Phytochemical and Anti-Microbial Analysis of the Leaves of Cola Gigantea (Sterculiaceae)

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Abstract

Phytochemical and antimicrobial analyses were carried out on the purified leaves extract of Cola gigantea. The Harbone method was used in the extraction and the extract separated using a combination of column chromatography and preparative thin layer chromatography resulting in the isolation of two fractions with Rf values of 0.4467 and 0.7067 for leaves fractions 1 and 2 respectively. The isolated fractions were subjected to structural elucidation using the combination of appropriate spectroscopic instruments; FTIR, UV-Visible, GCMS, H^1 and C^{13}- NMR which gave rise to the following suggested compounds; 1,2-benzenedicarboxylic acid,dioctyl-dodecanoic acid, 2-propenoic acid, 2-methyl ethenyl ester and 1,2-benzene dicarboxylic acid, diisooctyl ester,2-hydroxy cyclo pentadecanone found in leaves fractions 1 and 2 respectively. Results of the phytochemical analysis showed the presence of some secondary metabolites such as Alkaloids, Carbohydrates, Cardiac glycosides, flavonoids, steroids, tannin, terpenoids in various concentrations while cyanogenic glycosides and saponin were conspicuously absent. The values of Mineral elements; Cd (0.40mg/g), As (0.03mg/g), Cr (0.90mg/g), Co (0.43mg/g), Fe (0.04mg/g) etc in the leaves all fell below the WHO recommendations thus showing its overall safety for therapeutic purposes. The antimicrobial analyses (the antifungal and anti bacterial analyses) using the Punched Agar diffusion method was carried out on the two isolated fractions comparatively with a standard drug cipromax fort (a broad spectrum antibiotic). A total of fourteen test organisms consisting of eleven bacteria strains and three fungi were used with the leaves fractions being active on all the test organisms given their average diameter zones of inhibition which ranged between 10mm and 28mm. Comparatively, the standard drug cipromax fort was of better antimicrobial effect than the leaves extracts. However, these fractions can serve as antimicrobial to diseases caused by these test organisms as acclaimed by ethnomedical practitioners given their MIC, MBC and MFC results.

Key Words: Cola gigantea, Phytochemical analysis, antimicrobial analysis, cipromax fort

Introduction

There has been man's unending desire for good and healthy living from ancient days which has led to his curiosity to examine all aspects of his environment by trial and error (Daziel, 1961). This gave rise to traditional medicine practice which was the only way of saving life in the olden days before the advent of modern medicine as earliest humans used various plants to treat illnesses (Ajiwe et al., 2008). Unfortunately, the misuse of these life saving medications coupled with bacteria's amazing ability to adapt has led to an increase in the number of drug resistant organisms (Nester et al., 2004). Some people even speculate that we are in danger of seeing an end to the era of antimicrobial medications. In response, scientists are involved in much current research devoted to the phytochemical investigation of higher plants such as *Cola gigantea* which have ethno botanical information associated with them. *Cola gigantea* (Sterculiacea) is the scientific name for a large forest tree found both in the relatively dry and wet parts of the rain forest. It is an ever green moderately sized tree often growing to a height of 20-25metres with glossy ovoid leaves up to 20cm, narrowly buttressed hole, stout to 5m girth, erect, non-cylindrical and bearing a dense spreading crown.

Cola gigantea had also been reported to have a high anti-microbial activity against *Staphylococcus albus, Bacillus subtilis, Aspergillus niger* and *Candida albicans* thus showing its potency as antibiotics. (Adeniyi et al.,2004; Agyare et al., 2012; Idu et al., 2000; Reid et al., 2005; Sonibare et al., 2009). Its leaves also are acclaimed to boast blood supply, cure anemia, sores, skin infection and other inflammatory conditions while the mature fruit of the Cola species known as Kolanut has a bitter flavour and high caffeine content used to ease hunger pangs. (Benjamin, 1991; Blades, 2000; Idu et al., 2010).

However, so far from the literature available, the isolation and structural elucidation of the active phytochemicals in the leaves of *Cola gigantea* has not been done hence this present study which aims at identifying the antimicrobials, isolating and structurally elucidating them.

Materials and Methods

Plant Collection, Identification and Preparation

The leaves of the plant *Cola gigantea* used in this study were collected from Okpuno in Awka North Local Government Area of Anambra State, Nigeria. It was identified by Mr Ugwuozor a taxonomist of the Department of Botany, Nnamdi Azikiwe University, Awka and authenticated by Prof J.C Okafor as *Cola gigantea* of the *Stercliacea* family. Fresh leaves samples were dried under shade for two weeks, pulverised and stored in a glass jar for subsequent analyses.

Extraction and Fractionation into different classes (Harbone, 1998)

300g of the pulverized leaves were soaked in 1500ml of methanol/water mixture in a ratio of 4:1 for about 1hour 30 minutes. The mixture was filtered and the filterate heated on a water bath to one-tenth (1/10) of the volume at temperature of about 40˚C. The filterate was then acidified with 2ml of 2M H_2SO_4 and then extracted with chloroform. The mixtures were separated using separating funnel. The chloroform extract was heated to dryness and re-dissolved with chloroform given the chloroform extract (Harbone, 1998). This extract was thereafter subjected to a combination of column chromatography and preparative thin layer chromatography to isolate two different active fractions from the leaves extract.

Phytochemical Screening

The crude leaves extract was evaluated for the presence of acidic components, flavonoids, saponins, reducing sugar, carbohydrate, tannins, resins, steroids, terpenoids, alkaloids, proteins, cardiac glycosides, cyanogenic glycosides and oil using standard procedures (Harbone, 1998).

Trace metal determination

Using Atomic Absorption Spectrophotometer model Varian AA 280, trace metals (Ar, Cd, Cr, Co, Fe, Pb, Mn,Hg, Ni and Zn) level of the sample were determined.

Anti-bacterial Assay

The sensitivity of the fractions and standard drug (Cipromax fort) against the selected test organisms (*Bacillus typhi, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, Staph albus, Staphylococcus aureus, Streptococcus muteus, Streptococcus pyogeus, Aspergillus flavis, Aspergillus niger* and *Candida albican*) was carried out using the punched agar diffusion method (Bryant, 1972).

The MIC and MBC were determined using the serial dilution method while MFC was determined using the Punched Agar diffusion method as recommended by Bryant,1972.

Structural Elucidation

Using a combination of these spectroscopic techniques such as FTIR, UV-visible, GCMS, H^1 and C^{13}-NMR structures and molecular formulae were proposed for the two isolated fractions of the leaves of *Cola gigantea*.

Results and Discussion

The results of the organoleptic examination of the leaves are as given in the table below

Table 1: Organoleptic Examination of the leaves of *Cola gigantea*

Parameter	Colour	Odour	Taste
Leaves	Green	Little Pungent	Bitter

The bitter taste observed in the leaves of the plant shows its ability to aid the toning of some vital organs especially the liver and kidney (Adodo, 1998).

Table 2: Result of Thin Layer Chromatography (TLC) of crude extract of the leaves of Cola gigantea

Parameter	R_f Value	Solvent Systems
Leaves fraction 1	0.4467	Chloroform: ethylacetate:water (70:20:5)
Leaves fraction 2	0.7067	Chloroform: ethylacetate:water (70:20:5)

The thin layer chromatography of the leaves extract showed two spots under iodine vapour with different Rf values as given in Table 2.

Table 3 : Phytochemical compositions of the leaves of *Cola gigantea*

Tests	Alkaloids	Cardiac Glycosides	Cyanogenic Glycosides	Flavonoids	Saponin	Steroids	Tannins	Terpenoids
Fresh Leaves	++	+	-	+++	-	+++	++	+

- Absent + Present in low concentration
++ Present in high concentration +++ Present in very high concentration

The results of the phytochemical analysis of *C.gigantea* showed the presence of flavonoids and steroids in very high concentration while alkaloids and tannins were in moderately high concentration with saponin and cyanogenic glycoside conspicuously absent. The above phytochemicals are the main basis for the plant's medicinal properties and starting materials in the synthesis of new drugs today. The presence of tannin in the leaves could be associated with the bitter taste recorded in the organoleptic test which also showed the antibacterial properties of the plant part (Adeniyi, 2004; Idu *et al.*, 2010; Reid, 2005; Sonibare, 2009).

Presence of alkaloids in high concentration in the leaves signified possession of antimicrobial activity, cyto-toxicity and sometimes neutralization ability of poisons within the herb. Flavonoids which are predominantly present help to reinforce capillary walls, improve the exchange of nutrients and oxygen between the blood and tissues (Harbone, 1998).

Table 4: Results of the Mineral Elements Found in the Leaves of *Cola Gigantea*

Element	As	Cd	Cr	Co	Fe	Pb	Mn	Hg	Ni	Zn
Leaves (mg/g)	0.003	1.60	0.90	0.43	0.04	2.41	1.77	0.57	5.10	2.03
WHO Standard	0.01	0.003	0.005			0.01	0.50	0.001	0.02	

The values of the elements found in the leaves of *C.gigantea* although below the standards except for Cd and Hg need to be check-mated to reduce the trace metal levels to acceptable levels before human consumption to mitigate the adverse effects of these on human body as a result of their gradual accumulation. Other useful elements like Zn were equally present in substantial amount as shown in table 4.

Table 5: Results of Antimicrobial Activity of Extracts/Fractions of the Leaves of *C.Gigantea*

Extracts	Vol.Used (cm^3)	Average Diameter (mm) Zones of Inhibition on Test Organisms										
		E.Coli (NCTC 10481)	S.Au L.C.I	P.A L.C.I	K.P L.C.I	P.V L.C.I	S.M L.C.I	S.P L.C.I	B.T L.C.I	S.T L.C.I	E.A L.C.I	S.A L.C.I
Cipromax	0.05	18	22	14	18	30	14	16	14	24	35	24
Leave Fraction1	0.05	22	24	18	20	26	14	14	10	16	28	16
Leave Fraction2	0.05	20	22	16	18	24	10	12	10	18	24	14

S.Au= *Staphylococcus aureus,* P.A=*Pseudomonas aeroginosa,* K.P = *Klebsiella pneumonia,* P.V=*Proteus vulgaris,* S.M= *Strept muteus,* B.T=*Bacillus typhi,* S.T=*Salmonella typhi,* E.A=*Enterobacter aerogenes,* S.A= *Staph albus,* S.P= *Strept pyogenes*

The results in Table 5 showed that all the fractions were positively active on all the tests organisms as confirmed from their zones of inhibition which ranged from 10mm-28mm showing their antimicrobial effect on the diseases caused by these various test organisms.

Table 6: Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the leaves extracts of *C.gigantea*

Extracts		Average Diameter (mm) Zones of Inhibition on Test Organisms									
		E.Coli (NCTC 10481)	S.Au L.C.I	P.A L.C.I	K.P L.C.I	P.V L.C.I	S.M L.C.I	S.P L.C.I	B.T L.C.I	S.T L.C.I	E.A L.C.I	S.A L.C.I
Cipromax	MIC	0.0625	0.0313	0.125	0.0625	0.0156	0.125	0.125	0.125	0.0313	0.0156	0.0313
	MBC	0.125	0.0625	0.250	0.125	0.0313	0.250	0.250	0.250	0.0625	0.0313	0.0625
Leave Fraction1	MIC	0.0625	0.0323	0.0625	0.0625	0.0314	0.250	0.250	0.250	0.125	0.0156	0.125
	MBC	0.125	0.0625	0.125	0.125	0.0625	0.500	0.500	0.500	0.250	0.0313	0.250
Leave Fraction2	MIC	0.0625	0.0623	0.125	0.0625	0.0313	0.250	0.250	0.250	0.0625	0.0313	0.250
	MBC	0.125	0.125	0.250	0.125	0.0625	0.500	0.500	0.500	0.150	0.0625	0.500

S.Au= *Staphylococcus aureus,* P.A=*Pseudomonas aeroginosa,* K.P = *Klebsiella pneumonia,* P.V=*Proteus vulgaris,* S.M= *Strept muteus,* B.T=*Bacillus typhi,* S.T=*Salmonella typhi,* E.A=*Enterobacter aerogenes,* S.A= *Staph albus,* S.P= *Strept pyogenes*

The result of the antibacterial activity on eleven bacteria species both gram positive bacteria (*Staphylococcus albus, Bacillus typhi, Streptococcus pyogenes* etc) and gram negative bacteria (*Escherichia coli* and *Pseudomonas aeroginosa*) showed that various fractions from this plant can serve as broad spectrum antimicrobial (Cunha, 2009). The very high presence of flavonoids in the plant part as shown from the preliminary tests could account for this high antimicrobial effect as one of the undisputed functions of flavonoids and related polyphenols is their role in protection against microbial invasion. Several recent papers report the regular presence of antibacterial activity among flavonoids (Alinnor, 2007; Penecilla et al., 2011). Specifically, the value of the fractions on *staphylococcus aureus* and *staphylococcus albus* confirmed the work done by Haraguchi et al.,(1998) on the effect of flavonoids on *Staphylococcus aureus* a causative organism for skin and wound infections, abscess and osteomyelitis which according to Greenwood et al., (1992) could account for its use in the treatment of the aforementioned diseases.

Table 7: Results of Antifungal Activities of the Leaves Fractions of *C.Gigantea*

Extracts	Vol. Used (cm^3)	Average Diameter (mm) Zones of Inhibition on Test Organisms		
		Candida Albican L.C.I	*Aspergillus flavis* L.C.I	*Aspergillus Niger* L.C.I
Cipromax	0.05	NA	NA	NA
Leave fraction 1	0.05	10	8	10
Leave Fraction 2	0.05	12	10	12

Table 8: Results of MIC and MFC of the Leaves Fraction of *C.Gigantea*

Extracts		Presence or Absence of growth on Test Organisms		
		Candida Albican L.C.I	*Aspergillus flavus* L.C.I	*Aspergillus Niger* L.C.I
Cipromax fort	MIC	-	-	-
	MFC	-	-	-
Leave Fraction 1	MIC	0.25	–	0.25
	MFC	0.50	-	0.25
Leave Fraction2	MIC	0.25	0.25	0.25
	MFC	0.50	0.50	0.50

Comparatively with the standard drug cipromax fort, the various fractions had different activities on the test organisms with leave fraction 1 totally inactive on *Aspergillus flavus* confirming the report by Ibeh *et al*., 2003 that an inhibitory zone diameter of 10mm or less indicated that the organism was resistant. An inhibitory zone diameter of 11-15mm shows intermediate effect while 16mm and above indicated that the organism was susceptible to the compound (Ibeh *et al*., 2003). Hence, the leaves of *Cola gigantea* had an intermediate anti microbial effect as most values fell between 11-15mm as shown in Table 7.

Spectroscopic Analysis and Structural Elucidation

Table 9: FTIR Results of Leave Fraction 1

Wave band (cm^{-1})	Description
3337.93	OH Stretch for carboxylic acids and esters
2964.69	C-H Stretch for aromatics and alkanes
2898.14	
1399.4	C=O stretch for esters
1064.74	C-O deformation bond of esters
880.53	C-H deformation bonds of aromatics and alkyl groups
435.93	C-H deformation of alkyl and methyl groups

Table 10: UV-Visible Results of Leave Fraction 1

λmax (nm)	Chromophore description
664.50	C=C of aromatic ($\pi \longrightarrow \pi^*$)
607.50	
535.00	C=C of alkenes ($\pi \longrightarrow \pi^*$)
504.50	
402.50	C=O of esters($n \longrightarrow \pi^*$)

Table 11: Summary of the H^1 and C^{13} NMR Results of leave fraction 1

H^1 δ (ppm) & Multiplicity	Coupling Constant (MHz)	Types of Proton	C^{13} δ (ppm)	Types of Carbon
7.5 (mutiplet)	1.0320	ArH	130.8972	C=O
5.5 (d)	4.1855	ArH	130.1992	C=O
4.2 (multiplet)	20.3126	RC=CH	128.8480	C-OH
3.5 (t)		C-C-H	128.3231	C=C
2.5 (t)		CH_2	128.2324	C=C
1.3575		CH_2	127.7825	C=C
1.3094		CH_2	77.3480	C-O
1.2820		CH_2	77.0905	C-O
0.9042		CH_3	76.7128	C-O
			71.8122	C-O
			70.2735	C-O
			65.1180	C-O
			63.3755	C-O
			62.8346	C-O
			34.1195	CH_2
			33.8697	CH_2
			31.9093	CH_2
			31.4238	CH_2
			30.2129	CH_2
			30.0195	CH_2
			29.6753	CH_2
			29.4498	CH_2
			29.3341	CH_2
			29.2470	CH_2
			29.1418	CH_2
			27.7285	CH_2
			27.3684	CH_2
			27.2119	CH_2
			25.6245	CH_2
			25.5312	CH_2
			24.8868	CH_2
			24.7665	CH_2
			22.6647	CH_2
			19.1353	CH_2
			14.0738	CH_3

The combination of the FTIR, UV-Visible, H^1 and C^{13} NMR results with the major fragments in GC-MS gave rise to the proposed structure for the compound (fig.1.0)

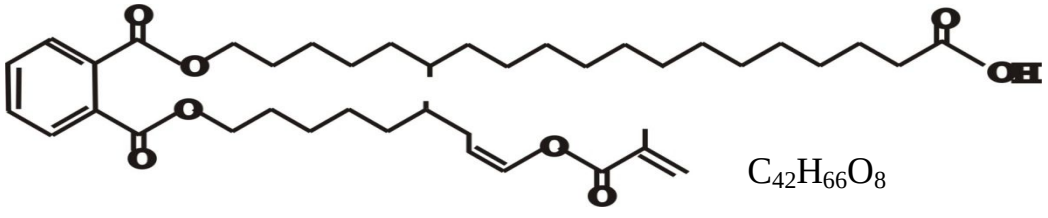


Fig 1.0 1,2-benzene dicarboxylic acid,dioctyl-dodecanoic acid,2-propenoic acid,2-methyl-ethenyl ester
Dodecanoic acid, a part of the above compound have been reported to have antimicrobial properties as confirmed by the anti microbial results (Table 5) of this work and by Hoffman, *et al.*, 2001 and Outtar *et al.*, 2000.

Table 12: FTIR Results of leave Fraction 2

Wave band (cm^{-1})	Description
3340.82	OH of alcohols, phenol and esters
2964.40	C-H Stretch of alkanes and aromatics
1063.78	C-O deformation bond of esters
879.57	C-H deformation bonds of aromatics and alkyl groups
430.14	C-H deformation of alkyl and methyl groups

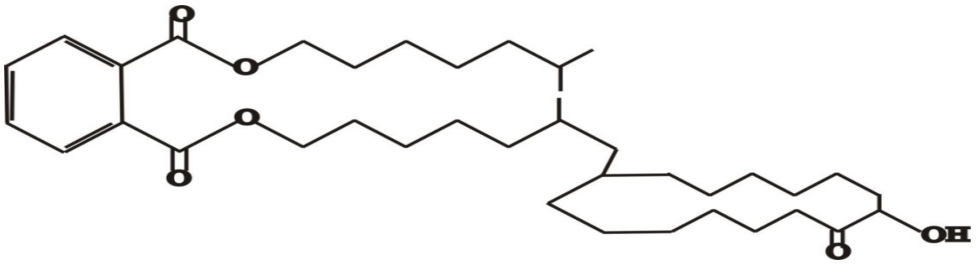
Table 13: UV-Visible Results of leave Fraction 2

λmax (nm)	Chromophore description
740.00	C-OH absorption bonds
666.50, 610.00, 529.00	C=C of aromatics ($\pi \longrightarrow \pi^*$)
499.50, 401.00	C=O of esters ($n \longrightarrow \pi^*$)

Table 14: Summary of the H^1 and C^{13} NMR Results of leave Fraction 2

H^1 δ (ppm) & Multiplicity	Coupling Constant (MHz)	Types of Proton	C^{13} δ (ppm)	Types of Carbon
9.75 (d)	0.0015	RCO_2H	130.8514	C=O
7.500 (t)	0.0130	ArH	130.0176	C=O
7.2916 (s)		ArH	128.8461	C-OH
7.00 (t)	0.0091	ArH	77.3271	C-O
5.5 (d)	0.0594	ArH	77.0099	C-O
4.20 (multiplet)	0.1625	RCH	70.3115	C-O
2.0665		CH_2	65.1568	RCH
1.3594		CH_2	65.0380	RCH
1.3115		CH_2	63.3654	RCH
1.3007 (multiplet)	1.0472	CH_2	60.3965	RCH
1.2831		CH_2	34.1600	RCH
1.2650		CH_2	33.8517	RCH
0.9069		CH_3	32.1059	RCH
			31.9084	RCH
			31.4239	RCH
			30.2103	RCH
			29.6721	RCH
			29.4352	RCH
			29.3352	RCH
			29.2311	RCH
			29.1220	RCH
			29.0734	RCH
			28.9361	RCH
			27.7315	RCH
			27.3596	RCH
			27.1897	RCH
			25.6276	RCH
			24.9054	RCH
			24.7144	RCH
			23.7894	RCH
			22.9597	RCH
			22.6626	RCH
			20.9926	CH_2
			19.1354	CH_3
			14.1687	CH_3
			14.0671	CH_3

The combination of the FTIR, UV-Visible, H^1 and C^{13} NMR results with the major fragments in GC-MS gave rise to the proposed structure for the compound (fig 2.0)



$C_{39}H_{65}0_6$

Fig 2.0: 1,2-Benzene Dicarboxylic Acid, Diisooctyl Ester,2-Hydroxy Cyclopentadecanone

References

Adeniyi, B.A.,Groves, M.J., and Gangadharam, P.R.J., (2004). Invitro antimycobacterial activities of three species of cola plant extracts, (sterculiaceae). Phytotherap Res., 18(5), 414-418.

Agyare, C.,Koffuor, G.A., Boamah, V.E, Adu, F., Mensah, K.B, Adu-Amoah L., (2012). Evidence Based Complementary and Alternative Medicine,Hindawi Publishing Corporation, India, p.902394

Ajiwe,V.I.E., Dimonyejiaku, N., Ajiwe ,A.C., Chinweuba, A.J., and Chendo, N.M., (2008). Preliminary study on the pharmaceutical constituents of e*milia sonchifolia* leaf, Anachem Journal, 2(2), 302-309.

Alinnor, I.J., (2007). Preliminary Phytochemical and antibacterial activity screening of seeds of *Garcinia Cola*, Journal Chemical Society Of Nigeria, 32(2), 41-47.

Benjamin, L.T., (1991). Coca-Cola, Caffeine and Mental Deficiency- Harry Hollingworth and the Chattanooga trial of 1999, Journal Histol Behavioural Science, 27(1), 42-45.

Cunha, B.A., (2009). Antibiotic Essentials, 8th edn., Jones and Barlett Learning Publishers, United States, p.180.

Dalziel,J.M., (1961). The Useful Plants of West Tropical Africa, the Crown Agents, London, p.308.

Greenwood, D.S and Peuthee, C.B., (1992). Medical Microbiology: A guide to microbial infections, pathogenesis, immunity, laboratory diagnosis and control, 14th edn., Chucrch hill livingstone medical division of Longman group, United Kingdom, pp.72-73.

Haraguchi, H., Tanimoto, K, Tamura, Y, Mizutani,K and Kinoshto, T (1998). Mode of Antibacterial action of Retrochalcones from Glycyrrhiza Inflata, Journal of phytochemistry, 48, 125-129

Harbone, J.B., (1998). Phytochemical Methods- A guide to Modern Techniques of plant Analysis, 3rd edn., Chapman and Hall, London, pp.36-89.

Hoffman, K.L, Han, I.Y, and Dawson P.L., (2001). Antimicrobial effects of Corn Zein Films Impregnated with Nisin, Lauric acid and EDTA, Journal food protection, 64(6), 885-889.

Ibeh, I.N and Uraih, N. (2003). Practical Microbiology, Vol.1, Ambik Press Ltd, pp 82-93.

Idu, M., (2010). Documentationon Medicinal Plants sold in markets in Abeokuta, Nigeria, Tropical Journal of Pharmaceutical Research, 9 (2), 110-118.

Nestler, M.T and Hurley,D., (2004). Microbiology; A Perspective, 4th edn., Mc Graw-Hill, New York, pp 230-253.

Ouattar, B, Simard R.E. ,Piett, G., Begin, A., and Holley, R.A., (2000). Anti-listerial activity of a polymeric film coated with hybrid coatings doped with Enterocin 416K1 for use as bioactive food packaging, International Journal of food microbial, 62 (1-2), 139-148.

Penecilla, G.L., Magno, C.P.,(2011). Antibacterial activity of extracts of twelve common medicinal plants from phillipines. J.med.plants research, 5(16),3975-3981.

Reid, K.A., Jager,A.K., Light,M.E., Mulholland, D.A., and Staden, J.V., (2005). Phytochemical and Pharmacological screening of Sterculiacea Species and Isolation of antibacterial compounds, J. Ethnopharmacol, 97(2), 285-291.

Sonibare, M.A., Soladoye, M.O., Esan, O.O., and Sonibare, O.O., (2009). Phytochemical and Antimicrobial Studies of four Species of *cola schott and End*l. *(sterculiaceae)*. Afr J Trad Complementary Alternative Medicine, 6 (4), 518-525.