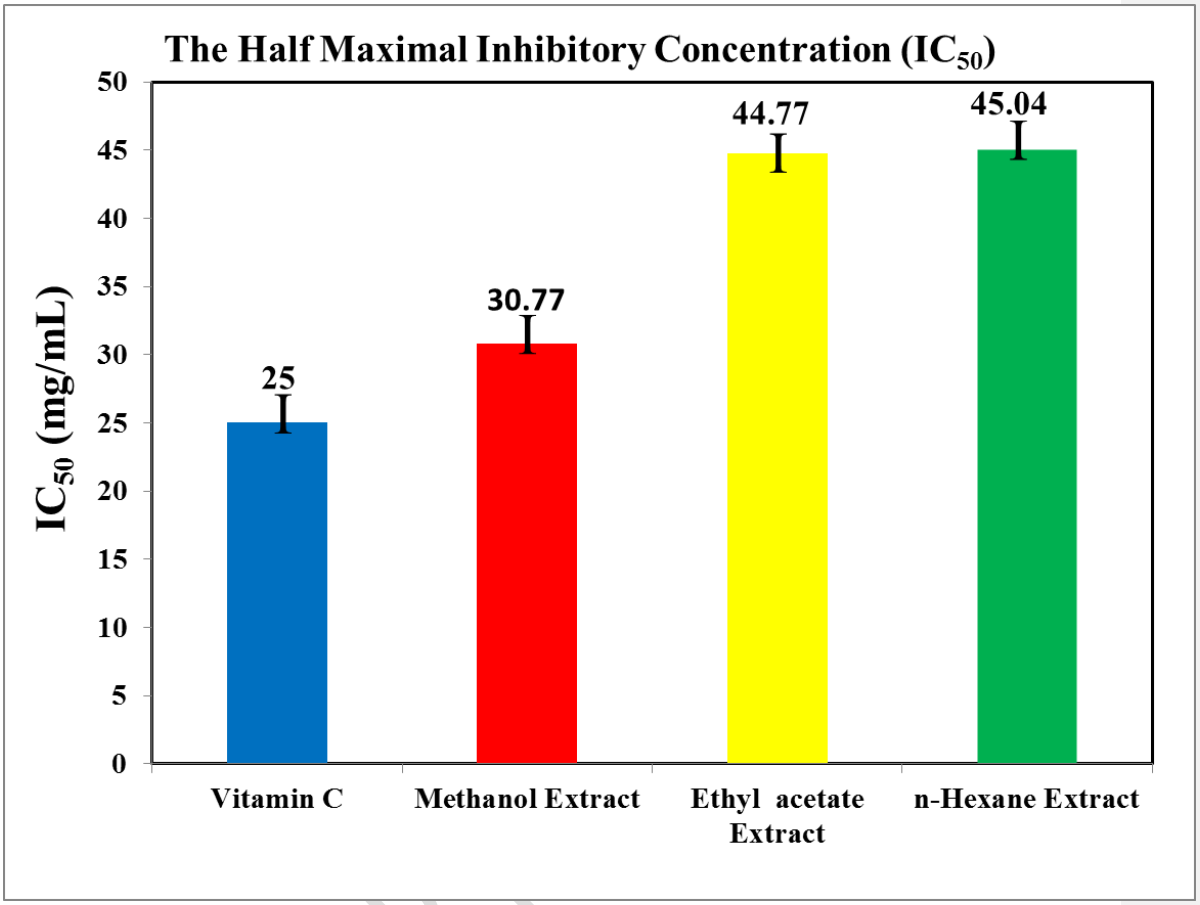

FIGURE 2: Bar graph showing IC_{50} values (mg/mL)of different solvent extracts *M. oleifera* seed and standard vitamin C from the DPPH assay.Values were statistically significant different ($P < 0.05$).

Table 3.Category of antioxidant activity strength invitro against DPPH

Intensity of IC_{50}	Value (µg/mL)
Very active	<50
Active	50-100
Medium	101-250
Weak	250-500
Inactive	>500

According to [25] Table 3, extracts which possess IC_{50} values < 50 µg/mL has very active antioxidant activity.

Interestingly, the IC_{50} value of all the extracts revealed very active antioxidant activity but lower than the standard vitamin C. This is due to their IC_{50} values < 50 mg/mL. The order of activity for all the assay was methanol > ethyl acetate > n-hexane.

Pharmacological activities of *moringaoleifera* are widely reported including antioxidant and antimicrobial activities. The type of extraction solvents has been shown to alter antioxidant activity. This is due to the vast range of chemical characteristics and polarity of varied solvent solubilities.On the basis of the result of this study, the methanol extract of *moringaoleifera* seeds was better able to inhibit free radicals than were the ethyl acetate extract and n-hexane extracts. This is in line with other research studies. One study on antioxidant activity of *moringaoleifera* extracts reported that themethanol extract had the highest antioxidant activity among the other leaves extract due to the fact that this extract may contain many phenolic compounds that contributed to its antioxidant activity [26].

Similar results were also found in which the methanol extract showed the highest antioxidant activity of DPPH assay among other extracts of *UncariaGambir*stems [27]and*Syzygiumpolynthum*leaves extracts [28].

4. CONCLUSION

Methanol, ethyl acetate and n-hexane extracts of *M.oleifera*seed exhibited potential antioxidant activity. They act in a concentration-dependent manner. Methanol extract has the most active antioxidant activity followed by ethyl acetate and n-hexane extract although still lower than the strength of standard vitamin C.Thus, indicating that the extracting solvent significantly altered the antioxidant property. The polarity-dependence increase in the antioxidant activity of *M.oleifera* seed extracts may be attributed to the high affinity of antioxidant compounds in *M.oleifera* seed towards solvent polarity.The results of this study confirm the therapeutic potency of the *M.oleifera* seeds, and scientifically validate the effectiveness of the medicinal plant as an antioxidant and an alternative to synthetic drug.

REFERENCES

Comment [m4]: Check referencing style

1). Christianto V, Smarandache F (2019) On the Efficacy of *MoringaOleifera* as Anticancer Treatment: A Literature Survey. BAOJ Cancer Res Ther 5: 069.

2). Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G, Singh HB (2009) Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringaoleifera*. Food ChemToxicol 47:1109–1116

3). Gopalakrishnan, L., Doriya, K. and Kumar, D. S. (2016) Moringaoleifera: A review on nutritive importance and its medicinal application. Food Sci. Hum. Wellness5:49–56.

4). Lalas S, Tsaknis J. Extraction and identification of natural antioxidant from the seeds of the *Moringaoleifera* tree variety of Malawi. J Amer Oil Chem Soc. 2002;79(7):677–683

5). Padla EP, Solis LT, Levida RM, et al. Antimicrobial isothiocyanates from the seeds of *Moringaoleifera* Lam. Z Naturforsch C J Biosci. 2012;67(11–12):557–564.

6). Singh RG, Negi PS, Radha C. Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of *Moringaoleifera* seed flour. J Funct Foods. 2013;5(4):1883–1891.

7). Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., &Bertoli, S. (2016). *Moringaoleifera* Seeds and Oil: Characteristics and Uses for Human Health. International journal of molecular sciences, 17(12), 2141. https://doi.org/10.3390/ijms17122141

8). Rafi, M.; Meitary, N.; Septaningsih, D.A.; Bintang, M. Phytochemical Profile and Antioxidant Activity of *Guazumaulmifolia*Leaves Extracts Using Different Solvent Extraction. Res. Artic.Indones. J. Pharm. Indones. J. Pharm 2020, 31, 171–180. [CrossRef]

9). Sharma, S.; Kumari, A.; Dhatwalia, J.; Guleria, I.; Lal, S.; Upadhyay, N.; Kumar, V.; Kumar, A. Effect of solvents extraction on phytochemical profile and biological activities of two *Ocimum* species: A comparative study. J. Appl. Res. Med. Aromat. Plants 2021, 25, 100348. [CrossRef]

10). Jaafaru, M.S., Nordin, N., Shaari, K., Rosli, R., and Abdull-Razis, A. F. (2018) Isothiocyanate from *Moringaoleifera* seeds mitigates hydrogen peroxide-induced cytotoxicity and preserved morphological features of human neuronal cells.

11). Oyetayo VO. Free radical scavenging and antimicrobial properties of extracts of wild mushrooms. Braz J Microbiol. 2009 Apr;40(2):380-6. doi: 10.1590/S1517-838220090002000031. Epub 2009 Jun 1. PMID: 24031376; PMCID: PMC3769741.

12). Xie, J. and K.M. Schaich, 2014. Re-evaluation of the 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. J. Agric. Food Chem., 62: 4251-4260.

13). Chanda, S., R. Dave and M. Kaneria, 2011. In vitro antioxidant property of some Indian medicinal plants. Res. J. Med. Plant,5: 169-179

14). Piang-Siong, W., P. Caro, A. Marvilliers, X. Chasseray, B. Payet, A.S.C. Sing and B. Illien, 2017. Contribution of trans-aconitic acid to DPPHC scavenging ability in different media. Food Chem., 214: 447-452.

15).Burgos, G.; Munoa, L.P.; Sosa, P.; Cayhualla, E.; Carpio, R.; Felde, T. zum. 2014. Procedures for chemical analysis of potato and sweet potato samples at CIP's Quality and

Nutrition Laboratory. Lima (Peru). International Potato Center (CIP). Global Program Genetics and Crop Improvement.ISBN 978-92-9060-444-0.24 p.

16). Jadid N., Hidayati D., Hartanti S.R., Arraniry B.A., Rachman R.Y., and Wikanta W. "Antioxidant activities of different solvent extracts of *Piper retrofractum Vahl*. using DPPH assay", AIP Conference Proceedings 1854, 020019 (2017) https://doi.org/10.1063/1.4985410

17). Mensor, L. L.; Menezes, F. S.; Leitao, G.G.; Reis, A.S.; Dos Santos, T.C.; Coube, C.S.; Leitao, S. G.; et al.. Screening of Brazilian plant extracts for antioxidant activity by the use of dPPH free radical method. Phytother.Res.. 2001,15, 127-130.

18). Sharma, K.R., Kalauni, S.K. and Awale, S. 2015. Antioxidant, phytotoxic and antimicrobial activities of methanolic extract of *Bauhinia variegata* Barks. Journal of Institute of Science and Technology. 20(2): 37-41. https://doi.org/10.3126/jist.v20i2.13946.

19). Khalaf, N.A., Shakya, A.K., Al-Othman, A., El-Agbar, Z. and Farah, H. 2008. Antioxidant activity of some common plants.Turkish Journal of Biology. 32(1): 51-55.

20). Sultana, Bushra& Anwar, Farooq& Ashraf, Muhammad. (2009). Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts.Molecules (Basel, Switzerland). 14. 2167-80. 10.3390/molecules14062167.

21). Tailor, Chandra Shekhar&Goyal, Anju. (2014). Antioxidant Activity by DPPH Radical Scavenging Method of *Ageratum conyzoides Linn.*Leaves.American Journal of Ethnomedicine. 1. 244-249

22). Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal. *Plants*. 2019; 8(4):96. https://doi.org/10.3390/plants8040096

23). Fatiha, M. and Abdelkader, T. (2019). Study of antioxidant activity of pyrimidiniumbetaines by DPPH radical scavenging method. Journal of Analytical and Pharmaceutical Research, 8(2), 33–36. https://doi.org/10.15406/japlr.2019.08.00308

24).Chaouche, TarikMohammeda,*; Haddouchi, Faraha; Ksouri, Riadhb; Atik-Bekkara, Fouziaa. Evaluation of antioxidant activity of hydromethanolic extracts of some medicinal species from South Algeria. Journal of the Chinese Medical Association: June 2014 - Volume 77 - Issue 6 - p 302-307
doi: 10.1016/j.jcma.2014.01.009

25). Fatmawaty F, Anggreni N. G. M, Fadhil N, Prasasty V. D. Potential In Vitro and In Vivo Antioxidant Activities from *Piper Crocatum* and *Persea Americana* Leaf Extracts. Biomed Pharmacol J 2019;12(2).

26). Fitriana, W. D., Ersam, T., Shimizu, K., &Fatmawati, S. (2016). Antioxidant activity of *Moringaoleifera* extracts. *Indonesian Journal of Chemistry*, *16*(3), 297-301. https://doi.org/10.22146/ijc.21145

27). Hidayati MD and Rahmatulloh A. Antioxidant Activity of *UncariaGambir* (Hunter) Roxb Extracts. Trop J Nat Prod Res. 2022; 6(8):1215-1218. doi.org/10.26538/tjnpr/v6i8.9

28). Hidayati, M.D., Ersam, T., Shimizu, K., &Fatmawati, S. (2017). Antioxidant Activity of *Syzygiumpolynthum*Extracts. Indonesian Journal of Chemistry, 17 (1) , pp. 49-53.