Chemistry and Materials Research
ISSN 2224- 3224 (Print) ISSN 2225- 0956 (Online)
Vol.6 No.2, 2014

Synthesis, ionization constant, toxicity, antimicrobial and antioxidant screening of 1-Phenyl-3-(phenylamino) propan-1-one and Phenyl (2-[phenyl amino) methyl] phenyl) peroxyanhydride Mannich bases

Ganiyat K Oloyede (Corresponding author)
Natural products/Medicinal Chemistry Unit, Department of Chemistry, University of Ibadan, Nigeria.
Tel: +234 803 562 2238 E-mail: oloyedegk@gmail.com
Omoboboye O. Omisakin
Natural products/Medicinal Chemistry Unit, Department of Chemistry, University of Ibadan, Nigeria.

The research is financed by University of Ibadan Senate Research Grant (2010) No SRG/ES/2010/16[A]

Abstract

Synthesis of 1-Phenyl-3-(phenylamino) propan-1-one (BO1) and Phenyl (2-[phenyl amino) methyl] phenyl) peroxyanhydride (BO2) both of which are Mannich bases were achieved in this study. Analysis and characterisation of the Mannich Bases were also achieved by UV, IR, and NMR Spectroscopy. The ionization constant (pKa) of the products is also reported. The pKa at 8.1 obtained by non-aqueous titration may enable the lone pair of electron on nitrogen in the compounds to be protonated at physiological pH. The result of the Brine shrimp lethality test showed that both BO1 and BO2 with LC_{50} values 17345.1µg/ml and 33520µg/ml respectively, were non – toxic. The result of the antimicrobial screening of BO1 and BO2 using *Escherichia coli*, *Staphylococcus aereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiellae pneumoniae*, *Salmonellae typhi*, *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillum nonatum* showed that the two compounds possess significant antimicrobial activity when compared with the standards, Gentamicin and Tioconazole. *In vitro* antioxidant screening of both compounds by DPPH free radical scavenging method, scavenging effect on Hydrogen peroxide and peroxide oxidation by Ferric thiocyanate method showed that the compounds possessed significant antioxidant activity when compared with antioxidant standards Ascorbic acid, BHA and α-Tocopherol . They possessed the highest %inhibition in peroxide oxidation by Ferric thiocyanate method.

Keywords: Mannich bases, 1-Phenyl-3-(phenylamino) propan-1-one, Phenyl (2-[phenyl amino) methyl] phenyl) peroxyanhydride, ionization constant, antimicrobial, antioxidant

1. Introduction

Organic compounds are important constituents of many products such as drugs, petrochemicals, food, explosives, plastics and paints. They are usually carbon-based compounds, hydrocarbons, and their derivatives. They may therefore contain other elements like hydrogen, nitrogen, oxygen, phosphorus, silicon and sulphur. Organic compounds can be formed through a series of organic reactions of which Mannich reaction, an important tool for the synthesis of novel compounds is one and involves an amino alkylation of an acidic proton placed next to a carbonyl functional group with formaldehyde and ammonia or any primary or secondary amine to give a β-amino-carbonyl compound known as Mannich base (Mannich et al, 1912; Blatt, 1964; Lindert et al, 1990; Shriner et al, 1997; Roman et al, 2000; Xu et al, 2004; Josephsohn et al, 2004; Eftekhari-Sis et al, 2006; Tanaka et al, 2008; Hatano 2008). Mannich reaction is also a nucleophilic addition of an amine to a carbonyl group followed by dehydration to the Schiff base. The Schiff base is an electrophile which reacts in the second step in an electrophilic addition with a compound containing an acidic proton (Hayashi 2003; Pittelkow and Christensen 2005; Liu et al, 2007; Hatano et al, 2010). In Mannich reaction, ammonia or primary or secondary amines are employed for the activation of formaldehyde. Mannich bases can be employed directly or used as intermediates in chemical synthesis and have been reported to have series of pharmacological activities, as antibacterial, antifungal, anticonvulsant, antihelminthic, anticancer, antiviral amongst others (Sheela et al 2005; Salter et al. 2006; Aytenir and Calis 2007; Dotsenko et al 2007; Huang et al 2008; Abdul Rahman et al, 2008, Chhonke et al, 2009; Shivananda and Shet Prakashi, 2011).

The aim of this research work is to synthesize Mannich bases 1-Phenyl-3-(Phenylamino) propan-1-one (BO1) and Phenyl(2-[Phenyl amino)methyl]phenyl) peroxyanhydride (BO2), to determine the purity of the synthesized

Mannich bases by analytical procedures such as melting point determination and thin layer chromatography, elucidate their structures by using spectroscopy methods such as Ultra-violet/Visible, Infra-red and Nuclear Magnetic Resonance Spectroscopy, to determine the ionization constant of the synthesized compounds by non-aqueous titration and to determine the toxicity level of the synthesized Mannich bases using Brine shrimp larvae eggs (*Artemia salina* nauplii). The antimicrobial effect on multi- resistance microbes was determined by Agar well diffusion method while the antioxidant screening was assayed using three free radical scavenging methods; scavenging activity on 2,2-Diphenyl picryl hydrazyl radical (DPPH), scavenging effect on hydroxyl radical generated from hydrogen peroxide and peroxide oxidation by Ferric Thiocynate method.

2. Experimental section

2.1 Materials: Chemicals and Reagents

The following are the major chemicals and reagents used; dimethyl suphoxide (DMSO), methanol, chloroform, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Vitamin C, hydrogen peroxide, α-Tocopherrol, acetophenone, formaldehyde, aniline, conc hydrochloric acid, benzoyl peroxide, ammonia solution, butanol, tetrahydrofuran, isopropanol, trioxonitrate (V) acid, acetone, diethyl ether, ethyl acetate, 1,4-dioxan, ammonium thiocyanate, ferrous chloride, linoleic acid, dimethylformamide, perchloric acid, potassium hydrogen phthalate, sodium tetraborate, hexane, butylated hydroxyl anisole (BHA).

All solvents used were BDH analar grade. Assessments of the degree of purity of the final products obtained were achieved by determination of melting point using Gallemkamp Melting point Apparatus Model MFB 595 and also analytical Thin Layer Chromatography. Thin Layer Chromatography was carried out using Silica Gel F_{254}, hexane, ethylacetate as the mobile phase. Ionization constant by pH and pKa determination was also carried out via non-aqueous titration using the pH meter 7020 (Electronic Instrument Ltd London). These compounds were further characterized by spectroscopic analysis, UV-Visible, Infra-red and NMR spectrometry. The UV/Visible Spectra of 0.01% w/v of the samples were determined with the aid of Spectro UV/Visible double beam Pc scanning spectrophotometer (UVD- 2960). The samples were scanned between 190nm and 1100nm. Data from chart/recorder gave a graph of Absorbance against wavelength (nm). V_{max} (cm^{-1}) from IR data also confirmed the structures. The Infrared spectra of the two synthesized compounds were recorded as KBr discs on Perkin-Elmer FT-IR Spectrophotometer in the range 4000-400 cm^{-1}. The spectrophotometer determines the relative strength and position of all absorption in the infrared region and plots the intensity (Transmittance against wave number). The ^1H NMR spectra of the synthesized compounds were determined using a 200MHz machine for 10% (w/v) solutions in deuteriodimethylsulphoxide. Pulse irradiation technique employed was FT NMR at ambient temperature. The ^1H NMR signals of the compounds appeared at δ scale relative to TMS.

2.1.1 Test Organisms: *Escherichia coli*, *Staphylococcus aereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiellae pneumoniae*, *Salmonellae typhi*, *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillum nonatum* (Micro organisms were collected from the stock of the Dept of Pharmaceutical Microbiology, Faculty of Pharmacy of University of Ibadan). The test organisms were maintained on nutrient agar slopes and kept in a refrigerator at 4 ^oC. 100 ml aliquots of nutrient broth were inoculated with the culture of test micro-organisms using a loop and then incubated at 37 ^oC for 24 hrs.

2.1.2 Reference Standards: Gentamicin (5 mg/ml) for bacteria and Tioconazole (70%) for fungi both for antimicrobial activity; Ascorbic acid, Butylated hydroxyanisole (BHA) and α-Tocopherol for antioxidant activity. Dimethylsulphoxide (DMSO) for toxicity test.

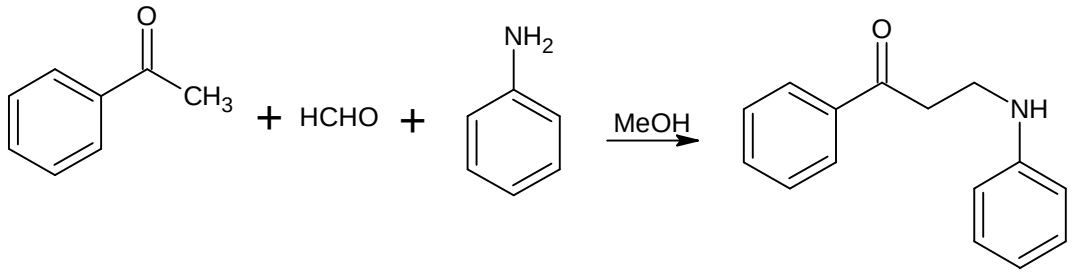
2.2 Methods

The preparation of Mannich bases 1-Phenyl-3- (phenylamino) propan-1-one (BO1) and Phenyl (2-[(phenylamino) methyl] phenyl) peroxyanhydride were carried out based on the procedure used for the synthesis of Mannich bases from substituted aminophenol and acetophenone. The lead compounds in both preparations are medicinally active and non-toxic (Roman et al, 2000; Xu et al, 2004; Josephsohn et al, 2004; Liu et al, 2007; Zhao et al, 2009; Hatano et al, 2010; Muthumani et al, 2010).

2.2.1 Preparation of 1-Phenyl-3-(Phenylamino) propan-1-one (BO1)

A mixture of 1M acetophenone, formaldehyde and aniline in the ratio 2:1:2 were refluxed in a basic medium for 13hrs; the reaction was monitored using Thin Layer Chromatography. The solution was cooled for 72 hours at 0^0c, the crystals were precipitated in chloroform and the crystals were filtered under pressure using a suction pump, the crystals obtained were re-crystallized with warm ethanol.

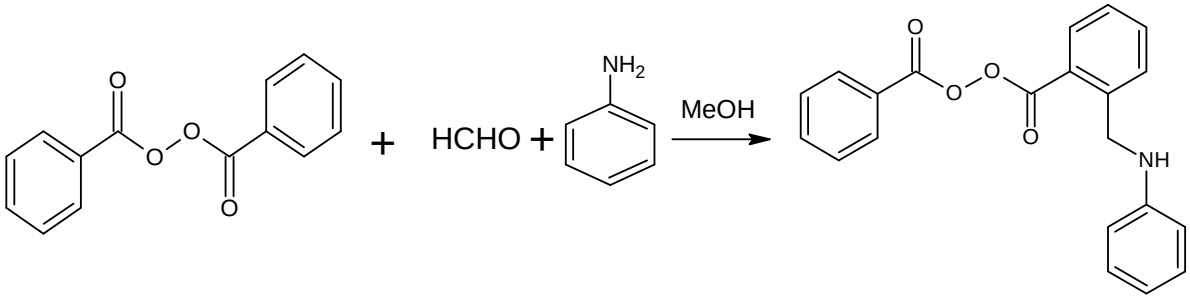
The equation for the reaction is as follows:



Scheme 1.1: Preparation of 1-Phenyl-3-(Phenylamino) propan-1-one **(**BO1)

2.2.2 Preparation of Phenyl (2-[(Phenyl amino) methyl] phenyl) peroxyanhydride (BO2)

A mixture of 1M benzoyl peroxide, formaldehyde and aniline in the ratio 2:1:2 was refluxed in a basic medium for 16 hrs, the reaction was monitored using Thin Layer Chromatography assay. The product was then cooled at 0^0C for 72 hours, after which the crystals obtained were precipitated with chloroform, filtered under pressure using a suction pump and re-crystallized with warm ethanol. The equation for the reaction is as follows:



Scheme 1.2: Preparation of Phenyl (2-[(Phenyl amino) methyl] phenyl) peroxyanhydride (BO2)

2.2.3 Analysis of the synthesized compounds

In other to assess the degree of purity of the compounds, Thin layer chromatography and melting point determination of the synthesized compounds (BO1 and BO2) were carried out. Spectroscopic analysis of the synthesized compounds was carried out to ascertain the structures using Ultra/violet, Infra-red and NMR spectrophotometry. The ionization constant, toxicity, antimicrobial and antioxidant activities of the synthesized compounds were also determined.

2.2.4 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a technique used in separation and analysis of the components of a mixture thereby confirming the presence or absence of by-products, contaminants depending on the number of spots viewed on the plate. Silica gel F_{254} precoated plates (Merck, Germany) were used and solvent systems Hexane 100% and Ethylacetate : Hexane 1:3. Visualisation was aided by the use of iodine vapour.

2.2.5 Determination of Ionization constant (pKa) of BO1 and BO2 via potentiometric titration.

Biological availability, metabolism and excretion of drugs are factors that influence both the absorption and passage of the drug through cell membranes. The ionization constant (pKa) is one of the physiochemical properties that can provide information that can be used to predict these factors. This has also shown that certain drugs are absorbed in their undissociated state either directly or by ion pair or complex formation.

Procedure

The pH was standardised using a standard buffer solution of pH4 (0.05M potassium hydrogenphthalate) and pH9 (0.01M borax solution). Standardized perchloric acid was used to titrate 2.5ml of solution of BO1 and BO2 respectively in 60% 1,4-dioxan. The pKa was then determined from a graph of pH vs volume (ml) of titrant using the Henderson-Hasselbalch equation. The physiochemical importance of the acid-base properties of

synthetic compounds has been summed up by pH – partition theory equation as shown below:
pH= pKa + log [salt]/[acid]
The pH beyond the equivalence point was calculated from the excess titrant added using the formula:
pH = pKw - pOH
pOH = 14 – log [H^+]
pOH = -log [OH]
(pH = -log of hydrogen ion concentration; pKa = -log ionization constant; pKw = ionic constant of water). The pKa results represent mean values of three determinations carried out near pH equivalence at 30^{o}C. The results were statistically analyzed and the limit of experimental error found to be ±0.001.

2.2.6 Toxicity analysis (Brine shrimp lethality test)

The toxicity level of the Mannich base crystals (BO1 and BO2) was conducted according to Falope et al, (1993) and Oloyede et al, (2010). The brine shrimp lethality test (BST) was used to predict the presence in the fractions, cytotoxic activity using *Artemia salina* (Brine shrimp) nauplii. The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (10000, 1000, 100, and 10 ppm) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at fifty percent mortality of the larvae (LC_{50}) was determined using the Finney computer programme.

2.2.7 Antimicrobial Screening BO1 and BO2

Preparation of samples for Antimicrobial analysis

One gram (1g) of each sample (BO1 and BO2) was weighed and dissolved in 5 mls of the solvent (DMSO) to give 200 mg/ml. Five other test tubes containing 2.5 mls of the same solvent were serially diluted until a concentration of 6.25 mg/ml of the content was obtained in the sixth test tube. The seventh test tube contained the solvent of dissolution only (negative control). The eighth test tube served as the positive control and contained gentamicin (5 mg/ml) for bacteria, tioconazole (70%) for fungi.

Agar diffusion: Pour plate method for bacteria

Cultures of the following organisms *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiellae pneumonae* and *Salmonellae typhi* were each prepared overnight. 0.1 ml of each of the organism was taken into 9.9 ml of Sterile Distilled Water (SDW) to give 10 ml of 1: 100 (10^2) dilution. 0.2 ml was taken into the prepared molten Nutrient Agar (NA) at 45 ^{0}C and this was aseptically poured into the sterile plates and allowed to set on the bench for 45 minutes. The stock was maintained on nutrient agar slant and sub-cultured in nutrient broth for incubation at 37 °C prior to each antimicrobial testing. Inoculation of the test organisms on nutrient agar-prepared plates was achieved by flaming a wire loop on a spirit lamp, cooling the wire loop (air cooling) and fetching the test organisms. The discs were prepared using a Grade No. 1 Whatman filter paper. 100 discs were obtained by punching and putting in vials-bottles and sterilizing in an oven at 150 °C for 15 min. Thereafter the cups (9 mm diameter) were aseptically bored into the solid nutrient agar using a sterile cork borer. That is a sterile cork-borer was used to create wells (or holes) inside the set plates. The test solutions (50µL) at concentration of 40 mg/ml were then introduced into each of the designated cups on each plate ensuring that no spillage occurred. The same amount of the standard antimicrobial agent and solvents were introduced were introduced using syringes into the remaining cups on each plate to act as positive and negative controls respectively. The plates were left at room temperature for 2 hours, allowed to diffuse into the medium, turned upside-down and thereafter incubated at 37 ^{0}C for 24 hrs in an incubator. Clear zones of inhibition were observed. Activity or inactivity of each extract was tested in triplicate and the diameters of zones of inhibition were measured in millimetre (mm) using a transparent well-calibrated ruler. The positive control for bacteria is Gentamicin at the concentration of 5 mg/ml. The analysis was done in triplicates and the average readings were calculated (Cushine and Lamb, 2005; Duraipindiyan et al, 2006).

Agar diffusion: Pour plate method for fungi

Into sterile plates was poured molten sterile Sabouraud Dextrose Agar (SDA) aseptically and allowed to cool down for 45 minutes. 0.2 ml of 1:100 dilution of the organisms *Candida albicanas, Rhizopus stolonifer, Aspergillus niger* and *Penicillum nonatum* were spread on the surface using a sterile spreader. Then, a sterile cork-borer was used to create wells inside the plates. The same procedure described for anti-bacterial activity above was followed from this stage. The positive control for the fungi is 70% tioconazole. All the plates for the fungi were incubated at 28 ^{0}C for 48 hours unlike that of bacteria that was incubated at 37 ^{0}C for 24 hours. The clear zones of inhibition were observed and recorded using the same method as described in the case of bacteria (Bayer et al, 1986; Hadecek and Greger 2000).

2.2.8 Antioxidant Activities of the Synthesized Compounds

Free radical Scavenging activity using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH)

The capacity to scavenge the "stable" free radical DPPH or antioxidant activity was determined using the DPPH free – radical scavenging activity (Mellors and Tappel, 1996; Lugasi et al, 1999). A 3.94 mg of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH which is a stable radical), was dissolved in 100 ml methanol to give a 100 µM solution. To 3.0ml of the methanolic solution of DPPH was added 0.5ml of the crystals dissolved in Dimethyl sulphoxide (DMSO) taken from the stock solution. The stock solution was prepared by dissolving 1.0 mg of the crystals in 100 ml DMSO. The mixture was shaken well and left to stand for 10 minutes, after which the decrease in absorption at 517 nm of DPPH was measured. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. Other concentrations were prepared from the stock solution by serial dilution and analyzed the same way as the stock solution. The radical scavenging activity (RSA) or % Inhibition was calculated as the percentage inhibition of DPPH discolouration using the equation below:

$$\% \text{ Inhibition} = \{(A_{DPPH} - A_S)/A_{DPPH}\} \times 100$$

Where A_S is the absorbance of the solution after the sample has been added at a particular concentration to the DPPH solution and A_{DPPH} is the absorbance of the DPPH solution (Hatano et al, 1998; Gulcin et al, 2002; Mutee et al, 2010). All tests and analyses were carried out in triplicates and the results obtained were averaged. The analysis was carried out for all the synthesized compounds with doses ranging from 1.0mg/ml to 0.0625mg/ml. The same experiment was carried out using Butylated Hydroxyl Anisole (BHA), Vitamin C and α-Tocopherol, which are known antioxidant standards.

Hydroxyl radical Scavenging Effect of BO1 and BO2 using Hydrogen Peroxide

The ability of the synthesized compounds (BO1 and BO2) to scavenge hydroxyl radical generated from hydrogen peroxide (H_2O_2) was determined spectrophotometrically at 285nm by the method of Oloyede and Farombi, 2010. A solution of 2 mM hydrogen peroxide was prepared in phosphate – buffered saline (PBS) at pH of 7.4. BO1 and BO2 at the following concentrations 1.0mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0.0625mg/ml was added to the hydrogen peroxide solution. Decrease in absorbance of hydrogen peroxide at 285nm was determined spectrophotometrically, 10 minutes later against the blank solution containing the test drug in PBS without hydrogen peroxide. All tests were run in triplicates. (Soares et al, 1997; Oloyede and Farombi, 2010)

$$\% \text{ Inhibition} = 1 - (A1/A2) \times 100$$

Where A1 was the absorbance of the test sample and A2 was the absorbance of control reaction.

Peroxide oxidation by Ferric thiocyanate method

The antioxidant activities of samples BO1 and BO2 were also determined by the ferric thiocyanate method. 10 mg of each sample was dissolved in dimethyl sulphoxide and various concentrations (0.8-0.00625 mg/ml) were prepared. A mixture of 2 ml of sample in DMSO, 2 ml of 2.51% linoleic acid in 99.5% ethanol, 4 ml of 0.05M phosphate buffer (pH 7.0) and 2 ml of water was placed in a vial with a screw cap and placed in an oven at 60^0C in the dark. To 0.1 ml of this sample solution, 10ml of 75% ethanol and 0.1ml of 30% ammonium thiocyanate was added. After the addition of 0.1ml of 0.002M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour that developed was measured in 3 minutes at 500 nm. The control and the standard were subjected to the same procedures as the sample, except that for the control, only solvent was added and for the standard, sample was replaced with the same amount of Butylated Hydroxyl Anisole (BHA), Vitamin C and α- Tocopherol (Oloyede et al, 2010[a,b]). All test and analysis were run in triplicates and the results obtained were averaged. The % inhibition was calculated using the equation below:

$$\% \text{ Inhibition} = 1 - (A1/A2) \times 100$$

Where A1 was the absorbance of the test sample and A2 was the absorbance of control reaction.

2.2.9 Statistical analysis

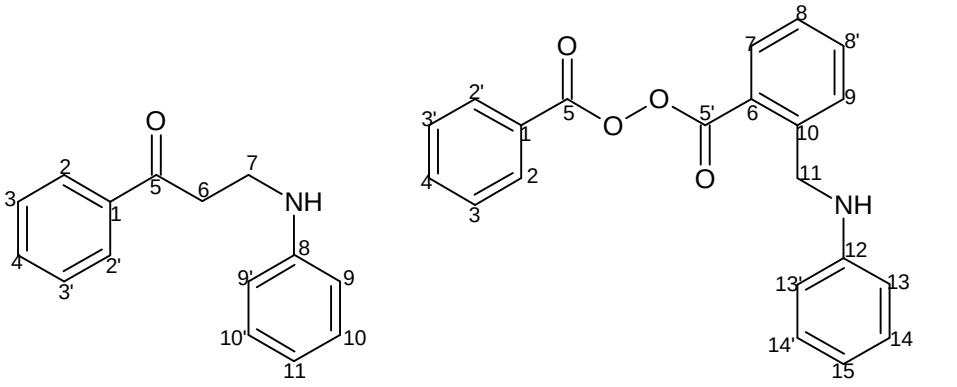
Data (Absorbance measurements) are expressed as mean absorbance ± SD of triplicate analysis. Statistical analysis was performed by a one-way analysis of variance (ANOVA) processed on SPSS 15 windows software for more than two means while Student's t-test was used for comparison between two means. Values of $p<0.05$ were taken to be statistically significant. The LC_{50} after 48 h was determined by probit analysis tested using the Finney computer programme

3. Results and Discussions

3.1 Mannich Base of 1-Phenyl-3-(Phenylamino) propan-1-one (BO1): Yellow crystals; yield: 14% (on dry weight basis); m.p: 180 – 182^0C. Soluble in dimethyl sulphoxide and dimethylformamide, sparingly soluble in tetrahydrofuran, 1,4-dioxan, hydrochloric acid, hexane, ethylacetate and 2-propanol. Insoluble in water, methanol, ethanol, acetone, acetonitrile, chloroform, n-butanol and diethyl ether. R_f 0.38 and 0.64 (Silica gel $F_{254,}$ 100% Hexane and Ethylacetate: Hexane 1:3). UV nm (EtOH, λ_{max}nm): 273.00 (0.024), 297.00 (0.200), 351.00 (0.033), 367.00 (0.024), 402.00 (0.447), 505.00 (0.084), 533.00 (0.085), 669.00 (0.150). IR (KBr) V_{max} cm^{-1}: 3381 (N-H stretch), 3025(C-H aromatic stretch), 2840, 2564 (C-H aliphatic stretch), 1604 (C=O stretch), 1466, 1405 (C-H aromatic out of plane bend). ^{1}H NMR (200 MHz; DMSO): δ 7.91 (2H, d, *J* = 8.5 Hz, H-2, 2′), 7.53

(2H, dd, *J* = 10.5, 9.5, H-3, 3′), 7.05 (1H, dd, *J* = 12.5, 10.5, H-4), 2.29 (2H, t, *J* = 9.5, H-6), 3.14 (2H, t, br, *J* = 12.5, H-7), 4.12 (1H, s, NH), 7.98 (2H, dd, *J* = 13.5, 10.5, H-9, 9′), 7.61 (2H, dd, *J* = 10.5, 6.5, H-10, 10′), 7.11 (2H, dd, *J* = 11.5, 7.5, H-11), Molecular weight (calc): measured for $C_{15}H_{15}NO$ 225. pKa=10.4 and 8.1.

3.2 Mannich Base of Phenyl (2-[(Phenyl amino) methyl] Phenyl) peroxyanhydride (BO2): Brownish - Yellow crystals; yield: 79.9% (on dry weight basis); m.p: 184 – 186^{0}C. Soluble in dimethyl sulphoxide, tetrahydrofuran, sparingly soluble in acetone, hexane, ethylacetate and 1,4-dioxan. Insoluble in water, methanol, ethanol, hydrochloric acid, chloroform, acetonitrile, dimethyl formamide, n- butanol, diethyl ether and 2-propanol. R_f 0.42 and 0.70 (Silica gel $F_{254,}$ 100% Hexane and Ethylacetate: Hexane 1:3). UV (EtOH, λ_{max}nm): 297.00 (0.458), 351.00 (0.038), 402.00 (0.363), 532.00 (0.142). IR (KBr) V_{max} cm^{-1}: 3387 (N-H stretch), 3025(C-H aromatic stretch), 2840, 2558 (C-H aliphatic stretch), 1604 (C=O stretch), 1489, 1408 (C-H aromatic out of plane bend). ^{1}H NMR (200 MHz; DMSO): δ 8.10 (3H, m, Hz, H-2, 2′, 7), 7.69 (2H, dd, *J* = 9.5, 7.5, H-3, 3′), 6.98 (1H, dd, *J* = 8.5, 6.5, H-4), 2.49 (1H, dd, *J* = 9.5, 6.5, H-8), 3.19 (1H, t, H-8′), 3.12 (1H, d, *J* = 12.5, 10.5, H-9), 3.14 (2H, m, H-11), 4.22 (1H, s, NH), 7.94 (2H, dd, *J* = 10.5, 4.5, H-13, 13′), 7.76 (2H, dd, *J* = 13.5, 3.5, H-14, 14′), 7.01 (2H, dd, *J* = 11.5, 7.5, H-15), Molecular weight (calc): measured for $C_{21}H_{17}NO_4$ 347. pKa= 8.1and 7.2.



1a 1b

Figure 1a and 1b: Structures of BO1 and BO2 showing the Protons Chemical Environment.

3.3 Toxicity test (Brine Shrimp lethality test)

Toxicity to living cells was carried out using Brine shrimp lethality test. The two synthesized compounds BO1 and BO2 with lethal concentration (LC_{50}) of 17345.1 and 33520.9 µg/ml respectively were non-toxic (Table 1).

Table 1**:** Brine Shrimp lethality test of BO1 and BO2*

CONC./	10000ppm		1000ppm		100ppm		10ppm		
Sample	Survivor	Dead	Survivor	Dead	Survivor	Dead	Survivor	Dead	LC_{50} µg/ml
BO1	16	14	19	11	23	7	25	5	17345.1
BO2	20	10	22	8	28	2	30	0	33520.9

*LC_{50} < 1000µg/ml =Toxic, LC_{50} > 1000µ/ml = Not Toxic

3.4 Antimicrobial screening of BO1 and BO2

Agar well diffusion method was used to screen the samples for protective effect against gram positive and gram negative bacteria and fungi. The zones of inhibition (mm) were measured in triplicate and the average results obtained is shown in Tables 2-3. It was observed that all the tested samples possessed antimicrobial activities which make them to have broad spectrum activities, the activity was pronounced with *Bacillus subtilis* for both BO1 and BO2. They also showed effective antimicrobial activity at 6.25 - 50 mg/ml though lower than the activities of the controls at the same concentration.

Table 2: Antimicrobial Screening of BO1*

Conc.	Zones of Inhibition (mm)									
	S.a	*E.coli*	*B.sub*	*Ps.a*	*Kleb*	*Sal.*	*A.n*	*C.a*	*Rh.s*	*Pen*
1	20	18	24	16	18	16	16	14	18	14
2	18	14	18	14	16	12	14	12	14	12
3	14	12	16	12	12	10	12	10	12	10
4	12	10	12	10	10	-	10	-	10	-
5	10	-	10	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
Negative	-	-	-	-	-	-	-	-	-	-
Positive	36	36	34	36	36	38	26	26	24	24

*Integers 1-6 represent various concentration of BO1 viz: (1) 50mg/ml, (2) 25mg/ml, (3) 12.5mg/ml, (4) 6.25mg/ml, (5) 3.125mg/ml, (6) 1.5625mg/ml -ve= negative control (DMSO), +ve = positive control {Gentamicin at 5 mg/ml for bacteria or Tioconazole (70%) for fungi}, "–'' = no inhibition, *S.a= Staphylococcus aureus, E. coli= Escherichia coli, B. Sub= Bacillus subtilis, Ps.a= Pseudomonas aeruginosa, Kleb= Klebsiellae pneumoniae, Sal= Salmonellae typhi, C.a= Candida albicans, A.n= Aspergillus niger, Rhs – Rhizopus stolonifer, Pen= Penicillum nonatum.*

Table 3: Antimicrobial Screening of BO2*

Conc.	Zones of Inhibition (mm)									
	S.a	*E.coli*	*B.sub*	*Ps.a*	*Kleb*	*Sal.*	*A.n*	*C.a*	*Rh.s*	*Pen*
1	16	16	20	18	18	18	18	16	16	14
2	14	12	16	14	16	14	16	14	14	12
3	12	10	12	12	14	12	14	12	12	10
4	10	-	10	10	10	10	12	10	10	-
5	-	-	-	-	-	-	10	-	-	-
6	-	-	-	-	-	-	-	-	-	-
Negative	-	-	-	-	-	-	-	-	-	-
Positive	36	36	34	36	36	38	26	26	24	24

*Integers 1-6 represent various concentration of BO2 viz: (1) 50mg/ml, (2) 25mg/ml, (3) 12.5mg/ml, (4) 6.25mg/ml, (5) 3.125mg/ml, (6) 1.5625mg/ml -ve= negative control (DMSO), +ve = positive control {Gentamicin at 5 mg/ml for bacteria or Tioconazole (70%) for fungi}, "–'' = no inhibition, *S.a= Staphylococcus aureus, E. coli= Escherichia coli, B. Sub= Bacillus subtilis, Ps.a= Pseudomonas aeruginosa, Kleb= Klebsiellae pneumoniae, Sal= Salmonellae typhi, C.a= Candida albicans, A.n= Aspergillus niger, Rhs – Rhizopus stolonifer, Pen= Penicillum nonatum.*

3.5 Antioxidant analysis

3.5.1 Free Radical Scavenging Effect on DPPH

A rapid, reliable and quantitative method for testing for antioxidant activity is by the use of 2,2-diphenylpicryl hydrazyl radical (DPPH); a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction in absorbance of DPPH at 517nm caused by the samples was measured in triplicate after 10min. BO1 and BO2 have only moderate activities as free radical scavengers when compared with controls, ascorbic acid, butylatedhydroxylanisole (BHA) and α –Tocopherol (Table 4). The percentage inhibition of BO1 and BO2 are 35.1 and 50.7% at 1.0 mg/ml respectively (Figure 1). Even though the activity is higher than that of α-tocopherol, but is lower than that of Ascorbic acid and BHA at the same concentration.

Table 4: Free Radical Scavenging Activity of BO1 and BO2 on DPPH*

CONC (mg/ml)	BO1	BO2	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
1.0	0.206±0.002	0.210±0.009	0.0843±0.010	0.0370±0.006	0.6800±0.029
0.5	0.166±0.001	0.187±0.001	0.2893±0.128	0.0460±0.006	0.7207±0.003
0.25	0.136±0.001	0.120±0.000	0.2977±0.124	0.0490±0.004	0.7207±0.012
0.125	0.095±0.004	0.078±0.001	0.32±0.08200	0.0483±0.002	0.7047±0.007
0.0625	0.068±0.001	0.048±0.001	0.5147±0.015	0.0650±0.003	0.7070±0.012

*Absorbance measurement of BO1 and BO2, Ascorbic Acid, BHA and α- Tocopherol at 517nm. Absorbance measurement of DPPH standard is 0.8037 at 517nm.

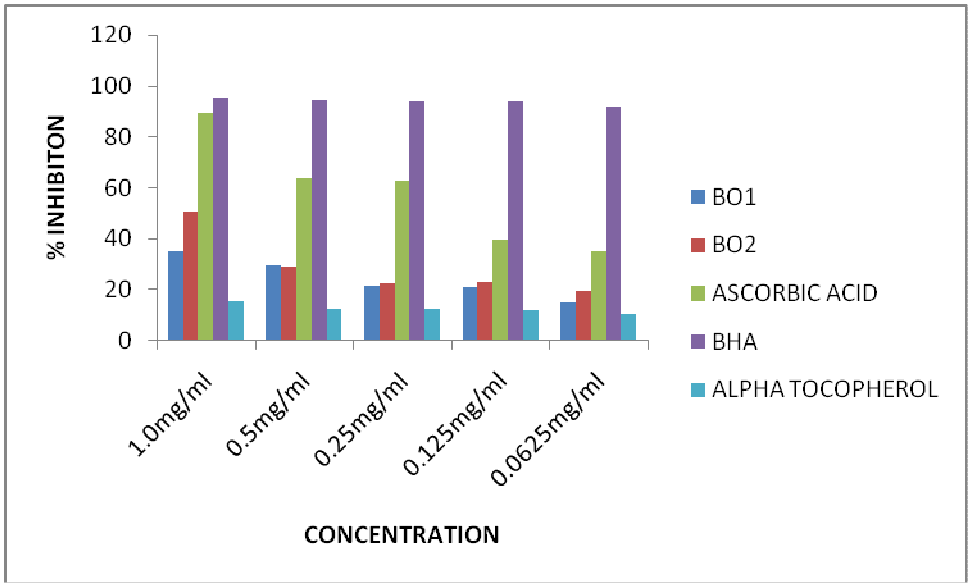


Figure2: DPPH Free Radical Scavenging Activity on BO1and BO2

3.5.2 Hydroxyl radical scavenging effect of BO1 and BO2 using H_2O_2

Highly reactive hydroxyl radical through the Fenton reaction has been observed to participate in free radical chain reactions thereby initiating lipid peroxidation and resulting in harmful disorders. Antioxidant agents which can stop or reduce the production of hydroxyl free radicals will terminate free radical reaction in biological systems. For BO1 and BO2, hydroxyl radical scavenging activity using H_2O_2 was measured in triplicates after 10min of incubation at 285nm. Table 5 shows the results of the analysis. There were comparable activities with standards used and activity was better than that of Ascorbic acid at 1.0 mg/ml. The % inhibition for BO1 and BO2 was 95.0 and 94.7% respectively at this concentration. For BO2 however, inhibition increases as concentration is decreasing unlike for BO1 (Figure 2).

Table 5: Hydroxyl Radical Scavenging Activity of BO1 and BO2 using H_2O_2

CONC (mg/ml)	BO1	BO2	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
1.0	0.1870±0.0019	0.1996±0.0038	0.1952±0.001	0.0413±0.016	0.0321±0.045
0.5	0.2389±0.0023	0.1367±0.0013	0.2078±0.012	0.0617±0.019	0.0633±0.032
0.25	0.2528±0.0022	0.1034±0.0051	1.2645±0.119	0.0740±0.015	0.1552±0.061
0.125	0.3447±0.0013	0.0576±0.0011	2.7586±0.049	0.0947±0.003	0.1807±0.015
0.0625	0.3899±0.0012	0.0331±0.0013	2.9236±0.211	0.1126±0.014	0.4940±0.017

*Absorbance measurement of BO1 and BO2, Ascorbic Acid, BHA and α- Tocopherol at 285nm. Absorbance measurement of Hydrogen peroxide standard is 3.7692 at 285 nm

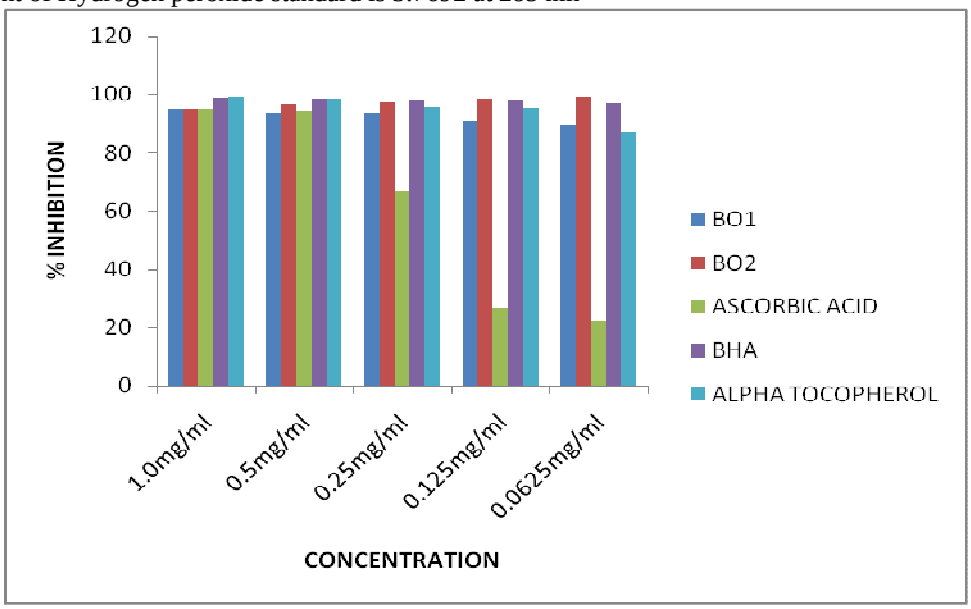


Figure 3: Hydrogen Peroxide Free Radical Scavenging Activity of BO1 and BO2

3.5.3 Peroxide Oxidation by Ferric Thiocyanate method

In this analysis, the Ferric Thiocyanate method was used to determine the amount of peroxide which oxidized ferrous chloride ($FeCl_2$) to a reddish ferric chloride ($FeCl_3$) pigment. Peroxide is oxygen reactive specie which is known to be involved in free radical chain reaction thereby causing damages to biological macromolecules. At various concentrations (0.00625 – 0.8 mg/ml), BO1 and BO2 showed antioxidant activities in a concentration dependent manner. The concentration of peroxide decreases as the antioxidant activity increases for BO1and BO2 (Table 6). At 0.8mg/ml, BO1 had percentage inhibition of 88.9% while BO2 gave 91.6% inhibition, but at the lowest concentration 0.00625 mg/ml the % inhibition was 78.2 and 82.9% respectively. These activities were better than those of the standards used, Ascorbic acid (76.95%), BHA (56.56%) and α-Tocopherol (82.28%) at 0.8mg/ml (Figure 4).

Table 6**:** Free Radical Scavenging Activity of BO1 and BO2 on Ferric Thiocyanate

CONC (mg/ml)	BO1	BO2	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
0.8	0.083±0.000	0.063±0.000	0.173±0.008	0.326±0.006	0.133±0.004
0.4	0.088±0.002	0.071±0.002	0.173±0.008	0.375±0.008	0.164±0.006
0.2	0.098±0.000	0.079±0.001	0.245±0.008	0.431±0.008	0.184±0.009
0.1	0.118±0.001	0.091±0.001	0.275±0.006	0.616±0.005	0.195±0.023
0.05	0.128±0.001	0.098±0.002	0.287±0.050	0.647±0.004	0.294±0.004
0.025	0.139±0.001	0.107±0.000	0.367±0.004	0.653±0.008	0.340±0.069
0.0125	0.156±0.001	0.113±0.001	0.516±0.008	0.747±0.003	0.360±0.005
0.00625	0.164±0.000	0.128±0.001	0.668±0.002	0.750±0.001	0.377±0.008

*Absorbance measurement of BO1 and BO2, Ascorbic Acid, BHA and α- Tocopherol at 500 nm.
Absorbance measurement of Ferric thiocyanate standard is 0.7505 at 500 nm.

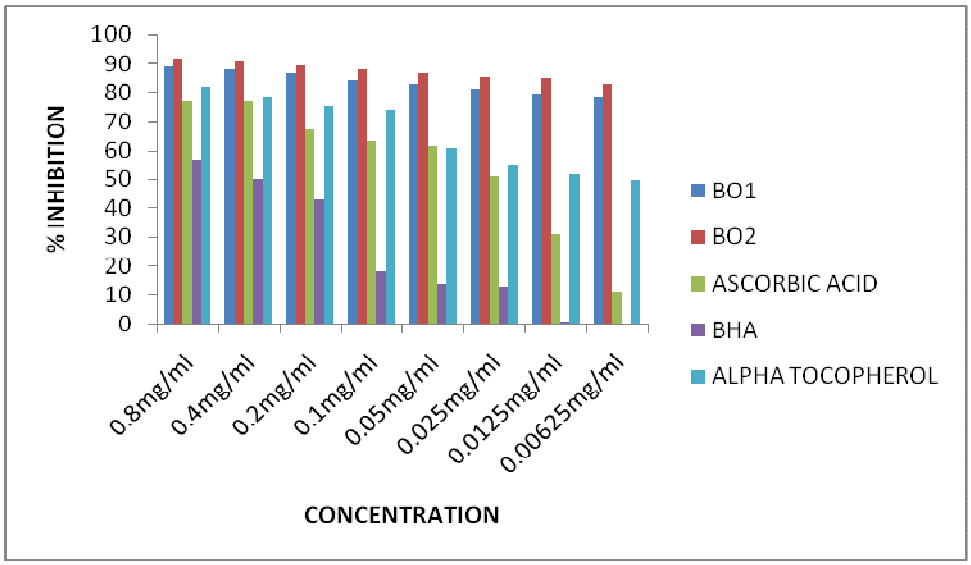


Figure 4: Free Radical Scavenging Activity of BO1 and BO2 on Ferric Thiocyanate

The preparation of Mannich bases 1-Phenyl-3- (phenylamino) propan-1-one (BO1) and Phenyl (2-[(phenylamino) methyl] phenyl) peroxyanhydride were achieved in this study. And was carried out based on the procedure used for the synthesis of Mannich bases from substituted aminophenol and acetophenone (Zhao et al, 2009; Muthumani et al, 2010). The two Mannich bases (BO1 and BO2) were characterized using UV/Visible and Infra-red spectrophotometry and NMR spectroscopy.

In other to assess the degree of purity of the synthesized compounds, thin layer chromatography (TLC) and melting point determination were carried out. TLC using solvent systems 100% hexane and hexane : ethylacetate, 1:3 showed a single spot for each of the two compounds aided by visualization in iodine vapour. The melting point of BO1 and BO2 were in the range 180-182^{0}C and 184-186^{0}C respectively which were sharp enough to confirm that the compounds were pure. Higher melting point of the products is on account of the increased molecular weight and H-bonding. The IR spectra confirmed the presence of certain functional groups, BO1 showed absorption peak (V_{max}) at 3626cm^{-1} and 3381cm^{-1} while BO2 showed absorption peak (V_{max}) 3387cm^{-1} which are assignable to the N-H stretching frequency. Absorption peak for both BO1 and BO2 at 3025cm^{-1} is assignable to the C-H aromatic stretching frequency and at 2840cm^{-1} for aliphatic C-H stretching, Both compounds also showed a strong absorption peak 1604cm^{-1} assignable to C=O. BO1 showed absorption peaks 1466cm^{-1} and 1405cm^{-1} assignable to C-H bending frequency while BO2 showed absorption peaks at 1489cm^{-1} and 1408cm^{-1} assignable to C-H bending frequency. The functional group C-N was seen at V_{max} 1236cm^{-1} for BO1 while that of BO2 was seen at V_{max} 1263cm^{-1}. Peaks at 974cm^{-1} and 810cm^{-1} for BO1 and at 984cm^{-1}and

$813cm^{-1}$ are assignable to aromatic C-H out of plane bending frequency. Both compounds have absorption peaks at $1405cm^{-1}$ and $1408cm^{-1}$ also due to aliphatic C-H bending which also confirmed the $-CH_2-$ in Mannich bases. The Ultraviolet-Visible absorption spectra for the two synthesized compounds with wavelength of absorption at 669 nm (BO1) and 532 nm (BO2) correspond to π-π* transitions and 297 nm and 351 nm respectively corresponding to n-π* transitions. These values imply that the two compounds (BO1 and BO2) are highly conjugated. The bands vibrational frequencies are affected by factors like conjugation effects, intra- or inter-molecular hydrogen bonding, and ring size effects. The π → π* transition of benzene appear in the region 273 - 297 (BO1) and 297nm (BO2) while the absorption in the region 351 nm confirmed the n - π* of the heterocyclic ring. The n-π* transition is due to the presence of n-electrons which are non-bonding electrons, such as those of the lone pairs on Oxygen and Nitrogen which are present in the two synthesized compounds. The π-π* transition is also due to be presence of double bonds. The various absorptions observed in the visible region shows that the two compounds are coloured. The ^1H NMR spectra of BO1 and BO2 give characteristic signals in the range δ 7.91-7.05 and 8.10 and 6.98 corresponding to the aromatic protons in differing electronic environments respectively. These facts were also supported by characteristic peaks in NMR data at δ 4.12 (BO1) and 4.22 (BO2) for NH protons. The $-CH_2-$ protons occurred at δ 3.14 for both BO1 and BO2 characteristic of the methylene protons. In order to predict the biological activity of the synthesized compounds, a very important physiochemical properties which provide information that can be used to predict biological availability, distribution, metabolism and excretion of drugs since it influences both absorption and passage of the drug through the cell membrane is to determine the ionization constant (pKa). Weak bases for instance are reabsorbed on the renal tubules (pH 5-7), depending on their degree of ionization and are eliminated at a rate depending on their pKa values. The rate is faster when the pH of urine is low and slower when the pH is high. The biological half life of drugs may also increase or decrease as the pH changes (Beckett, 1959; Beckett and Stenlake, 1986; Olaniyi, 1989). The results obtained from the non-aqueous titration of BO1 and BO2 using standardized perchloric acid as titrant gave two Ionization constant (pKa) values 10.4; 8.1 and 8.1;7.2 respectively. BO1 and BO2 at pKa 8.1 may enable the lone pair on nitrogen in the compounds to be protonated at physiological pH and then be able to interact with the anionic site as postulated by Beckett.

The result of the brine shrimp lethality/toxicity test showed that both BO1 and BO2 with LC_{50} values 17345.1µg/ml and 33520µg/ml respectively, are non - toxic, they both have LC_{50} value greater than 1000 unlike some Mannich bases which have been reported to be toxic. The result of the antimicrobial screening of BO1 and BO2 using *Escherichia coli*, *Staphylococcus aereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiellae pneumoniae*, *Salmonellae typhi*, *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillum nonatum* showed that the two compounds possess significant antimicrobial activity when compared with the standards Gentamicin and Tioconazole. Other synthesized Mannich bases have also been observed to have antimicrobial and filaricidal activities (Pandeya et al, 1999; Oke and Achife, 1999; Roman et al, 2000; Altintas et al, 2005; Bhasin et al, 2005; Ali and Sharharyar, 2007; Saraswathi et al, 2010; Oloyede et al, 2011).

Antioxidant activity of BO1 and BO2 as determined by three complementary tests, namely DPPH free radical scavenging effect, scavenging effect on Hydrogen peroxide and peroxide oxidation by Ferric thiocyanate method showed that the compounds have significant antioxidant activity. For the DPPH scavenging effect, the percentage inhibition of BO1 and BO2 are 35.1 and 50.7% at 1.0 mg/ml respectively. Even though the activity is higher than that of α-tocopherol, it is lower than that of Ascorbic acid and BHA at the same concentration. The result of the scavenging effect on Hydrogen peroxide showed that for BO1, the % inhibition increased with increase in concentration, at 1.0mg/ml, the % inhibition was 95.04% which is also the same for BHA but higher than that of Ascorbic acid and α-tocopherol. Also at 0.0625 mg/ml, % inhibition was 89.66% which is greater than that of Ascorbic acid and α- tocopherol. BO2 on the other hand gave % inhibition of 94.7% and 99.12% at 1.0 mg/ml and 0.0625 mg/ml respectively. For peroxide oxidation by Ferric thiocyanate method, % inhibition increased with increase in concentration. BO1 at 0.8 mg/ml gave % inhibition of 89.9% and 78.2% at 0.00625 mg/ml. For BO2, % inhibition was 91.6% at 0.8mg/ml and 82.9% at 0.00625 mg/ml. % inhibition increased with increase in concentration for both compounds. Activity is better here than with the standards used at all concentrations. These results also confirmed the report of antioxidant activities of some synthesized Mannich bases of 4- Piperidones which have also been observed to possess anticancer activity (Shivananda and Shet Prakash, 2011; Ali and Sharharyar, 2007). The antioxidant screening result obtained is also justified by the pKa result as protonation will reduce free radical chain reactions.

4. Conclusion

Mannich bases 1-Phenyl-3-(phenylamino)propan-1-one (BO1) and Phenyl(2-[(phenylamino) methyl] phenyl)

peroxyanhydride (BO2) were synthesized and the structures confirmed by spectroscopic analysis UV/Visible, IR and NMR spectrophotometry indicating the presence of the functional groups C=O, N-H, C-H and C-C. The ionization constant value obtained showed that the compounds will be readily metabolized in cell membranes. The synthesized compounds were not toxic to Brine shrimp larvae eggs (*Artemia salina* nauplii) as LC_{50} results gave values above 1000 µg/ml in the toxicity test. BO1 and BO2 were also moderately effective as antimicrobial agents when compared with the activities of Gentamicin and Tioconazole. The two compounds possessed significant antioxidant activity in the three antioxidant screening method employed when compared with control antioxidant standards Ascorbic acid, Butylated hydroxylanisole (BHA) and α-tocopherol. They possessed the highest inhibition effect in peroxide oxidation by Ferric thiocyanate method. The results of the present study, shows that these Mannich bases can be used as starting materials for drugs used to cure microbial infections and diseases resulting from oxidative stress.

Acknowledgement
The authors gladly acknowledge University of Ibadan for the award of Senate Research Grant (2010) No SRG/ES/2010/16^{A} in respect of this work and the staff of Multi Research Central Science Laboratory, University of Ibadan and Central Science Laboratory Obafemi Awolowo University for the use of spectroscopic equipment. The authors will also like to thank Mr. Festus of the department of Pharmaceutical Microbiology University of Ibadan, Nigeria for assisting in carrying out the antimicrobial analysis.

References

AbdulRahman M., Balakrishina A.& Kalluraya M. 2008. Synthesis, Characterization, Antimicrobial and Anthelmintic Activity of Some Sydnone-N-Mannich Bases. *J. Ind. Council Chem.* 25(1): 10-14.

Afsah EM., Kandeel M., Khalifa MM & Hammouda WM. (2007). Mannich Bases as Synthetic Intermediates; synthesis of 3- and 4- Functionalized 2- Pyrazolines. *Z. Naturforsch* 62b: 540-548.

Ali MA. &Sharharyar M. (2007). Oxadiazole Mannich bases. Synthesis and antimycobacterial activity. *Bioorganic and medicinal chemistry letters* 17: 3314-3316.

Altintas H., Ates O., Birteksoz S., Otuk G., Uzun M. & Santana D. (2005). Synthesis of Mannich Bases of some 2, 5-Disubtituted 4-thiazolidinones and Evaluation of their Antimicrobial Activities. *Turk . J. Chem*. 29: 425-435.

Aytenir M.D &Calis U. (2007). Synthesis of some Novel Mannich bases derived from Allomaltol and Evaluation of their Anticounvulsant activities. *Hacettepe University Journal of the faculty of Pharmacy*. 27: 1-10.

Bayer AW., Kirby MDK., Sheris, JC., & Trick M. (1986). Antibiotic Susceptibility Testing by Standard Single Disc Diffusion Method. *Am. J. Clinical Pathol*. 45: 493-496.

Bhasin, PS., Sachdeva N., Pandeya SN., Nath, G. and Singh SK. (2005). Synthesis and Evaluation of Schiff and N- Mannich Bases of Isatin as Potential Antimicrobial agents. *Acta Pharmaceutica Turcica* 47: 21-29.

Blatt AH. (1964). Organic Synthesis Collective Vol 1, 2 Ed. Published by John Wiley and Sons. NY.

Beckett A. (1959). Stereochemical factors in biological activity. *Progress in Drug research*, 1: 455-530.

Becket A.H. and Stenlake J.B. (1986). Pratical Pharmaceutical Chemistry 3rd edition Vol 1. The Athione Reis London. Pulblished by S.K. Jain for CBS Pulblishers and Distributors Delhi, India.

Chhonke YS., Veenu B., Hasim SR., Niranjan K., Devendra K., & Pradeep K. (2009). Synthesis and Pharmacological Evaluation of Some New 2-Phenyl Benzimidazoles Derivatives and their Schiff's Bases. *European Journal of Chemistry.* 6(SI): S342-S346.

Cushine T.P., & Lamb A.J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents.* 26(5): 343-356.

Duraipandiya V., Ayyanar, M., & Ignacimuthu, S. (2006). Antimicrobial Activity of Some Ethnomedicinal Plants Used by Paliya Tribe from Tamil Nadu, India, BMC Comp. *Alt. Med*. 6: 35-41.

Dotsenko VV., krivokolysko SG. & Litvinor VP. (2007). The Mannich reaction in the Synthesis of N, S-containing heterocycles 6* New approaches to the synthesis of pyrimdo [4, 3-b][1,3,5] thiadiazine derivatives. *Russian Chemical Bulletin*, International Edition 56(7): 1474-1476.

Eftekhari-Sis B., Abdollahifar A., Hashemi MM., & Zirak M. (2006). Stereoselective Synthesis of β-Amino Ketones via Direct Mannich –Type Reactions Catalyzed with $ZrOCl_2.8H2O$ under Solvent Free Conditions. *European Journal of Organic Chemistry*. 5152-5157.

Falope MO., Ibrahim H., & Takeda Y., (1993). Screening of higher plants requested as pesticides using the brine shrimp lethality assay. *International Journal of Pharmacognosy* 37 (4), 230-254.

Gulcin IM., Oktay OK., & Aslan A. (2002). Determination of antioxidant activity of Linchen *Cetraria islandica* (L). *Ach. Journal of Ethnopharmacology.* 79, 325-329.

Hatano M., Horibe T., & Ishiara K., (2010). Chiral Lithium (l) Binaphtholate Salts for the Enantioselective Direct Mannich –Type Reaction with a Change of Syn/Anti and Absolute Stereochemistry. *J. Am. Chem Soc*. 132: 56-57.

Hatano, T., H., Kagawa, T., Yasuhora & T., Okuta. (1988). Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bullettin* 36, 200-209.

Hatano M., Maki T., Moriyama K., Arinobe M., & Ishihara K. (2008). Pyridinium 1, 1'-Binaphthyl-2, 2'-Disulfonates as Highly Effective Chiral Bronsted Acid-Base Combined Salt Catalyst for Enantioselective Mannich –Type Reaction. *J. Am. Soc*. 130:16858-16860.

Hayashi Y., Tsubo W., Ashimie I., Urushima T., Shoji M., & Sakai K. (2003). The Direct and Enantioselective, Three Component , Cross Mannich Reaction of Aldehydes. *Angew Chem. Int. Edu.* 42:3677-3680.

Hadecek F. &, Greger H. (2000). Testing of antifungal on Natural products: Methodologies, Comparability of result and assay choice. *Phytochemical Analysis*.11: 137-141.

Huang PJ., Youssef D., Cameron TS. & Jha A. (2008). Microwave-assisted Synthesis of novel 2- naphtol bis –Mannich Bases *ARKIVOC* (xvi): 165-177.

Josephsohn NS., Snapper ML., & Hoveyda AH. (2004). Silver Catalyzed Asymmetric Mannich Reactions of Enol Ethers with Aryl, Alkyl, Alkenyl, and Alkynyl Imines. *J. Am. Chem. Soc*. 126: 3734-3735.

Lindert A., Wolpet A., &Stephen M. (1990). Tannin Mannich Adducts for Corrosion Resistance Pripmering of Metals PCT. Int. Appl. W090 05, 794 (cl L 23c22/00) 1990 USb Appl. 272, 172, 1988 23pp.

Liu TY., Cui HL., Long J., Li Y., Wu LS., & Ding YC. (2007). Organocatalytic and Highly Stereoselective Direct Vinylogous Mannich Reaction. *J.Am. Chem. Soc.* 129: 1878-1879.

Lugasi A., Honvahorich P., & Dworschark A. (1999). Additional Information to the *In-vitro* Antioxidant Activity of *Ginkgo Biloba L. Phytotherapy Research* 13: 160-162.

Mannich C., & Krosche W. (1912). Ueber ein Kondensationsprodukt aus Formaldehyd, Ammoniak und Antipyrin. *Archiv der pharmazie* 250:647-650.

Mellors H. & Tappel AL. (1996). The Inhibition of Mitochondrial Peroxidation by Ubiquinone and Ubiquinol. *Journal of Biology and Chemistry*. 1: 151-217.

Mutee AF., Salhimi SM., Yam MF., Lim CP., Abdullah GZ., Ameer OZ., Abdulkareem MF., & Asmawi MZ. (2010). *In vivo* anti-inflammatory and *in vitro* antioxidant activities of *Peperomia pellucida*. *International Journal of Pharmacology* 6: 686-690.

Muthumani P., Neckmohammed1 R., Vekataraman1 S., Chidambaranathan N., Devis P., & Suresh Kumar CA. (2010). Synthesis and Evaluation of Anticonvulsant and Antimicrobial Activities of Some Mannich Bases of Substituted Aminophenol and Acetophenone. *International Journal of Pharmaceutical and Biomedical Research.* 1(3): 78-86.

Nicolau KC., Sarlah D., & Shaw M. (2007). Total Synthesis and Revised Structure of Biyouyanagin. *Angew. Chem. Int*. 46: 4708-4711.

Oke JM., &Achife JC. (1999). Filaricidal activity of Oxamide Derivatives of Mebendazole. *African J. Biomedical Research* 2(2): 75-82.

Olaniyi AA. 1989. Essential Medicinal Chemistry, Shaneson CI Ltd Ibadan, Nigeria ISBN 978-240-017.

Oloyede GK., Farombi OE. 2010[a]. Antioxidant Properties of *crinum ornatum* Bulb Extract. *World Journal of Chemistry*, 5(1): 32-36.

Oloyede GK., Onocha PA., Soyinka J., Oguntokun O. & Thonda E. 2010[b]. Phytochemical screening, antimicrobial and antioxidant activities of four Nigerian medicinal plants. *Annals of Biological Research*, 1 (2): 114-120.

Oloyede GK., Onocha PA., & Oke JM. (2011). Mannich Synthesis of 2-(Thiazol-4-yl)-1H-benzimidazole-2-methylene Piperazinyl (2,5 -Diamino-1,3,4-thiadiazolyl) Oxamide and Evaluation of the Filaricidal Activity. *American-Eurasian Journal of Scientific Research* 6 (2): 116-122.

Pandeya SN., Sriram D., Nath G. & De Clercq E. (1999). Synthesis, antibacterial, antifungal and anti-HIV evaluation of Schiff and Mannich bases of Isatin derivatives with 3-amino-2-methyl mercaptoquinazolin-4(3H)-one. *Pharmaceutica Acta Helvetiae* 74: 11-17.

Pittelkow M., & Christensen B. Jrn.(2005). Convergent Synthesis of Internally Branched Panam Dendrimers. *Org. Letter*. 7(7):1295-1298.

Roman G., Nanu D., Comanita E., & Comanita B. (2000). Synthesis and Reactivity of Some Mannich Bases. *Turk. J. Chem*. 24: 67-71.

Salter MM., Kobayashi Y., Shimizu S., & Kobayashi S. (2006). Direct-Type Catalytic Three-Component Mannich Reactions Leading to an Efficient Synthesis of α, β-Diamino Acid Derivatives. *Org Lett*. 8: 3533-3536.

Sarawathi M., Rohini RM., Ranaprathapsingh A., & Naira N., (2010). Synthesis and Evaluation of Mannich Bases of Benzimidazo [1, 2, c] Quinazolin- 6(5H)- Thione for Antimicrobial Activity. *Pak. J. Pharm. Sci*. 23: 159-462.

Sheela J., Navita K., Deepak K & Rakesh S. (2005). Synthesis and *In vitro* study of Novel Mannich Derived from Allomaltol and Evaluation of their Anticonvulsant Activities. *Hacettepe University J of Faculty of Pharmacy* 27: 1-10.

Shivananda MK., & Shet Prakash M. (2011). Antifungal Activity Studies of some Mannich Bases Carrying Nitrofuran Moiety. *Journal of Chemistry and Pharmaceutical Research*. 3(2): 302-307.

Shriner RL., Hermann CKF., Morrill TC., Curtin DY., & Wiley John Fuson RC. (1997). The Systemic Identification of Organic Compounds. ISBN 0-471-59748-1.

Soares JR., Dinis TC., Cohn AP., & Almeida LM. (1997). Antioxidant activity of some extracts of *Thymus zygis*. *Free Radical Research* 26: 469-478.

Tanaka Y., Hasui T., & Suginome M. (2008). Diaryborinic Acid Derivatives as a Catalytic Iminium Ion Generator in the Mannich-Type Reaction Using Secondary Amines, Aldehydes and Ketene Silyl Acetals. *Syn lett*. 1239-1241.

Xu LW., Xia CG., & Li L. (2004). Efficient Synthesis of N-Protected β-Aryl-β-Amino Ketone Compounds. *Journal of Organic Chemistry*. 69: 8482-8484.

Zhao YJ., Wei W. Zhi- Guo S., & Guang –Hui M. (2009). Poly(ethylene glycol) prodrug for anthracyclines via N-Mannich base linker; Design, Sythesis and biological evaluation. *Int J of Pharmaeutics*. 379: 90-99.