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Phytochemical Screening and Antibacterial/Antifungal Activities of *Ginkgo biloba* Extract EGb 761

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Abstract: *Ginkgo biloba possesses a variety of biological and pharmacological activities. The present study was carried out to evaluate the phytochemical components and antimicrobial activities of Ginkgo biloba extract (EGb 761). Phytochemical screening revealed the presence of alkaloids, glycosides, tannins, steroids, terpenes, flavonoids and saponins in the extract. The extract was examined for antimicrobial activity using Agar Diffusion method. It was effective against Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Candida albicans, Saccharomyces cerevisiae and Geotrichum candidum. The zone of growth inhibition ranged from 22-27 mm with variable MIC, and MBC/MFC values. These results support the use of the Ginkgo biloba extract as herbal medicine for the treatment of various diseases.*

Keywords: *Ginkgo biloba, EGb 761, phytochemical screening, pharmacological activities, antimicrobial activity, herbal medicine.*

I. Introduction

Kingdom Plantae is a source of great amounts of biologically active compounds with various chemical properties and diverse protective/disease preventive properties. Herbal medicine is still patronized by a large section of the human race, mainly in developing countries, for primary healthcare because of better cultural acceptability and fewer side effects [1]. Also primary healthcare facilities are generally inadequate in the management of patients that present with various ailments. The high cost of orthodox (conventional) pharmaceuticals and healthcare services discourage patients that have little means, thereby making traditional healthcare service highly sought after by a large segment of the population[2].Different parts of plants and herbs, including leaves, flowers, stems, roots, seeds, fruits, bark and pigments are all constituents of herbal medicines [1, 3]. The contribution of these traditional constituents in the fight against disease dates back to several centuries and has, to a certain extent, been documented by the ancient Chinese, Indian and North African civilizations [4, 5].

These medicinal plants and herbs contain phytochemical components which produce requisite physiological actions on the human body [1]. The most important of these components are alkaloids, tannins, flavonoids and phenolic compounds [6, 7]. Phytochemicals are extensively found at different levels in various medicinal plants and used in herbal medicine to treat diverse ailments such as cough, malaria, wounds, and rheumatism [8].

Researchers, especially those working on infectious diseases, are interested in the antimicrobial, particularly anti-bacterial and anti-fungal, activities of phytochemicals [7]. This interest has heightened over the past decade due to development of drug resistance by the various disease causing agents leading to increase in therapeutic problems [9]. Natural antimicrobials that are found in plants are effective in the treatment of infectious diseases and often devoid of many of the side effects that are often associated with synthetic antimicrobials [10].

Ginkgo biloba is an ancient Chinese gymnosperm tree and the only surviving member of Ginkgoaceae family that appeared more than 250 million years ago [11]. For many centuries now, the Chinese have used *Ginkgo biloba* as herbal remedy for various ailments, and the demand for its extracts in many herbal products is on the rise, especially in the United State and in Europe [12, 13]. *Gingko biloba* extract is used as an alternative medicine for the treatment and/or the prevention of different pathological conditions and in some cases, its usage goes hand in hand with contemporary medicines [14]. Over the past decades, there was an appreciable growth in the demand for these alternative medicines. Concentrated and partly purified products made from *Ginkgo biloba* constituents, have been marketed widely under different trade names, for the treatment and management of cognitive deficiencies, other age-related impairments, and many other chronic and acute diseases such as cardiovascular and bronchial pathologies [15, 16].

The use of different parts of this plant, including the leaves and nuts (seeds), in traditional medicine has been documented, especially in Chinese literature. It was first mentioned in herbals in Yuan dynasty [1280 to 1368 AD], and published in 1350AD [13]. The seeds have been extensively used for the treatment of pulmonary disorders (like asthma and cough), alcohol abuse and bladder inflammation while the leaves have been mainly used to treat heart and lung dysfunctions in addition to its use in skin infections [13]. *Ginkgo biloba* extract

stimulates blood circulation to the brain, Ginkgo extract is used to treat patients with memory loss, dizziness, sleep disorders, dementia, tinnitus and peripheral circulatory disorders. These and other benefits (anti-inflammatory, antitumor, anti-proliferation, cognitive, neuropsychiatric, hepatoprotective, cardiovascular, anti-diabetic and other miscellaneous roles) of *Ginkgo biloba* have been extensively reviewed [5]. The standardization of *Ginkgo biloba* leaf extract, EGb 761, was originated in Germany about 30 years ago.

The knowledge of the chemical constituents that are present in plants is a gateway to knowing their biological activities and hence medicinal uses. Consequently, it becomes necessary to investigate the chemical composition of these plants in order to find out their possible medicinal values. Since the specific function of many phytochemicals found in crude extracts of plants is still unclear [7], antimicrobial screening of these plant extracts provides a starting point for antimicrobial drug discovery [17, 18].

The objectives of this work were to determine the phytochemicals present in *Ginkgo biloba* extract and to study its antimicrobial activities against selected species of bacteria and fungi, namely *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Candida tropicalis* and *Candida krusei*.

II. Materials And Methods

Chemicals and Reagents

Ginkgo biloba extract (EGb 761) was obtained from Gmb H& Co (Germany). All chemicals used were of analytical grade.

Microbial Isolates

Microbial strains used in this study (Table 1) were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika, Kaduna State, Nigeria.

Table 1: Microbial species for testing the anti-microbial activities of *Ginkgo biloba* extract

Microbial group	Test species
Bacteria	*Staphylococcus aureus* *Klebsiella pneumoniae* *Bacillus subtilis* *Escherichia coli* *Salmonella typhi*
Fungi	*Candida albicans* *Candida tropicalis* *Candida krusei* *Saccharomyces cerevisiae* *Geotrichum candidum*

Phytochemical Screening

Phytochemical screening to detect the presence of secondary metabolites including alkaloids, flavonoids, saponins, tannins, glycosides, anthraquinones, cardiac glycosides, steroids and terpenes were carried out according to standard procedures as reported by Sofowora [19] and Trease and Evans [20].

Test for glycosides (Ferric chloride test)

Concentrated H_2SO_4 (5 ml)was added to about 0.5 g of the standardized leaf extract in a test tube and boiled for 15 min. This was then cooled and neutralized by 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution was added to one of the portions, a green black precipitate indicated presence of phenolic aglycone as a result of hydrolysis of glycoside.

Test for free anthraquinones (Bontrager's test)

About 0.5 g of the standardized leaf extract was shaken with 10 ml of benzene and filtered. Exactly 5 ml of 10% NH_3 solution was added to the filtrate and stirred. The formation of a pink-red or violet color indicated the presence of free anthraquinones.

Test for saponins (frothing test)

About 0.5 g of the standardized leaf extract was dissolved in 10 ml of distilled water; this was then shaken vigorously for 30 seconds and was allowed to stand for 30 min. A honey comb-likestructure that formed for more than 30 min indicated the presence of saponins.

Test for steroids and triterpenes (Lieberman-Burchard's test)

Acetic acid (10 ml) was added to about 0.5 g of the standardized extract. This was followed by the addition of 1 ml of conc. H_2SO_4 downside the tube and the color change was monitored. Red, pink or purple color indicated the presence of triterpenes while blue or blue-green indicated steroids.

Test for tannins (ferric chloride test)

About 0.5 g of extract was dissolved in 10 ml of distilled water and then filtered. Few drops of ferric chloride solution were added to the filtrate. Formation of a blue-black precipitate indicated presence of hydrolysable tannins and green precipitate indicated the presence of condensed tannins.

Test for flavonoids (Shinoda's test)

About 0.5 g extract was dissolved in 1.5 ml of 50% methanol and warmed on a steam bath. Exactly 0.1 g of metallic magnesium and 5 drops of concentrated HCl were added. A red-orange colour indicated the presence of flavonoids aglycone.

Test for alkaloids (Dragendoff's test)

To about 0.5 g of the standardized leaf extract, 1% of diluted HCl (20 ml) was added in a conical flask, heated on a steam bath and filtered. The filtrate was made alkaline with 28% NH_3 solution and then extracted with chloroform ($3\times5cm^3$). The combined $CHCl_3$ extracts were concentrated and treated with equal volume of 1% HCl. Dragendoff's reagent (2 ml) was added and formation of orange-red precipitate indicated the presence of alkaloids.

Determination of antimicrobial activity (Sensitivity test)

The antimicrobial activities of the *Ginkgo biloba* extract was determined using Agar Diffusion method. Exactly 0.6 g of the extract was weighed and dissolved in 10 ml of dimethyl sulfoxide (DMSO) to obtain a concentration of 60 g/ml. This was the initial concentration used to check the activities of the extract. Mueller Hinton agar was the medium used as the growth medium for the microorganisms. The medium was prepared according to the manufacturer's instructions, sterilized at 121^{o}C for 15mins, poured into sterile petri dishes and was allowed to cool and solidify. The sterilized medium was seeded with 0.1 ml of the standard inoculum of the test microbes; the inoculum was spread evenly over the surface of the medium by the use of a sterile swab. A well was cut at the center of each inoculated medium by the use of standard cork barrier of 6 mm diameter. Exactly 0.1 ml of the solution of the extract of the concentration of 60 mg/ml was then introduced into each well on the inoculated medium. Antibiotic discs of ciprofloxacin and fluconazole were used as positive control whereas the disc of the extracting solvent was used as negative control. Incubation was done at 37^{o}C for 24 h, after which each plate of the medium was observed for the zone of inhibition of growth. This zone was measured with a transparent ruler and the result recorded in millimeters.

Minimal inhibitory concentration (MIC)

Search for the minimal inhibitory concentration (MIC) was carried out using micro-broth dilution method as outlined by National Committee for Clinical Laboratory Standards [21]. Mueller Hinton broth was prepared according to manufacturer's instructions; 10 ml was dispensed into test tubes and sterilized at 121^{o}C for 15mins, and the broth was allowed to cool. McFarland turbidity standard scale number 0.5 solution was prepared according to manufacturer's instruction. Normal saline was prepared according to the manufacturer's instruction; 10 ml was dispensed into sterile test tube, inoculated with the test microbe, and incubated at 37^{o}C for 6 h. Dilution of the test microbe was done in the normal saline until the turbidity reached that of the McFarland scale by visual comparison, at this point the test microbe has a concentration of 1.5×10^8cfu/ml. Two-fold serial dilution of the extract in the sterile broth was made to obtain the concentration of 60 mg/ml, 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.25 mg/ml; the initial concentration was obtained by dissolving 0.6 g of the extract in 10 ml of the sterile broth. Exactly 0.1 ml of the test microbe in the normal saline was then inoculated into the different concentrations of the extract; incubation was made at 37^{o}C for 24 h, after which the test tubes of the broth were observed for turbidity (growth). The lowest concentration of the extract in sterile broth which showed no turbidity was recorded as the minimal inhibitory concentration.

Minimal bactericidal concentration (MBC)/ Minimal fungicidal concentration (MFC)

Experiments were carried out to determine whether the test microbes were killed or only their growth was inhibited during the MIC experiments. Mueller Hinton agar was prepared, sterilized at 121^{o}C for 15mins, poured into sterile petri dishes and allowed to cool and solidify. The contents of the test tubes which showed no turbidity during the MIC experiments were then sub cultured in the prepared Mueller Hinton medium and

incubated at 37^{o}C for 24 h, after which each medium was observed for colony growth. The MBC and MFC were the plates with lowest concentration of the medium without bacterial and fungal colony growth respectively.

III. Results And Discussion

Many natural compounds are produced by plants as secondary metabolic products, many of which are involved in plant defense and may possess antimicrobial activities. These include terpenoids, saponins, tannins, flavonoids, steroids, glycosides and alkaloids [7]. In this study, the phytochemical analysis of *Ginkgo biloba* extract revealed the presence of alkaloids, flavonoids, tannins, saponins, triterpenes, steroids and glycosides while anthraquinones were found absent as shown in Table 2.

It was earlier reported that *Ginkgo biloba* leaf extract was a complex product containing different active compounds used as a phytomedicine to increase peripheral and cerebral blood flow [1]. This is also consistent with the findings of Goh and Barlow [22], and Defeudis and Drieu [23].

Table 2: Result for the phytochemical screening of *Ginkgo biloba* extract EGb 761

Test	Result
Glycosides	**+**
Saponins	**+**
Steroids	**+**
Triterpenes	**+**
Tannins	**+**
Flavonoids	**+**
Anthraquinones	**-**
Alkaloids	**+**

+ = present, - = absent

The antimicrobial activities (sensitivity test), zone of minimal inhibitory concentration (MIC) and minimal bactericidal/ fungicidal (MBC/MFC) concentration of the *Ginkgo biloba* extract were studied using ten microorganisms: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Candida tropicalis* and *Candida krusei*.The antimicrobial activities of *Ginkgo biloba* extract were investigated using Agar diffusion method. The result showed marked antimicrobial activities on 6 (six) out of 10 (ten) microorganisms namely: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Candida albicans*, *Saccharomyces cerevisiae* and *Geotrichum candidum* (Table 3) with the zone of inhibition ranging from 22-27mm (Table 4) (Figures 1-3). The large zones of inhibition signifies that the extract EGb 761 was very active and this might be due to presence of variety of active compounds in the extract such as tannins, saponins and alkaloids as suggested by Abo et al.[24]. Interestingly, the extract was active against *S. cerevisiae* while the organism was resistant to fluconazole. Even though *S. cerevisiae* is generally considered non-pathogenic, diseases associated with this baker's yeast are on the increase [25]. Of particular mention is that this organism has been implicated in allergic vaginal yeast infection [26], fungemia [27] and allergic bronchopulmonary mycosis [28].

Table 3: Antimicrobial activities (Sensitivity test) of EGb 761 compared with Ciprofloxacin and Fluconazole

Test organisms	Extract EGb 761	Ciprofloxacin	Fluconazole
Staphylococcus aureus	**S**	**S**	**R**
Bacillus subtilis	**R**	**S**	**R**
Escherichia coli	**S**	**S**	**R**
Klebsiella pneumonia	**S**	**S**	**R**
Salmonella typhi	**R**	**S**	**R**
Geotrichum candidum	**S**	**R**	**S**
Candida albicans	**S**	**R**	**S**
Candida tropicalis	**R**	**R**	**S**
Candida krusei	**R**	**R**	**S**
Saccharomyces cerevisiae	**S**	**R**	**R**

S=sensitive, R=resistant

Table 4: Zone of inhibition (mm) of the EGb 761 extract against test micro-organisms

Test organisms	Extract EGb 761	Ciprofloxacin	Fluconazole
Staphylococcus aureus	**24**	**35**	**0**
Bacillus subtilis	**0**	**32**	**0**
Escherichia coli	**26**	**37**	**0**
Klebsiella pneumoniae	**22**	**34**	**0**
Salmonella typhi	**0**	**41**	**0**

Geotrichum candidum	**27**	**0**	**31**
Candida albicans	**25**	**0**	**35**
Candida tropicalis	**0**	**0**	**34**
Candida krusei	**0**	**0**	**30**
Saccharomyces cerevisiae	**24**	**0**	**0**

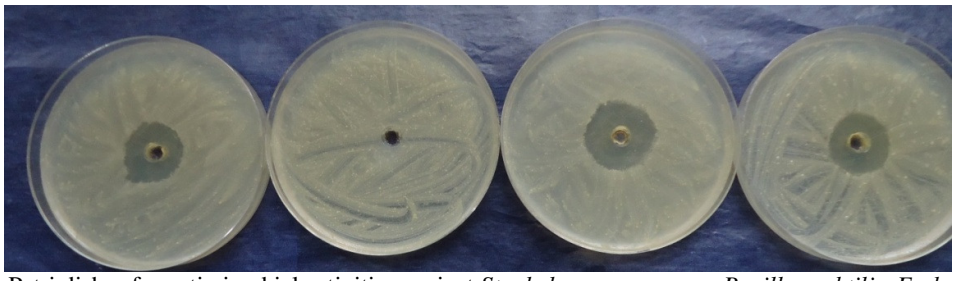

Figure 1: Petri dishes for antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* (From left to right)

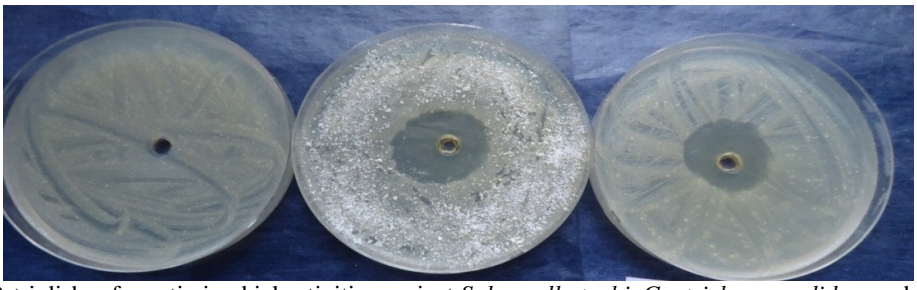

Figure 2: Petri dishes for antimicrobial activities against *Salmonella typhi*, *Geotrichum candidum* and *Candida albicans* (From left to right)

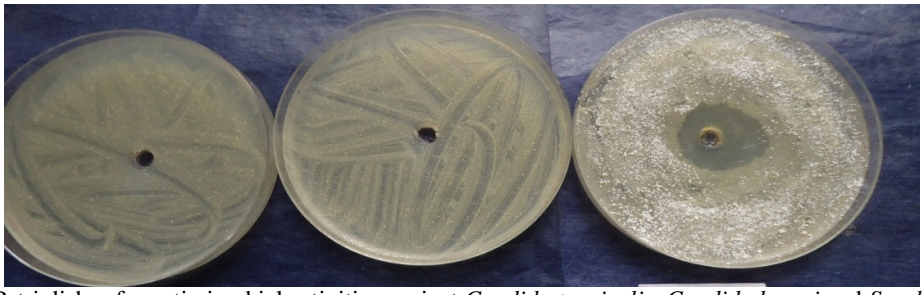

Figure 3: Petri dishes for antimicrobial activities against *Candida tropicalis*, *Candida krusei* and *Saccharomyces cerevisiae*. (From left to right)

The minimal inhibitory concentration was found at 15mg/ml for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida albicans* and *Saccharomyces cerevisiae* while for *Geotrichum candidum* it was found at 7.5mg/ml (Table 5). The result shows that Ginkgo extract has variable inhibition effect against pathogenic bacteria and fungi. This finding is consistent with that of Hossam and Mona [1] who observed that the *G. biloba* contained active compounds that showed considerable activity on pathogenic bacteria and fungi. This also agreed with the previous finding of Sati and Savita [29]. The antimicrobial activities against both Gam positive and Gram negative bacteria suggest the presence of broad spectrum antibiotic compounds or simply metabolic toxins [1].

Table 5: Minimal inhibitory concentrations (MIC) (mg/ml) of the EGb 761 extract against test microorganisms

Test organisms	**60**	**30**	**15**	**7.5**	**3.25**
Staphylococcus aureus	**-**	**-**	**MIC**	**+**	**++**
Escherichia coli	**-**	**-**	**MIC**	**+**	**++**
Klebsiella pneumonia	**-**	**-**	**MIC**	**+**	**++**
Salmonella typhi	**-**	**-**	**-**	**-**	**-**
Geotrichum candidum	**-**	**-**	**-**	**MIC**	**+**
Candida albicans	**-**	**-**	**MIC**	**+**	**++**
Saccharomyces cerevisiae	**-**	**-**	**MIC**	**+**	**++**

- =no turbidity (no growth), + =low turbidity (light growth), ++ = moderate turbidity.

The MBC/MFC was found at 60mg/ml for *Klebsiella pneumoniae* while for *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae* it was found at 30mg/ml, and 15mg/ml for *Geotrichum candidum* (Table 6).This result shows that the various microorganisms responded in different ways to the treatment with *G. biloba* extract, but as small as 7.5 mg/ml concentration of the extract may be enough to elicit inhibitory activity against some microorganisms, as exemplified by MIC of 7.5 mg/ml against *Geotrichum candidum*. This is consistent with the findings of Sati and Savita [29]. Our findings support the justification for the ethnobotanical uses of *G.biloba*. Based on the findings of Singh *et al.* [30], bioactive compounds in *G. biloba* extract, especially the terpene trilactones and flavonoid glycosides, might be responsible for its antimicrobial potential.

Table 6: Minimal bactericidal/fungicidal concentration (MBC/MFC) (mg/ml) of the extract against test microorganisms

Test organisms	60	30	15	7.5	3.25
Staphylococcus aureus	**-**	**MBC**	**+**	**++**	**+++**
Escherichia coli	**-**	**MBC**	**+**	**++**	**+++**
Klebsiella pneumoniae	**MBC**	**+**	**++**	**+++**	**++++**
Salmonella typhi					
Geotrichum candidum	**-**	**-**	**MFC**	**+**	**++**
Candida albicans	**-**	**MFC**	**+**	**++**	**+++**
Saccharomyces cerevisiae	**-**	**MFC**	**+**	**++**	**+++**

- = no colony growth, + = scanty colony growth, ++ = moderate colony growth, +++ = heavy colony growth.

IV. Conclusions

The antimicrobial activities of the *Ginkgo biloba* extract showed that the extract has marked antimicrobial activities against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Candida albicans*, *Saccharomyces cerevisiae* and *Geotrichum candidum*. As small as 7.5 mg/ml MIC was enough to inhibit the growth of *G. candidum* while maximum MBC/MFC required to completely kill the sensitive test microorganisms was 60mg/ml. The extract was, interestingly, potent against *S. cerevisiae* while well-recognized commercial antifungal drug fluconazole gave negative test. The result of the phytochemical screening of the *Ginkgo biloba* revealed the presence of quite a number of chemical constituents such as alkaloids, saponins, flavonoids, glycosides, tannins, steroids and triterpenes. These constituents may be responsible for the pharmacological activities associated with *G. biloba* extract.

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