

**Original Article****PRELIMINARY BIOAUTOGRAPHIC ANALYSIS OF THE SEEDS OF *GLYPHAEA BREVIS* (SPRENG) MONACHINO FOR ANTIOXIDANT AND ANTIBACTERIAL PRINCIPLES.**Michael Lahai<sup>1</sup>, Tiwalade Adewale Olugbade<sup>2</sup>

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**ABSTRACT**

*Glyphaea brevis* (Spreng) Monachino (*G. brevis*) is widely distributed in West Africa with its various parts used in traditional medicine. In Sierra Leone, the leaves of the plant are used traditionally to enhance labour while the seeds are used in the treatment of skin infections. The present study is aimed at screening the seeds for phytochemical groups, antioxidant principles and antibacterial principles. The plant extracts were examined for phytochemical groups by standard spot tests. Chromatographic separation of seed extract was performed on reverse phase column and on silica at medium pressure chromatographic set-up. Thin layer chromatographic plates were visualised by UV light and vanillin sulphuric acid spray reagent. The crude extract and chromatographic fractions were examined for radical scavenging properties using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH). Antibacterial bioautographic evaluation was performed using *B. subtilis* NCTC 8236 as test organism and Methylthiazolyl tetrazolium chloride as detecting reagent. Phytochemical screening of the seeds of *G. brevis* indicated the presence of phenolics and saponins. Pink colour reaction of polar Thin Layer Chromatographic (TLC) spots with vanillin-sulphuric acid spray (V/SA) suggested the presence of terpenoid glycosides. Screening with DPPH showed prominent antioxidant spots on silica at R<sub>f</sub> 0.8, 0.5, 0.4 Ethyl acetate (EtOAc). The three spots reacted purple to V/SA. Bioautographic analysis revealed antibacterial spots at R<sub>f</sub> 0.8(EtOAc) and 0.7(EtOAc-MeOH, 9:1).

The seeds of *Glyphea brevis* contain specific antioxidant and antibacterial principles which have been described by their chromatographic properties in this study.

**Keywords:** antioxidant, phytochemical, antibacterial,

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## INTRODUCTION

Medicinal plants are being used in the treatment of diseases such as malaria, diabetes, sickle-cell anaemia, mental disorders (Elujoba, Odeleye & Ogunyemi, 2005) as well as microbial infections (Okigbo & Mmeka, 2006).

The use of traditional therapy is not restricted to developing countries alone; the Food and Agricultural Organisation reported in 2002 that at least 25% of drugs used in modern pharmacopoeia are derived from plants while many others are synthetic analogues based on prototype compounds isolated from plants. According to the World Health Organization (WHO, 2001), 80% of the worlds' population use medicinal plants in the treatment of diseases. This rate is much higher with an estimated 90% of the population in developing countries relying on the use of medicinal plants to help meet their primary health care needs (WHO, 2002).

In the developed countries, medicinal plants serve as the basis for drug development making traditional medicine an essential commodity in both the developed and developing countries. Medicinal uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoctions from the plants (Ogbulie, Ogueke, & Okorundu, 2004). *Glyphaea brevis* (Spreng) Monachino a Tilaceous shrub which produces fruits in duplets is widely distributed in West Africa. The various parts of the plant are used in traditional medicine across West Africa. In Sierra Leone, the leaves of the plant are used traditionally to hasten labour, and the leaves and stem bark are good fodder for goats. It is also used in the treatment of sleeping sickness, an aphrodisiac, as an antibacterial for an eye infection, and cleaning of the gum (Ogbonnia, Van-Staden, Jager & Coker, 2003). It is also known for its carminative effects and its use as an anticonvulsant in children.

The plant was first collected by Monachino, Joseph Vincent and is widely distributed in different countries across West Africa such as Liberia, Cote d'Ivoire, and Nigeria; filed as *Glyphaea brevis* (Spreng) Monachino, family Malvaceae. However unresolved controversy still surrounds the classification of the family Malvaceae. While the narrower circumscription of the family is monophyletic, the broader or *sensu lato*

circumscription of Malvaceae is polyphyletic. In particular, the genus *Glyphaea* has been severally classified into the Malvaceae, Tilaceae, Gramicidaceae or Spermamiaceae (Heywood, Brummitt, Culham & Seberg, 2007).

The local name in Sierra Leone is Eh lontho ya Eh-Wor (Themne) or niki-gui (Gola), or Lamden (Kissi), or gbondo-kpokwe (Kono) and in bulontokawari (Limba). The Local name of *Glyphaea brevis* in Nigeria is Aloanyasi (Ibo) or Atori (Yoruba) (Ogbonnia, Van-Staden, Jager & Coker, 2003). In Senegal, it is called Tukulor Keki (Fula) or bola pane (Wolof). In Ghana, the local name is Foto (Akan) (Boateng, Bennet-Lartey, Opoku-Agyeman, Mensah & Fjeiser, 2004). The traditional uses of *G. brevis* are summarised in Table 1

The literature report on the chemistry of *G. brevis* is limited to the lipophilic contents of the leaves. Thus, a mixture of n-alkanes, Tetracosane (primary isolate), a mixture of fatty acid esters of primary alcohols, dotriancotanol and a mixture of Oleanolic and Echinocystic acids have been reported from the leaves of the plant (Mbosso, Ngouela, Ngueda, Beng, Rohmer & Tsamo, 2010).

When a free radical reacts with a non-radical, a free-radical chain reaction results and new radicals are formed. Attack of reactive radicals on membranes or lipoproteins starts lipid peroxidation. This can disrupt the natural balance and lead ultimately to oxidative stress (Halliwell, Gutteridge & Cross, 1992).

The external sources of oxidative stress can be drugs, carcinogens, hyperoxia, ozone, radiation, cigarette smoke and the cellular sources can be inflammatory cells, fibroblasts, xanthine, oxidase, NADPH, and endothelial cells.

Oxidative stress is known to contribute significantly to the process of inflammation, which underpins conditions like rheumatoid arthritis, inflammation, metabolic syndrome and diabetes, as well as to neurodegenerative diseases like Alzheimer's (Ding, Dimayuga, & Keller, 2007). Superoxide dismutase (SOD) along with catalase and glutathione peroxidase, form the front line of

the body's antioxidant enzyme defences. (McCord & Fridovich, 1969).

However, previous work have focus more on phytochemical screening and biological activity of the leaves and stem bark, with little or none on the seeds (Mbosso, Ngouela, Ngueda, Beng, Rohmer Tsamo, 2010). The present study is thus designed as a preliminary bioautographic screening of the seeds of *G. brevis* for anti-oxidant and antimicrobial principles.

**Table 1: Traditional uses and Biological activities of *G. brevis***

Parts of plant	Ethno-medicinal uses	Chemistry	Biological activity
<b>Leaves</b>	Fodder for goats and sheep. Juice or Poultrice to treat cuts and wounds (Nigeria) (Isidore, Akpan-Ebe, Olajide & Okon, 2011). Leaf enema is used to quicken labour in pregnant women, treatment of ulcers (Nigeria) (Gobah & Liang, 2011). Decoction for the treatment of sexually transmitted infections. Used as abortifacient, ecboic, antiemetic, for eye treatment, liver problems and naso-pharyngeal affections, and as genital stimulants, pain-killers, treatment of pulmonary troubles, oedema and Gout (Nigeria) (Burkill, 1985)	Phenolic, Reducing Sugar, Phytosterols, Triterpenoids Alkaloids, Cardiac glycosides and Tannins detected. (Borokini & Omotayo, 2012)	Anticonvulsant effect in mice. Antioxidant effect. Antibacterial, anti-inflammatory (Ogbonnia, Van-Staden, Jager & Coker, 2003) (Dickson, Annan & Komlaga, 2011).
<b>Roots</b>	Decoction is used to treat stomach upset, indigestion and to increase appetite. Also used to treat diarrhea, dysentery, as febrifuge, for paralysis, epilepsy, convulsions and spasms (Cuendet, Hostettmann, Potterat & Dyatmiko, 1997).		.
<b>Stem bark</b>	Aphrodisiac, appetizer, laxative, and as a remedy for chest pains, diarrhea, dysentery, and sleeping sickness.	Phenolic, Reducing Sugar, Triterpenoid, Saponins and Flavonoids detected (Dakam, Oben & Ngogang, 2008).	Anti-inflammatory, Antioxidant and antibacterial effect (Dickson, Annan & Komlaga, 2011).
<b>Seed</b>	A Paste of the dry powder mixed with ashes for Ringworm Infection. (Boateng, Bennet-Lartey, Opoku-Agyeman, Mensah & Fjeiser, 2004).	-	-

## MATERIALS AND METHODS

The sample of seeds, leaves and stem bark of *Glyphaea brevis* were collected on the 12<sup>th</sup> of May 2012 at Mayenkinah Road, Calaba Town in Freetown, Sierra Leone and authenticated at the Department of Pharmacognosy and Phytochemistry, University of Sierra Leone.

### Reagents

Reagents used included, Vanillin sulphuric acid spray (V/SA) comprised of vanillin (1g) in concentrated sulphuric acid (100mL) that is used for detection of higher alcohols, phenols, terpenoids, steroids, essential oils and esters generally after heating at 120°C for 1minute (Borokini & Omotayo, 2012).

Other reagents used were: 2, 2- Diphenyl-1-picrylhydrazyl radical, DPPH, (1g) in methanol (100mL) which can detect antioxidant principles

(radical scavenging) (Borokini & Omotayo, 2012); Dragendorffs Reagent, which is a mixture of solution A and B diluted to 100mL with H<sub>2</sub>O; Solution A is comprised of Bismuth nitrate (0.17g) in AcOH (2mL) and H<sub>2</sub>O (8mL) and added to Solution B: KI (4g) in AcOH (10mL) and H<sub>2</sub>O (20mL); it is used to detect alkaloids. One other reagent used was ferric chloride (FeCl<sub>3</sub>) solution: 1% iron (III) chloride solution is neutralised with sodium hydroxide until a slight precipitate of FeO(OH) is formed. The mixture is then filtered before use. It is used to detect the presence of phenol.

For the TLC, four solvent systems were used: Solvent A = EtOAc (Ethyl acetate), Solvent B = EtOAc/ MeOH ratio 9:1 (Ethyl acetate/Methanol),

Solvent C = MeOH- (Methanol), Solvent D= MeOH (50%)

An accelerated gradient chromatography (AGC) system was used comprising the set-up of AB-Separo (Sweden). This is a medium pressure chromatography under pressure with accelerated solvent flow to allow for the separation of bulk plant material into various fractions. It is used for preparative work.

**Comparative Phytochemical Screening of Leaves, Stem Bark and Seeds of *G. brevis*** Plant parts were air-dried for 14 days and macerated with methanol (20mL) for five days. The extract (1mL) of each plant part obtained after maceration with methanol was treated with a drop of ferric chloride solution and observed for any colour change (blue-black). The extract (1mL) was treated

with 2 drops of Dragendorff solution and observed for any reddish-brown precipitate, indicating presence of alkaloids. To each Plant part (200mg) was added distilled water (10mL) and the mixture shaken vigorously. This was observed for any frothing persisting longer than 30 minutes. Different solvent compositions were tried on both the normal phase (Ethylacetate/ MeOH) and the Reverse phase plates (methanol/water) to resolve the components of *G. brevis*. The TLC plates were first observed under UV-light and then sprayed with Vanillin Sulphuric Acid (V/SA).

The powdered dried seed of *G. brevis* (580g) were extracted in cold methanol (1L) for 48 hours. The extract was clarified by filtration and evaporated to dryness *in-vacuo*.

### Chromatography of Seed Extracts

Reverse-phase column chromatography of the bulk extract from the seeds of *G. brevis* was carried out. The crude extract (6g) was dissolved in MeOH (7ml) and loaded on Lobar reverse phase (C-18) glass column and then eluted in gradients of Water-Methanol. The process was repeated with the remaining crude extract (9g). Fractions that reacted pink after reverse phase chromatography were pooled (1.5g) and subjected to AGC using Silica gel and gradients of n-hexane and ethyl acetate.

## Bioautography for Antioxidant and

### Antimicrobial Principles

Preparation of the TLC plate

Solutions of the test samples (GB) were spotted on reverse phase TLC plates and silica phase TLC and developed in solvent systems C, D and A, B respectively. The plates were then allowed to dry in air. The DPPH spraying reagent was used to spray the dried TLC plates and observed for any decolourisation over a period of 10 minutes.

Nutrient agar (14g) granules was weighed and suspended in distill water (500mL) in a flat bottom flask. The mouth of the flask was covered with foil

and the mixture was shaken together. This was heated to dissolve the particles completely. The clear solution was dispensed using a dispenser (15mL) into Mc Cartney bottles before allowing the mixture to cool. The bottles were tightly capped and placed in an autoclave for sterilization at 121<sup>o</sup> C for 15mins. The prepared nutrient agar was then inoculated with *Bacillus Subtilis* NCTC 8236.

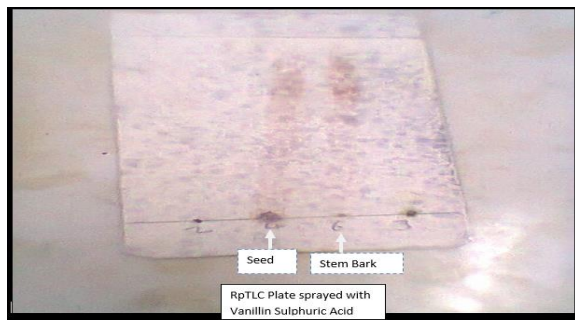
Solution of the test samples were spotted on silica TLC plates in duplicate. The plates were then developed using solvent system B and then allowed to dry in air. A spreader was used to overlay the seeded agar on the TLC plates.

The spread agar was allowed to set on the plates and thereafter incubated at 37<sup>o</sup>C for 18 hrs. After incubation, inhibition zones were made visible by spraying the plates with aqueous solution (2.5 mg/mL) of thiazolyl blue (Methylthiazolyltetrazolium chloride MTT) and incubated for 2h.

### RESULTS

Seeds and Stem bark of *G. brevis* showed a blue black colour with ferric chloride in a test tube. The Leaves of *G. brevis* showed no change in colour with ferric Chloride. The seeds also showed reddish brown precipitate with Dragendorff reagent. No reddish-brown precipitate was observed for the Leaves and Stem bark when mixed with Dragendorff reagent. Frothing persisted for 24h when the seeds were shaken with water. No persistent frothing was observed for the leaves or the stem bark.

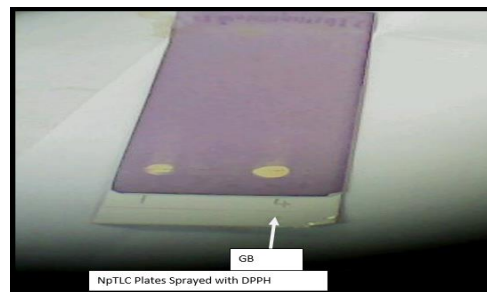
No mobile spot was detected on silica with Solvent Systems A (ethylacetate) or B (10% methanol in ethyl acetate) (NpTLC). However, the reverse phase TLC with methanol (RpTLC) yielded 0.8 UV and pink (V/SA reaction) for seeds; 0.8UV, pink (V/SA reaction) for stem bark and in 50% methanol the average yield for seeds was 0.19 UV and pink (V/SA) (Fig. 1).



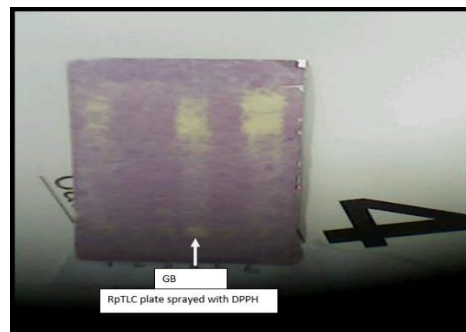
**Figure 1: RpTLC Plate (solvent system D) sprayed with Vanillin Sulphuric Acid.**

The combined total weight of the crude extract obtained after extraction and re-extraction is 18.9g which is 3.26 % of the total weight of the dried seeds of *G. brevis*. The crude extract of the seeds (GB) (6g) subjected to medium pressure chromatographic separation on reverse phase gave fractions, collected in test tubes (15mL). A Pinkish broad spot with vanillin sulphuric acid weighing 33mg was obtained in one of 15 tubes with 10% methanol solvent. A similar spot weighing 352mg was obtained with methanol. The remaining crude extract (9g) subjected to chromatographic separation on reverse phase as shown in Table 2. The combined fractions obtained from the reverse phase column chromatography, Fraction M, E and L were pooled and eluted on silica gel as shown in Table 3.

When the NpTLC plate (silica-solvent system B) of the extract of the seeds of *G. brevis* was sprayed with DPPH a reaction was observed at the origin as shown in Figure 2A. When the RpTLC plate (reverse phase-50% methanol) of the extract of the seeds of *G. brevis* was sprayed with DPPH a reaction was observed at a broad area around  $R_f$  0.8 as shown in Figure 2B.



**Figure 2A: GB on Normal Phase TLC (NpTLC) Plates Sprayed with DPPH**



**Figure 2B: GB on Reverse phase TLC Plate sprayed with DPPH**

**TABLE 2: Repeated Reverse Phase Column Chromatographic Separation**

Solvent System/Volume	Collection of Fraction	Remarks
10% methanol(900ml)	Flask 1- 3	No detectable colour
20% methanol (500ml)	4- 10	Fraction 4- 11 were orange after spraying; spot turned ash after heating and became black after prolong heating (1.69g)
30% methanol	11- 12	Flask 12 - 19 showed pink colour observed only at the origin (Not DPPH reacting) The quantity was 366mg.
40% methanol	13- 14	--
50% methanol	15	--
60% methanol	16	--
70% methanol	17	--
100% methanol (washing)	18- 20	Flask 20 had weak antioxidant property, it's a mixture of 5 pink spots (Fraction E-999mg). (V/SA) Flask 18 had 3 pink spots (Fraction L-156mg-).

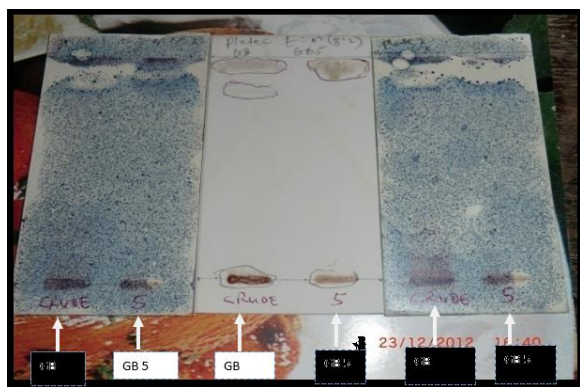
**TABLE 3: Silica Gel-Chromatography Separation of Fractions Obtained from Reverse-Phase Chromatography**

Solvent system	Tube Number
n-hexane (100%)	1-3
n-hexane/EtOAc (50%)	4-6
n-hexane/EtOAc (30%)	7-10
EtOAc(100%)	11-15
EtOAc/methanol (90%)	16-20
EtOAc/methanol (80%)	21-25
EtOAc/methanol (70%)	26-30
EtOAc/methanol (60%)	31-36
EtOAc/methanol (50%)	37-41
methanol (100%)	42-43

The various column fractions of the seed extract were re-examined for homogeneity by TLC (V/SA) and antioxidant properties (DPPH), as shown in Table 4.

### Antimicrobial Bioautography

Samples tested for antimicrobial activity, by bioautography, are the crude extract GB and the final silica column fractions GB 5, GB 21 and GB 43. Antibacterial activities were seen as a very whitish spot against a purplish background. GB 5 showed antibacterial activity at  $R_f$  0.9. The antibacterial activity of the spot was observed at corresponding  $R_f$  0.9 in the crude extract (GB). GB 21 was active against the tested organism (*B. subtilis*) with a very clear whitish spot against a purplish background as shown in Figure 3. GB 21 showed an antibacterial spot at  $R_f$  0.7. The duplicate plate



**Figure 3: Antimicrobial Activity (*Bacillus Subtilis* NCTC 8236) of Crude Extract GB and GB 5**

sprayed with V/SA, however, indicated three spots at  $R_f$  0.7, 0.8, 0.9.

### DISCUSSION

The Crude extract (GB) of the seeds of *G. brevis* gave a reddish brown precipitate in Dragendorff's reagent indicating the presence of alkaloids. Ferric chloride test gave a blue-black colour indicating the presence of phenolics. Frothing was also observed with crude extract suggesting the presence of saponins.

The leaves of *G. brevis* did not show any reddish brown precipitate with Dragendorff's reagent. Ferric chloride test did not show blue-black colour and no frothing was observed. Thus, indicating the absence of alkaloids, phenolics and saponins.

The Stem bark of *G. brevis* showed a blue-black colour with ferric chloride test indicating the presence of phenolics. No reddish brown precipitate was observed for Dragendorff reagent and no frothing was observed indicating the absence of alkaloids and saponins.

Preliminary TLC studies of the different parts of *G. brevis* on silica with mobile phase as ethylacetate or 10% methanol in ethylacetate did not show mobility of components beyond the origin. However, reverse-phase TLC with 50% methanol revealed pink-reacting spots (V/SA spray) for both the seed extract ( $R_f$  0.8, broad) and the stem bark extract ( $R_f$  0.7 and 0.8, both broad). This is suggestive of the presence of polar terpenoid compounds (terpene glycosides) in both the seeds and the stem bark. The chemistry of the stem bark by Dakam, Oben & Ngogang in 2008 showed the presence of Phenolics, Triterpenoids and Saponins. 100% methanol showed a better resolution of the seed extract, indicating pink-reacting spots at  $R_f$  0.36, 0.13, and 0.07.

As a follow-up to the preliminary TLC studies attempts were made to isolate pure components of the seed extract by repeated medium-pressure chromatography (reverse-phase and normal-phase) as discussed subsequently. Reverse phase column chromatography eluted a 33mg fraction with MeOH (10%) that reacted pink with vanillin Sulphuric acid as shown in Table 4. This 33mg sample gave a pink spot (lipophilic, non-uv detectable). Fractions from tubes 15 to 23 reacted light brown and became ash after heating (Supposed sugar present). Methanol as solvent system eluted fraction M (352mg) that also reacted pink with vanillin Sulphuric acid.

On further reverse phase column chromatography separation of the remaining crude extract (9g) with Methanol, fraction L (999mg) and fraction E (156mg) was obtained. Fraction E showed five pink spots, whilst fraction L showed three pink spots. Fraction E and L was also found to be weakly antioxidant. This indicated that the three fractions M, E and L are not pure fractions but mixtures.

The three Fractions M, E, and L were pooled (1.5g) and subjected to silica gel chromatography as shown in Table 4 showing evidence of the presence of antioxidants in *G. brevis*.

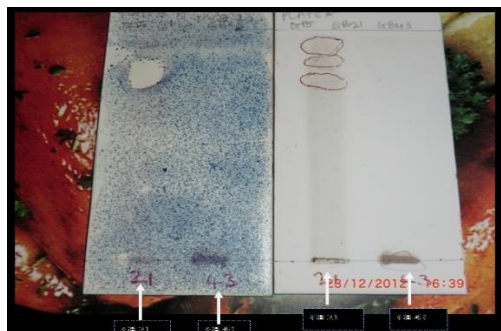
The crude extract (GB) of *G. brevis* was poorly resolved on NpTLC (showing decolourisation at the origin). On RpTLC the crude extract (GB) showed reaction with DPPH at  $R_f$  0.8 (solvent D). This is also consistent with the reaction of eluted fractions of *G. brevis* as shown in Table 7. Thus, GB 1, GB 5, GB 6, GB 9, GB 26, and GB 43 all showed immediate reaction with DPPH, on silica. The prominent antioxidant spots in the fractions were observable at  $R_f$  0.8, 0.5, 0.4 (solvent A); 0.2 (solvent B) and 0.14 (solvent C). This infers the presence of antioxidant

**Table 4: Bulking and TLC Profile of the Various Fractions of *G. brevis***

Bulked Fractions	Mobile phase, R <sub>f</sub> , (UV detection) (V/SA colour detection),	DPPH reacting spots*	Quantity
Fractions 1- 4 (GB 1)	A; 0.8 (Purple)	Immediate	68mg
Fraction 5(GB 5)	A; Two spots 0.8 (uv detectable) (purple) ; 0.5 (non-uv detectable), (Purple)	Immediate	125mg
Fractions 6-8(GB 6)	A; two spots; 0.8 (uv detectable; 0.5 (non-uv detectable), (Purple)	0.5 Immediate	317mg
Fractions 9- 13(GB9)	A; 0.4 (non-uv detectable) (purple)  (only slightly impure)	0.4 (immediate)	35mg
Fractions 15-17 (GB 15)	A; 0.2 (single pure spot), (Purple)	Weakly antioxidant	12mg
Fractions 21- 25 (GB 21)	B; Three spots; 0.7; 0.8; 0.9	-Weakly antioxidant	45mg
Fractions 26-27 (GB 26)	B; Two spots; 0.4; 0.2(Purple)	0.2 (immediate)	17mg

principles in the seeds of *G. brevis*(Borokini & Omotayo, 2012)..

The crude extract (GB) of *G. brevis* showed antibacterial activity when tested with *B. subtilis*. This activity was replicated by GB 5 at R<sub>f</sub> value about 0.9 (solvent A), as shown in figure 4.



**Figure 4: Antibacterial Activity (*Bacillus Subtilis* NCTC 8236) of G.B 21**

GB 21 and GB 43 were also tested for antibacterial activity. GB 21 showed antibacterial activity with spot at about R<sub>f</sub> 0.7 (solvent B). However, a duplicate TLC plate sprayed with V/SA showed that GB 21 is a mixture with three spots at R<sub>f</sub> 0.7, 0.8 and 0.9. GB 43 had no antibacterial activity but showed prominent antioxidant activity. Work done on *G. brevis* by Ogbonna, Van-Staden, Jager & Coker in 2003 and Dickson, Annan & Komlaga in 2011 also reported biological activity that includes antibacterial and antioxidant activity.

## CONCLUSION

Phytochemical screening of the seeds of *G. brevis* indicated the presence of phenolics and surface-active agents presumed to be saponins. No alkaloid reacting spot was detected on TLC in spite positive colour reaction of the crude extract in the spot test. False alkaloid reactions to Dragendoff's reagent are known. Pink colour reaction of polar TLC spots with vanillin-sulphuric acid spray (V/SA) suggested the presence of terpenoid glycosides.

Screening of the various column fractions with DPPH showed three prominent antioxidant spots on silica at R<sub>f</sub> 0.8, 0.5 and 0.4 (solvent system A). The three spots reacted purple to V/SA. Another purple reacting antioxidant spot was observable at R<sub>f</sub> 0.2 (10% methanol in ethylacetate - solvent system B). The fifth prominent antioxidant spot reacted pink to V/SA at R<sub>f</sub> 0.14 (solvent system C).

Bioautographic analysis revealed two antibacterial spots on silica at R<sub>f</sub> 0.8 (solvent system A) and 0.7 (solvent system B).

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