Extraction, Isolation and Characterization of Bioactive Compound from *G. diversifolia* Methanolic Extract

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Abstract

Plant-derived ingredients almost contribute to 25% of prescription pharmaceuticals yet only a small percentage of the plants in the world have been assessed for their prospective pharmaceutical use. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. The present work focused on the analytical methodologies, which include the phytochemical screening, extraction, isolation and characterization of bioactive compound from the *G diversifolia* methanolic leaf extract. One phyto-constituent Caffeic acid was isolated from *Girardinia diversifolia.* The analysis of bioactive compound present in the *G diversifolia* extract involving the applications of chromatographic techniques such as HPLC and, TLC as well as non-chromatographic techniques such as MS and Fourier Transform Infra-Red (FTIR).

Keywords

Bioactive compound; Plant Extraction; Isolation; Girardinia diversifolia; Phyto-constituent

Introduction

The consumption of the herbal medicines is growing day by day for the treatment of the ailments and it can be supported by the fact that 80% of the worldwide population in the developing countries depends on the medicinal herbs. India has rich repository of medicinal plants that possess 8% of biodiversity in the world with 12500 species including 2500 different medicinal plant species and host of the four hot- spots of biodiversity in the Western Ghats, the Himalayas, Sunderland and the North-eastern region (Chikezie *et al*., 2015). Though in India the medicinal plants are used in the management of different diseases since ages but its use is decreased as it takes a long time to show its pharmacological action, therefore a large number of people start using the allopathic medicines which gives a quick relief (kumar *et al*, 2019). In the current scenario again in India, the use of herbal medicines is increasing continuously owing to lesser side effects as compared to conventional allopathic medications. However, the compounds responsible for the physiological actions of these traditional remedies were not known yet (Yuan *et al*., 2016). This has not only hindered the standardization and development of these herbs, but also made its recognition; acceptance and utilization to remain locally restricted. Therefore, the current study was performed to isolate, characterize and identify the bioactive compounds in the leaf extracts of *G. diversifolia,* commonly known as allo (in eastern part Nepal) or puwa (in western Nepal), Dans Kandali (in Uttarakhand), Himalayan Nettle or Bicchu Butti (in Himalaya) belongs to the family *Urticaceae*. It is having lots of commercial and therapeutic value and commonly grown in open forests and moist riverside habitats (Nand and Naithani, 2018).

Collection

The methanolic leaf extract of *G.diversifolia* was procured from Green Heaven, Maharashtra, India.

Phytochemical studies

Procured methanolic leaf extract of *G. diversifolia* were subjected to phytochemical analysis for the detection of different constituents including carbohydrates, saponins, alkaloids, phenolic compounds, terpene, etc. (Abdulwahab et al., 2011).

Determination of Total Phenolic Content

Total phenolic content of the extracts of *G.diversifolia* was calculated by the Folin–Ciocalteu reagent method. The leaf extract *G. diversifolia* were dissolved in distilled water to prepare

stock solutions of 1 mg/ml. Thereafter, 1 ml of the extracts were combined with 1 ml of Folin–Ciocalteu reagent followed by addition of 4 ml of 7% sodium carbonate solution and 4 ml of distilled water after 5 min. The mixtures were left in the dark to incubate for 60 min and the absorbance was read at 750 nm using Perkin Elmer Lambda 25 UV-Vis spectrophotometer against a reagent blank. Different dilutions of 2, 4, 6, 8 and 10 µg/ml were made from the stock solution of gallic acid (GA) in water (100 mg/ml) and prepared in the same way as the samples to obtain the standard curve. The total phenolic content was calculated from the following formula (kumar *et al*., 2018).

Total phenolic content= (C×V/N),

Where, C=concentration of GA established from the standard curve (mg/ml), V= volume of the extract (ml), N= weight of the extract (g).

Total phenolic content was represented as mg of GA equivalents per g extract (mg GAE/g). The experiment was conducted in triplicate (Sharma & Agarwal, 2015).

Determination of Total Flavonoid Content

The leaves extracts of *G. diversifolia* were dissolved in distilled water to prepare stock solutions of 1 mg/ml. 0.5 ml of the extracts were admixed with 2 ml distilled water followed by addition of 0.15 ml of 5% sodium nitrite solution. To the above mixture, 0.15 ml of 10% aluminium chloride solution was added after 5 min and kept for additional 6 min. To this, 2 ml of 4% sodium hydroxide solution was added and the total volume was adjusted to 5 ml with 0.2 ml of distilled water. The mixtures in the tubes were mixed properly and kept for a period of 15 min and the absorbance was read against a reagent blank at 510 nm. Various dilutions of 2, 4, 6, 8 and 10 µg/ml were made from a stock solution of rutin (RU) in distilled water (100 mg/100 ml) and prepared in the same way as the samples to establish the standard curve. The total flavonoid content was calculated from the following formula.

Total flavonoid content= (C×V/N)

where, C= concentration of RU established from the standard curve (mg/ml), V= volume of the extract (ml), N= weight of the extract (g).Total flavonoid content was represented as mg of RU equivalents per g extract (mg RE/g).The test tubes were prepared in triplicate (Ahiakpa *et al*., 2013).

Fractionation of methanolic extract

2.5 gm of the methanolic extract was suspended in 30 ml distilled water and different fractions were obtained by treating it with different solvents such as hexane, ethyl acetate and butanol.

Isolation of Active Compounds

The isolation of the active constitutes were done based on the polarity, solubility and affinity of solvents.

Colum Chromatography (Principle)

It is used to isolate the active phytoconstituents using the principle of partioning coefficient.

Method: Wet packing of the column

Packing material: Silica gel

Procedure for packing: The column was packed with the silica gel using wet method and the extract was added into it.

Characterization of fractions obtained from *G. diversifolia:*

For characterization of compounds, Infra-Red spectroscopy (IR), Nuclear Magnetic Resonance (NMR) and Mass spectra (MS) were performed. IR spectra of isolated compounds were obtained by using a FTIR (Fourier transform Infrared spectroscopy; Model - Agilent Cary 630 FTIR spectrometer). They were recorded within the wave number range 4000–400 cm^{-1} in an FTIR instrument. NMR spectra were recorded on Bruker Avance AV-III type, 7.05 Tesla (300 MHz 1H frequency) to 11.74 Tesla (500 MHz 1H frequency). Isolated compound were dissolved in respective solvent $CDCl_3$/DMSO and about 600 µl was poured in NMR tube and observed on the applied magnetic field. Mass spectra were obtained by Bruker Electrospray Ionization instrument SL Dual Funnel Iontrap bench top with a mass accuracy of 0.1Da.

Result and discussion

Phytochemical screening of *G. diversifolia* extracts

The methanolic leaf extract of *G. diversifolia* showed the presence of all major plant secondary metabolites (Table 1), which proposes a multifaceted therapeutic approach like anti-inflammation, anti-hyperglycemia, and anti-diarrheal etc. However, presence of phenolic and flavonoids are also responsible in treating the life threatening diseases such as cardiovasular diseases, cancer, etc.

Table 1: Phytochemical Screening of *G. diversifolia* leaf extracts

S No.	Screening assay	Methanolic extract
1	Carbohydrates	Absent
2	Saponins	**Present**
3	Alkaloids	**Present**
4	Phenolic Compounds	**Present**
5	Flavonoids	**Present**
6	Proteins	**Present**
7	Purines	**Present**
8	Glycosides	**Present**
9	Terpene	**Present**

Total Phenolic and Flavonoid Contents

Total Phenolic and Flavonoid Contents represent the therapeutic potential of a plant. The methanolic of *G. diversifolia* methanolic leaf extract showed a significant amount of total phenolic and flavonoid content. The phenolic and flavanoid content was calculated as 316.25 ± 0.01(mg of GAE/g) and 382.45 ± 0.02(mg of QR/g) for *G. diversifolia* methanolic leaf extract respectively as shown in Table 2. The presence of flavonoid and phenolic content in the extracts proposes a multifaceted therapeutic approach of *G. diversifolia* since flavonoids showed anti-inflammation, anti-hyperglycemia, and anti-diarrheal activity potentials (Jung *et al*., 2014). In addition, flavonoids act as a powerful antioxidant by neutralizing free radicals (Pietta, 2000). Moreover, the presence of phenolic contents increases the acceptability in the treatment of various skin disorders, wounds, burns, inflammatory disorders, diabetic and

oxidative stress. Phenolic contents of plant are also responsible in treating various life threatning diseases including cardiovascular diseases, cancer, etc. (Działo *et al*., 2016).

Table 2: Total Phenolic and Flavonoid Contents of *G. diversifolia* in leaf extract

S No	Sample Extract	Phenolic content (mg of GAE/g DW)	Flavonoid content (mg of QR/g DW)
1	Methanolic extract (*G. diversifolia*)	316.25 ± 0.01	382.45 ± 0.02

Fractionation of the methanolic extract of *G. diversifolia* leaves

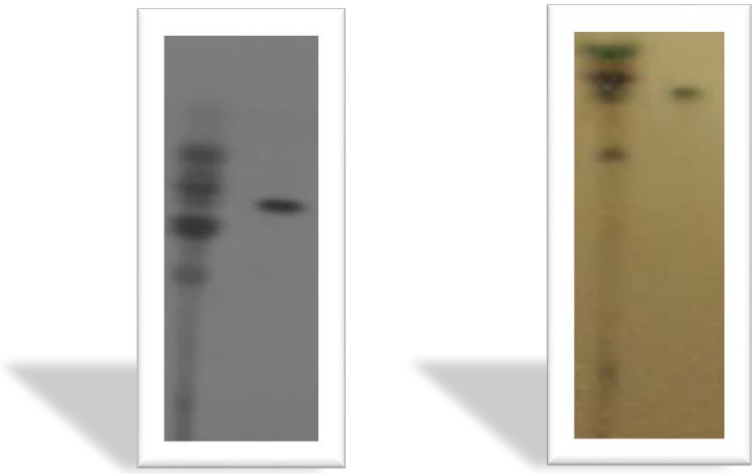
Fractionation of methanolic extract of *G. diversifolia* leaf was done according to the bioactive guided fractionation method using n-hexane, ethyl acetate and n-butanol as solvents (kumar *et al,* 2019). The ethyl acetate sub fraction of *G. diversifolia* methanolic extract (G-1) showed spot at R_f value 0.5, with the yield of 200 mg.

Thin Layer Chromatography of the methanolic extract of G. diversifolia leaves

Thin layer chromatography of sub fraction of methanolic extracts were done on pre-coated aluminium TLC plate using glass capillaries, optimized solvent system of various polarity such as for *E. binata* fraction C (EA: Methanol: Water 8.2:2:0.8) and fraction D (Benzene: Acetone: Formic acid (5:4:1)). Identification of phyto-constituents was done by Iodine and UV chamber. These pre-coated plates also used for purification of compounds from fraction by carefully scratching them using surgical blade and dissolved in ethanol and filtered using PTFE filter.

Analytical TLC of ethyl acetate sub fraction of methanolic leaf extract of *G. diversifolia* was carried out using Toluene: Ethyl acetate: Formic acid: Water (1:8:0.5:0.5) as mobile phase and Caffeic acid as standard. Identification was done by UV chamber (at 254 nm showed dark spot and at 366 nm showed blue colour) and spraying with 5% $FeCl_3$ green

colour was noted. Preparative TLC was carried out and clean spot was scrapped off using surgical blades, dissolved in ethanol, and filtered using PTFE filter.



A B

Figure 1: Thin Layer Chromatography of the ethyl acetate sub fraction of *G. diversifolia* methanolic extract A) Under UV Chamber 254 B) Spraying with $FeCl_3$

Characterization of fractions obtained from G. *diversifolia*:

IR spectrum analysis of Fraction

The IR spectral data of fraction (cm^{-1}) showed peaks at 3431 (O-H stretching), 3065 (C-H stretching (a), 1650 (C-O stretching), 2987, 3045 (C-H stretching (b), and 1600, 1530, 1400 (C-C stretching (a,b), here b and a stand for benzene moiety and acyclic chain respectively shown in figure 2.

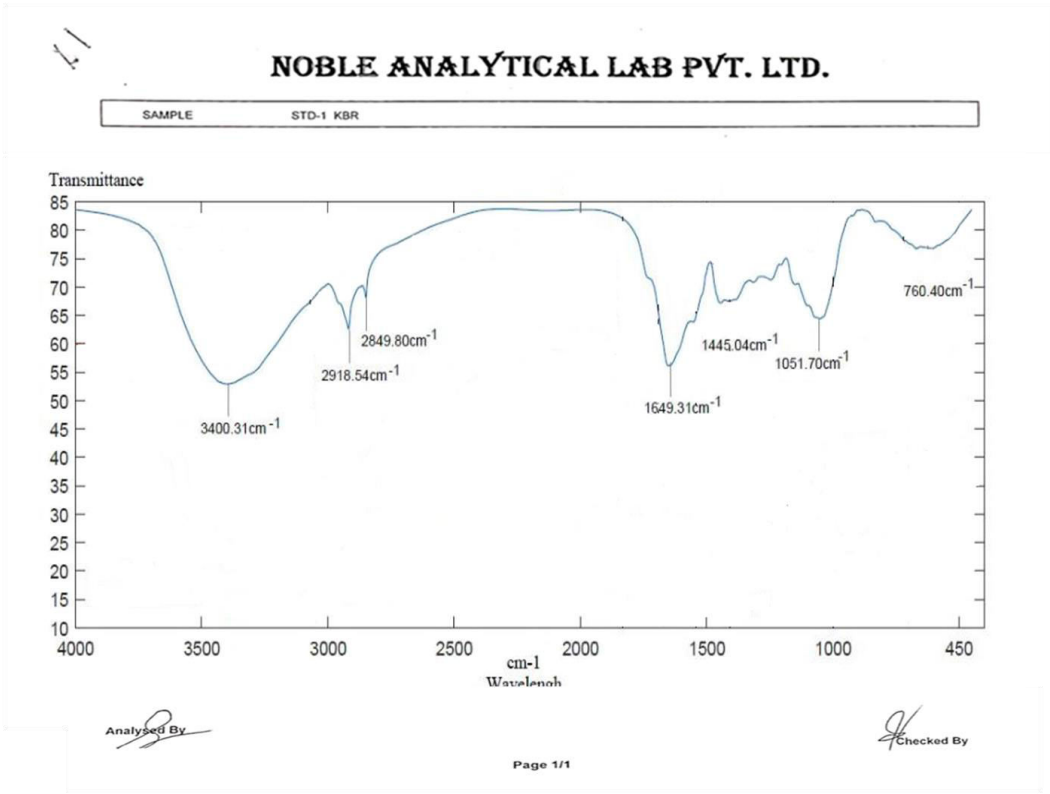


Figure 2: **FTIR spectra of fraction of *G. diversifolia***

Proton and Carbon NMR analysis

Proton NMR spectra (300 MHZ, DMSO-d6) δ (ppm) showed the peaks at 7.73, 7.6, 7.2 indicates Ar-H, 8.13 and 6.13 represent the Acyclic H and peak at 7.6 (2H) indicate Ar OH proton. The ^{13}C NMR spectrum showed δ ppm value 167, 146, 115 for Acyclic-C=C and 111 to 124 for aromatic carbon group shown in figure 3 and 4.

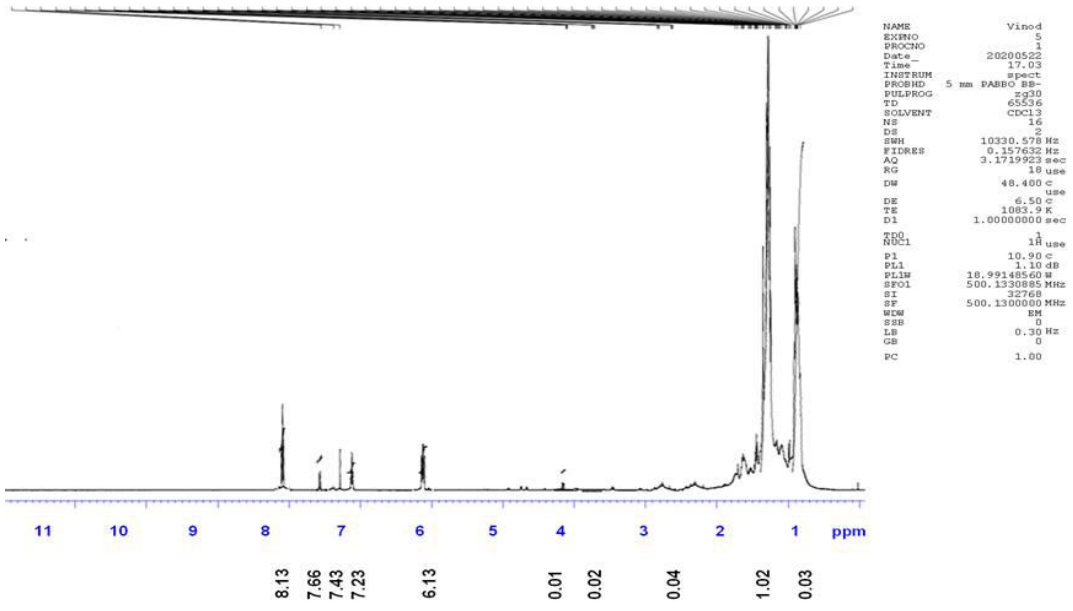


Figure 3: **Proton NMR (^{1}H NMR) spectra of fraction**

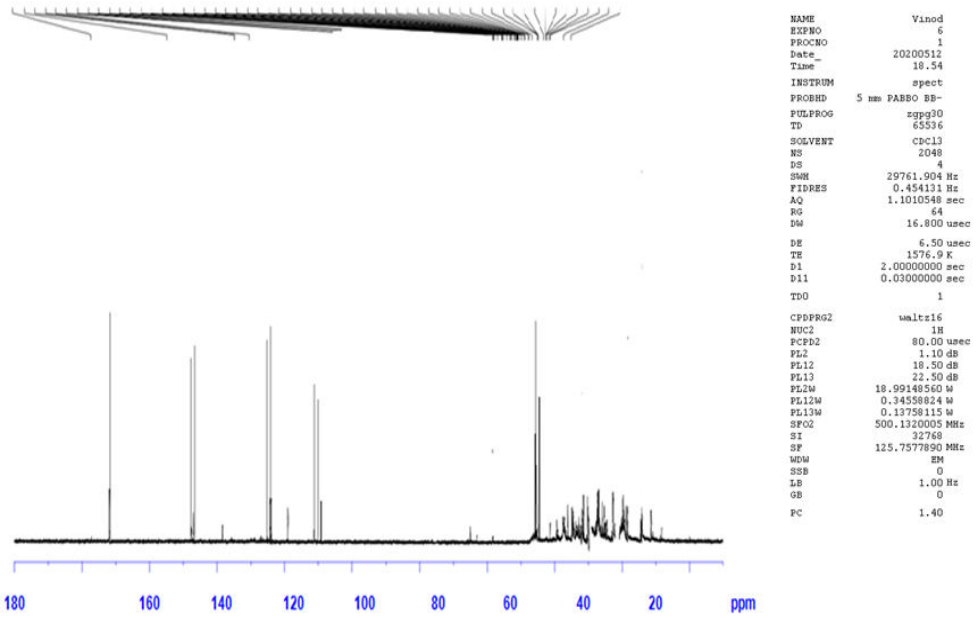


Figure 4: **Carbon NMR (C^{13} NMR) spectra of fraction**

LC-Mass spectroscopy spectra of fraction

The molecular ion peak M+ of the isolated fraction was observed at m/z 179.6 which in literature showed that it resembles Caffeic acid (3-(3,4-Dihydroxyphenyl)prop-2-enoic acid) figure 5. The IR, ^{1}H NMR and ^{13}C NMR and mass spectra were in agreement to the structure of Caffeic acid, confirmed that fraction was Caffeic acid.

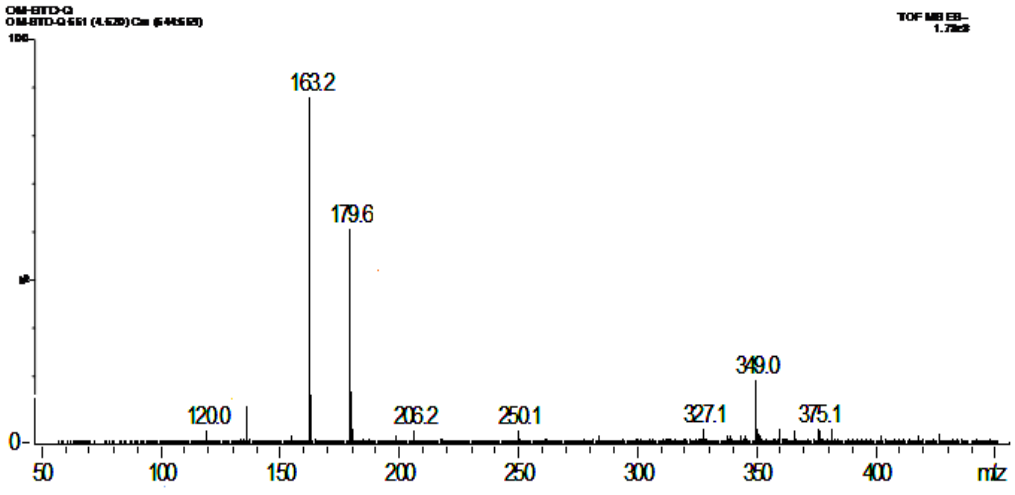


Figure 5: **Mass spectra of Fraction of *G. diversifolia***

Conclusion

From the fractionation of methanolic extract of *G. diversifolia*, one compounds was isolated i.e. caffeic acid. The phyto-constituent was confirmed by various spectroscopic methods i.e. IR, NMR and LC-MS. It was present in reasonable quantities sufficient to exhibit a promising pharmacological effect. The presence of the caffeic acid proved that the two plants are a rich source of efficacious phenolic and flavonoid aglycones thus making it a medicinal boon for the treatment of various disorders.

References

[1]. Abdulwahab, N. Z., Shahar, S., Abdullah, S. H., Pihie, A. H. and Ibrahim, N. (2011). Antioxidant, antibacterial and antiviral properties of *Goniothalamusumbrosus* leaves methanolic extract. *Afr. J.Micro. Res.* 5(20), 3138–3143.

[2]. Chikezie, P. C. and Ojiako, O. A. (2015). Herbal Medicine: Yesterday, Today and Tomorrow. *Altern Integr Med*. 4 (3), 195-200.

[3]. Dzialo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J. and Kulma A. (2016). The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. *Int J Mol Sci.* 17(2), 160-201.

[4]. Jung, M., Choi, J., Chae, H. S., Cho, J. Y., Kim, Y. D., Htwe K. M., Lee, W. S., Chin, Y. W., Kim, J. and Yoon, K. D. (2014). Flavonoids from *Symplocos racemosa*. *Molecules.* 20(1), 358-65.

[5]. Kumar **V**, Mohan C and Kumari S *et al*., "Synthesis, characterization and antibacterial studies of Semicarbazide based Schiff bases and their Pb(II), Zr(IV) and U(VI) complexes" Indian Journal of Chemistry -Section A, (Article Id: IJCA-2967) 2019, ISSN: 0376-4710

[6]. Kumar V, Sharma AK, Rajput SK, Pal M, Dhiman N. Evaluation of phytochemical, toxicological and pharmacological profile of Eulaliopsis binata leaf extracts. Toxicol Res. 2018; 7: 454-464

[7]. Kumar **V**., Pal M., and Dhiman, N., Determination of Sun Protection Factor in different extract of *Eulaliopsis binata*, Plant Archives, 2019; 19(2), 185-187

[8]. Nand, K. and Naithani, S. (2018). Ethnobotanical uses of wild medicinal plants by the local community in the Asi Ganga sub-basin, Western Himalaya. *J Complement Med Res.,* 9(1), 34-46

[9]. Pietta, P. G. (2000). Flavonoids as Antioxidants. *J. Nat. Prod. 63(7),*1035–1042.

[10]. Sharma, V. and Agarwal, A. (2015). Physicochemical and Antioxidant Assays of Methanol and Hydromethanol Extract of Ariel Parts of *Indigofera tinctoria* Linn. *Indian J. Pharm. Sci.* 77(6), 729-734.

Ahiakpa, J. K., Quartey, E. K., Amoatey, H. M., Klu, G.Y.P., Achel, D. G., Achoribo, E. and Agbenyegah S. (2013). Total flavonoid, phenolic contents and antioxidant scavenging activity in 25 accessions of okra (*Abelmoschus spp* L.). *Afr. J. Food Sci. Technol.* 4(5), 129-135.

[11]. Yuan, H., Ma, Q., Ye, L. and Piao, G. (2016). The Traditional Medicine and Modern Medicine from Natural Products. *Molecules.* 21(5), 559-576.