Purification, Isolation And Characterization Of Bioactive Components From *Breonadia Salicina* Exract (SABULU RAFI)

Cletus E Gilbert [1], H M Adamu [1], A A Mahmoud [1]

[1]*Student,Department of Chemistry, Abubarkar Tafewa Balewa University Bauchi, Nigeria.*

Corresponding Author: successclet74@gmail.com

Abstract: - *Breonadia salicina* extract has been used to treat sleeping sickness, and respiratory diseases in North central Nigeria, and Nigeria at large. In view of its usage, the aim of this research is to isolated, purify, and characterize the bioactive components from *B.salicina* extract. The ethyl acetate extracts of the leave shows promising potency against the test microbes and was subjected to thin layer chromatography and column chromatography. Which resulted in the isolation of pure fraction coded EAF8. The bioactivity of EAF8 was tested using agar diffusion technique, resulted to the growth inhibition of *S.aureaus, E.coli, A.niger, and C. albicans*as to significant extent. The fraction (EAF8) was evaluated using GC-MS and IR. The IR revealed the presence of some functional groups, which were 770 cm^{-1}(ortho disubstituted) for aromatic; 890 cm^{-1} (C-H deformation) for aromatic; 1150 cm^{-1} (C-O stretching) for ether; 1394 cm^{-1} (C-H bending) for CH_3; 1488 cm^{-1}(C-H bending) for CH_2; 1663 cm^{-1}(C=C)for aromatic; 1705 cm^{-1}(C=O stretching) for ketone;2885 cm^{-1}(C-H stretching) for methylene; 2951cm^{-1}(C-H stretching) for methyl; 3345 cm^{-1}(N-H stretching) for primary amine; 3462 cm^{-1} (O-H stretching) for alcohol/phenol. Gas Chromatogram revealed the spectra line 5, area 6250073, and retention time of 31.677. While the mass spectroscopy revealed the mass ratio of a fragments which were 253 (5 %), 236 (6 %), 191 (100 %), and 173 (2 %). Hence from the IR data and GC-MS, fraction EAF8 was proposed to be 7-amino-4,5-dihydroxy-3-(ethoxymethane) coumarin. The results of the antimicrobial activity obtained from this research justified the traditional uses of the plant. Meanwhile the research recommend that further studies should be carried out to isolate more bioactive compounds from stem bark and the root of the *Breonadia salicina.*

Key Words: — ***Breonadia salicina, Purification, isolation, and coumarin.***

I. INTRODUCTION

Plants have been used for various purposes since prehistoric times. Indian Ayurveda medicine used herbs as early as 1900 BC describing about 700 medicinal plants (Anders, 2007).

However, many more plants needs to be documented for immediate research and use for posterity, even more so when plant used varies highly from place to place. Typhoid fever, a common and sometimes fatal infection of both adults and children that causes bacteremia and inflammatory destruction of the intestine and other organs, is endemic in countries, especially throughout Asia and Africa.

Manuscript revised October 16, 2021; accepted October 17, 2021. Date of publication October 18, 2021.
This paper available online at www.ijprse.com
ISSN (Online): 2582-7898; SJIF: 5.494

Typhoid fever, caused by the bacterium *Serovar typhi* (*S. typhi*), has become rare in industrialized countries, yet it remains a major cause of enteric disease in children in developing countries, resulting in an estimated incidence of 50 cases per 100,000 persons per year, predominantly in young school-age children (WHO, 2001). Globally, it is estimated that typhoid accounts for 16 million cases each year, resulting in over 600,000 deaths (Rufaro, 2003).

Plants are either wild plant species; means those growing spontaneously maintaining population in nature or semi nature ecosystems, and could exist independently of direct action. Domesticated plant species means those that have arisen through human actions, which depend on maintenance for their existence, example *Aloe barbadensis* (Khaled, 2006).

II. MATERIALS AND METHODS

2.1 Isolation and purification of active components

Functional groups give the molecule distinctive chemical reactivity, as well play a vital role in physical properties of compounds. Different physical properties allow the separation of one component from the mixture. In some cases separation can be based on the solubility of the compound in a giving organic solvent, so that a compound can be recrystallized (Kwaji *et al*., 2019).

2.2 *Thin Layer Chromatography (TLC)*

The plant extract was dissolved in minimal amount of methanol and chloroform in different ratios (1:2 in 10 ml). The resulting chromatogram, after air drying was viewed by placing it in an isolated system containing iodine crystal for visualization. Finally a ratio of methanol and chloroform (9.5:0.5) was found as the most suitable mobile phase that provided an excellent resolution. This was obtained by the comparison of the resulting chromatograms, which emerge the best solvent system for column chromatography (Kwaji et al., 2019)

2.3 Column Chromatography

The separation of the ethyl acetate extract into different chemical components was carried out in two stages using column chromatography technique with silica gel (60-120 mesh) as the stationary phases.

2.4 Column packing

A glass column of 100cm3 long and internal diameter 3 cm3 was used. A 10 g silica gel (60-120 mesh) was activated in an ovun at 1000 C for 1hour. It was allowed to cool at room temperature in a desiccator. Slurry of silica gel was made with the solvent system and packed into the column. The system was preconditioned by passing the solvent system continuously for 30 minutes (Kwaji et al., 2019).

2.4.1 Loading of the extract into the column

A 2.0 g of the extract was dissolved in minimum volume of the solvent system and mixed with 3.0 g silica gel. This was loaded into the top of the column after draining the solvent to the level of the silica gel bed, followed by addition of 2.0 g silica gel and the solvent system was allowed to pass through the column.

2.4.2 Collection of the Eluent

Fraction of the eluents were collected using test tubes at a flow rate of 10 cm^3 per hour until the issuing eluents was clear of extract. All the fractions that were collected were concentrated in vacou using rotary evaporator, spotted on TLC plate and developed with the same solvent system. Similar fractions was pooled together and further purified using mini column chromatography technique, and the active fraction was coded EAF8.

2.5 Structural elucidation

The active compound (EAF8) was elucidated using Fourier transform-infrared (FT-IR) and Gas chromatography-mass spectrometry (GC-MS) to determine the structure.

2.6 Fourier Transform-Infrared Spectroscopy (FT-IR)

The FT-IR instrument consist of an IR light source, a sample holder, a means of selecting individual wavelength or frequencies of the light, some means of detecting the amount of incident light that the sample absorbs, and a device for plotting the amount of the light absorbed as a function of wave length or frequency.

A small quantity of the sample was grounded with potassium bromide to very fine powder (to remove scattering effects from large crystals). This powdered mixture was then pressed in a mechanical presser to form a translucent pellet (a special device to allow the sensor to shine through it) through which the beam of the spectrometer can pass. The machine was run to obtain the IR spectrum.

2.7 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-Mass spectrometer model was used in this analysis. The column size is 30 meter × 25µm film thickness. The injection volume was 0.5µl, injection temperature is 250 C. The auto-sampler injects 0.5µl of the sample, injection in un-split mode. The carrier gas was helium, at a working constant flow rate of 1.5 ml/min. Mass spectra were recorded in electron impact mode at 70 eV; electron multiplier 2500 V; ion source. Mass spectra data were acquired in the scan mode in m/z range 40-350 uma.

III. Result And Discussion

3.1 Purification of bioactive compound

Table.1. Column chromatography and TLC

Hexane	Ethyl acetate	Volume	Fraction	TLC Solvent System	PF	RfValue
Hexane Ethyl acetate						
100	0	200	F1-F2	1 : 1		
90	10	300	F3-F5	1 : 1		
80	20	300	F6-F7	1 : 1		
70	30	200	F8-F9	1 : 1		
60	40	100	F10	1 : 1		
50	50	200	F11-F12	1 : 1		
40	60	200	F13-F14	1 : 1		0.33
30	70	400	F15-F16	1 : 3		0.43,0.50
20	80	200	F17	1 : 3		0.51
10	90	400	F18-F19	Chloroform Methanol 9.5 : 0.5	F18	0.5,0.53
0	100	500	F20-F22	9.5 : 0.5		0.50,0.31,0.17
Ethyl acetate	Methanol	Volume	Fraction	TLC solvent system	PF	Rf
90	10	200	F23	9.5 : 0.5		0.22
80	20	500	F24-F25	9.5 : 0.5		0.36,0.42
70	30	200	F26	9.5 : 0.5		
60	40	400	F27-F28	9.5 : 0.5		
50	50	200	F29	9.5 : 0.5		0.12
0	60	500	F30-F31	9.5 : 0.5		0.19,0.26
Table 1						
30	70	200	F32	9 : 1		0.31
20	80	600	F33-F34	9 : 1		0.40,0.46
10	90	600	F35-F36	9 : 1		0.29,0.37
0	100	600	F37-F38	9 : 1		0.44,021

PF= Pool Fraction
Rf= Retention Fraction

3.2 Isolation of bioactive compound (EAF8)

3.2.1 Minimum Column Chromatography:

Table.2. data for mini column chromatography and TLC

Ethyl acetate	Chloroform	Volume	Fraction	TLC solvent system	PF	Rf
100	0	200	F1	Chloroform Methanol 9.5 : 0.5		
80	20	200	F2-F3	9.5 : 0.5		
50	50	400	F4-f6	9.5 : 0.5		0.36,0.23,0.50
20	80	300	F7-F9	9.5 : 0.5	F8	0.37,0.60,0.58
0	100	200	F10-F11	9.5 : 0.5		0.25

PF= Pool Fraction
Rf= Retention Fraction

Shai et al. (2013), used ethyl acetate: chloroform: methanol (55:5:40) as a suitable solvent for the purification and isolation of bioactive component (1,3-Oxo-28-hydroxylbetuli20(29)-ene), the bioactive component have carbonyl group, and hydroxyl group from B.salicina leaves, likewise in this research chloroform and methanol (9.5: 0.5) were the suitable solvents for isolation, while ethyl acetate and methanol (9.5 : 0.5) were suitable solvents for purification, where the isolated bioactive component have the same carbonyl group, and hydroxyl group Thus, this research work is in line with the result obtained by Shai (2013).

Mathabe et al. (2017) used column chromatography to isolated and purified compounds in form of colourless powder (terpenoids) from Spirostachy Africana. Likewise in this research column chromatography was used to purified and isolate compound (EAF8) in form of yellowish crystal from Breonadia salicina leaves.

Barnabas and Bawazeer (2019) isolated five compounds from B.salicina stem bark, obtaining Rf values (0.34, 0.50, 0.36) similar to this research. Thus this research is in line with the result obtained by Barnabas and Bawazeer (2019).

3.2.2 Antimicrobial activities of EAF8

The biological activities test carried out with the ethyl acetate fraction (EAF8) of Breonadia salicina leaves shows growth inhibition of Eschericha coli, Candidaalbicans, Aspergillus niger, and staphylococus aureaus to some extent

compared with standard drugs ciprofloxacin and amphotericin. Which is summarized in the following table.3.

Table.3. Antimicrobial activities of EAF8

Zone of inhibition		**Control**	
Microorganism	**EAF8**	**Cipro/amp**	**Distilledwater/ DMSO**
E. coli	16	26	00
C. albican	16	23	00
A.niger	11	24	00
S. aureaus	10	27	00

Note: Zone of inhibition ≥ 8mmis sensitive while<8 mm is resistant

KEY: E.coli =*Escherichia coli*

C. albican= *Candida albican*

A.niger=*Aspergillus niger*

S. aureaus= *Staphylococcus aureaus*.

Amp=Aphotericin B

Cipro= Ciprofloxacin

EAF8= Ethyl acetate fraction 8

This research indicated that the pure extract (EAF8) from *Breonadia salicina* inhibits the growth of *Escherichia coli*, *Candida albicans*, *Aspergillus niger*, and *Staphylococus* aureaus (Table 13). The inhibition of the pure extract against microbes might be due to the presence of the secondary metabolites observed, which is in line with the result obtained by Chakraborty *et al*. (2011).

3.2.3 FT-IR Analysis of isolated compound (EFA8)

The results of IR reveals the presence of Alkane (C-C), substituted benzene (C=C), ether (C-O-C), primary Amine (NH_2) with a wag band, and carbonyl group, absorption bands at 1394cm^{-1}, 1488 cm^{-1}, 2885-2951cm^{-1} for alkane, 770cm^{-1}, 890cm^{-1} for substituted benzene, 996-1150cm^{-1} for ether, 3462cm^{-1} for hydroxyl group, 3345cm^{-1} for primary Amine, and 770-770cm^{-1} for wag band of primary amine, and 1705cm^{-1} for carbonyl ester, respectively.

Table.4. FT-IR Analysis of EAF8 Fraction from Ethyl acetate extract

Absorption bands in cm^{-1}	**Vibration mode**	**Functional group**
770	Ortho disubstituted	Aromatic
890	C-H def	Aromatic
996	C-O-C bending	Heterocyclic ether
1150	C-O-C stretching	Ether
1394	C-H bending	CH_3-
1488	C-H bending	-CH_2-
1663	C=C stretching	Aromatic
Table 4 continued		
1705	C=O stretching	Ketonic
2885	C-H stretching	Methylene
2951	C-H stretching	Methyl
3345	N-H stretching	Primary amine
3462	O-H stretching	Alcohol

FT-IR spectrum analysis base on this outcome, ethyl acetate extract of B.salicina exhibited twelve function groups, shows in Table 16. Barnabas and Bawazeer (2019), isolated coumarin from B.salicina stem bark, and used FT-IR to characterize the functional groups, he reported the peak values 990cm-1, similar to 996cm-1 for ether, 1395cm-1 similar to 1394cm-1 for C-H bending, 1700cm-1 similar to 1705cm-1 for ketone carbonyl group, 3425cm-1 similar to 3462cm-1 for alcohol/phenol, 3340cm-1, Thus this research is in line with the result obtained by Barnabas and Bawazeer (2019).

Likewise, the FT-IR analysis of acetone/methanol of E.africana from ATBU Bauchi State studied by Kwaji et al. (2020), reveals the peak values 3332.60cm-1 slightly differs with 3462cm-1 for alcohol/phenol, 2915.27cm-1 similar to 2951cm-1 for C-H alkane stretching, 1632.27cm-1, similar to 1663cm-1 for Vinyl alkene, 1705.52cm-1 similar to 1705cm-1 forketone carbony group. Thus this research is in line with the result obtained by Kwaji et al. (2020).

Umashankar et al. (2015) reported the characterization of coumarin isolated from Crotalaria pallida using FT-IR analysis revealed the peak values of 1154cm-1 similar to 1150cm-1 for C-O-C stretching ether, 1715cm-1 similar to 1705cm-1 for ketone carbonyl group, 2963cm-1 similar to 2951cm-1 for C-H alkane stretching, 3440cm-1 slightly similar to 3462cm-1 for alcohol/phenol. Thus this research is in line with the result obtained by Umashankar et al. (2015).

3.3 GC-MS Analysis of the isolated compound (EFA8)

The GC-MS analysis of the isolated compound reveals a compound (line 5) with peak area of 6250073.69, height 364642.48, and retention time 31.677 and base peak is 191m/z. As shown in table.5.

Table.5. GC Analysis of EAF8

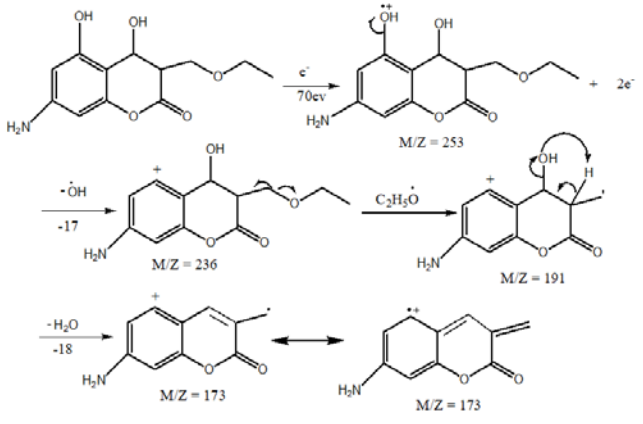
Spectrum line		**Ar**		
Line 5	6250073	31.677		

Compound (line 5) in appendix 3, in the GC of EAF8 shows area peak of 6250073.69, height of 364642.48 according to the data base of National institute standard and Technology (NIST) spectral library collection.

Table 6: MS Analysis of EAF8

Mass of fragment ion		**Proposed identity**
253 (5 %), 236 (6 %), 191 (100 %), 173 (2 %)		7-amino-4,5-hydroxyl-3-(ethoxymethane)coumarin

Line 5: RT= 31.697, peak area=6250073.69; height = 364642.48, and base peak=191.1 correspond to 7-amino-4,5-dihydroxy-3-(2-ethoxymethane) coumarin compare with the data base of National institute standard and Technology (NIST) spectral library collection. Molecular formula; C12H15NO5.The proposed fragmentation pattern is shown in scheme 1.



Scheme 1: Fragmentation Pattern for 7-amino-4, 5-dihydroxy-3-(-1-ethoxymethane) Coumarin.

The molecular ion (M+ =253) of the proposed compound, 7-amino-4,5-dihydroxy-3-(2-ethoxymethane) coumarin, loss hydroxyl radical leading to the fragment with m/z=236, follow by α-cleavage with loss of ethyl epoxide, giving rise to a fragment with m/z= 191 (base peak) , undergo abstraction of hydrogen by loss of water, leading to a fragment with m/z=173.

The IR spectrum of EAF8 justify the identification of the named compound 7-amino-4,5-dihydroxy-3-(2-ethoxymethane) coumarin, for there were characteristics absorption of an ether C-O-C stretching (996-1150 cm^{-1}), meta, and ortho disubtituted benzene (770-890 cm^{-1}), O-H stretching (3462 cm^{-1}), N-H stretching (3345 cm^{-1}) and C=O stretching (1705 cm^{-1}).

Save *et al.* (2015) isolated coumaric acid from the *T.peruviana*, and that its exerted positive anticancer effects on the prostrate, breasts, lungs, and pancreatic human cancer cell lines by inducing the loss of activity in most cancer cell lines. The presence of coumarin have been reported from variety of plants including; Ricinus communis Linn. (Euphorbiaceae) (Sani and Pateh, 2009), Ehretia leavis (Rasika *et al*., 2015), Ocimum americanum L.(Lamiaceae) (Shubhangi, 2016) and in honey (Monika and Kamaljit, 2016).

Sudha *et al.* (2014) obtained the structure of coumarin ((E)-5-(4-methyl-2-oxo-2H-chromen-8-yl)-2-(4-methylbenzyl)-3-oxopent-4-enenitrile) from *Ceropegia juncea* which has molecular ion peak; m/z = 360. The molecular ion fragment was observed at m/z 343. In his research the major fragment ions (m/z) were; 334,292, 262, 219,177,129 and 100, while in this research, the molecular ion peak ; m/z = 253, with the major fragments ion 236 (6 %), 191 (100 %), and 173 (2 %). The differences in both the molecular ion and fragmentation ions might be due to the present of daughters attached to the parent (coumarin).

IV. CONCLUSION

The sample plant was extracted using hexane, ethyl acetate, acetone, and methanol. Ethyl acetate extract from B.salicina leaves possess the highest antibacterial activity compare to other extracts. Ethyl acetate was further subjected to TLC and column chromatographic analyses for purification and isolation as shown in tables 1 and 2. Fraction EAF8 was found to have a good antimicrobial activity against the pathogenic organism, as shown in table 3. Structural elucidation was carried out using FT-IR, indicating the functional groups

as shown on table 4, and GC-MS indicating the retention time, area, height and the mass ratio of the different fragments this led to the propose structure,7-amino-4,5-dihydroxy-3-(1-ethoxymethane) coumarin.

REFERENCES

[1]. Adiaratou T., Ingvild A., Annette T., Drisss D.and Berit S.P(2008). Agriculture Sourcebook, Rohini Reddy SARRA; India pp.l – 3.

[2]. Adiaratou T. (2008). Ethnopharmacology, Phytochemistry and Biological Activities of Malian Medicinal Plants, thesis for the degree of philosophiae doctor Ph.D degree, printed in norway: ait e-dit as, oslo, 1-82.

[3]. Barnabas.J.Nvau, Bawazeer Sami.Adicardin and other coumarin from B.salicina Hepper. Tropic Journal Natural Product Resource 3(9):298-301.

[4]. Chakraborty A., K., Rambhade S. and Patil U.K., (2011). Chromolaena Odorata (L): An overview, Journal of Pharmacy Research, 4(3):573-576.

[5]. Kakali A.S (2020).Nigerian Trees.Published by the Department of Forest Research, Ibadan.Pp 252 – 254.

[6]. Katunku,D.,Ogunwolu,E.O.,andUkwela,M.U.(2014).Conta ct toxicity of Canarium schweinfurthii Engl.tissues against microbial activities.4(5):1234-1335.

[7]. Khaled A. Tawaha (2006). Cytotoxicity Evaluation of Jordanian Wild Plants using Brine Shrimp Lethality Test. Journal. Applied Science 8(1): 12-17.

[8]. Kumar S, Pandey AK (2013). Chemistry and biological activities of Flavonoids. An overview. The Science World Journal. 11:1-16.

[9]. Kwaji, A, H.M Adamu, I.Y. Chindo (2019). Isolation and partial characterization of alkylferulate from Entada Africana stem bark extract. Journal Applied 4(4): 97-99.

[10]. Monika G. and Kamaljit K. (2016).Quanlitative Analysis for free Radical Scavenging and Acid value of Honey Including GC-MS Spectra.Research Journal of pharmaceutical, Biological and Chemical Science 7(6):1998-2000.

[11]. Sahoo, J., Parween, G., Sahoo, S., Mekap, S. K., Sahoo, S., & Paidesetty, S. K. (2016). Synthesis, spectral characterization, in silico and in vitro antimicrobial investigations of some Schiff basemetal complexes derived fromazo salicylaldehyde analogues. Indian Journal of Chemistry, 55 (10): 1267-1275.

[12]. Saidu K., Onah j., Orisadipe A., Olusola A., Wambebe C., and Gamaniel K. (2018); Antipasmodial, analgesic, and anti-inflammatory activities of the aqueous extracts of the stem bark of Erythrina senegalensis. Journal of Ethnopharmacology71(1):275-280.

[13]. Sani U.M. and Pateh U.U (2009).Isolation of Coumarin from Methanolic extract of the variety minorseeds of Ricinuscommunis Linn.(Euphorbiaceae).Nigeria Journal Pharmacology Science 8(2):107-114.

[14]. Sanjit Karki, Kebindra Shrestha , Rajendra Gautam and Ram Narayan Jha (2020). Phytochemical Screening, FT-IR and GC-MS analysis of Euphorbia hirta. Journal of Pharmacognosy and Phytochemistry 9(1):1883-1889.

[15]. Sanne J.Wiersma, christiaan Mooiman, Martin Giera,Jack T.Prank,(2020). Squelene- Tetrahymanol cyclase L.Camara.Applied and Environmental microbiology. 86(17) 325-335.

[16]. Save, S. A. Lokhandel, R.S. and Chowdhary A.S. (2015). Determination of Coumarin compounds from the twigs of Thevetia peruviana as a Colwell Biomarker. Journal of Innovation in Pharmaceuticals and Biological.Science. 3(2):349-362.

[17]. Shai Lj, Chauke MA,Magano SR, Mogale AM, Eloff JN (2013). Antibacterial aaactivity of sixteen plant species from Phalaborwa, Limpopo Province, South Africa. Journal Medicinal Plants Research. 2013;7:1899-1906.

[18]. Sudha Karayil, Subhash Chandran K.p, Sudesh P.S.S Veriah.K (2014). Isolation and Structural Elucidation of novel bioactive molecule Coumarin from Ceropegia juncea.9(3) 19-22.

[19]. Suma, A. et al (2018): GC-MS and FTIR analysis on the methanolic extract of A.nilgiricum. World Journal Pharm. Science 6: 106-113.

[20]. Umashanka T, Govindappa M, Yarappa Lakshmikantha R (2015). Isolated and characterization of coumarin isolated from endophyte, Alternaria species-1 of Crotalaria pallidaand its Apoptotic Action of Hella Cancer cell line metabolomics 5:158.

[21]. Warner M (2007). Herbal Plants of Jumaica Macmillion Caribbeen: PortSmouth,NH,USA.

[22]. WHO (2001). Legal Status of Traditional Medicine and Complementary or Alternative medicine. Worldwide review. 1-3.

[23]. WHO Press, Geneva, 2013. Traditional Medicine Strategy 2014-2023.

[24]. Wise, R., Hart, T., Cars, O., Streulens, M., Helmuth, R., Huovinene, P., & Springer, M. (1998) Antimicrobial resistance. British Medical Journal, 317 (7159):609-610.

[25]. Wilson Obidah Henry L Badung Joseph Ajuiji Hakemo Peter Husseini Bello (2014): Effect of Erythrina senegalens is aqoeues leaf extract I rats. America Journal of Research Communication 2(4): 176-185.

[26]. Witabouna M. K., Kakou-N. E. S. and Mireille D., (2011). Assessing Sub-saharian Erythrina for Efficacy.Traditional Uses.Biological Activities and Phytochemistry. Pakist an Journal of Biological Science, 14 (1): 560-571.

[27]. Yuliana P, E.B. Loconi, E.Wina and A.Jayaneyawa.(2014). Extract of Tannins and Saponins from Plant Sources and their Effect on in Vitro Methanogenesis and Rumen Fermatation. Journal Indomesian Tropic Animal Agriculture 39(2):91-97.

[28]. Zhang, Q.; Lin, L.; Ye, W. (2018). Techniques for Extraction and Isolation of Natural Products: A comprehensive review. Chinese Medicinal. 2018, 13, 20.

[29]. Zuodong J., Chase K. and Joe C. (2016).Extraction and Analysis of Terpenes/Terpenoids.Current Protocol Plant Biology. 1: 345-358.