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PII: S0308-8146(14)00835-8
DOI: http://dx.doi.org/10.1016/j.foodchem.2014.05.119
Reference: FOCH 15902

To appear in: *Food Chemistry*

Received Date: 2 August 2013
Revised Date: 21 April 2014
Accepted Date: 21 May 2014

Please cite this article as: Oloyede, G.K., Willie, I.E., Adeeko, O.O., Synthesis of Mannich bases: 2-(3-Phenylaminopropionyloxy)-benzoic acid and 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one, their toxicity, ionization constant, antimicrobial and antioxidant activities, *Food Chemistry* (2014), doi: http://dx.doi.org/10.1016/j.foodchem.2014.05.119

Synthesis of Mannich bases: 2-(3-Phenylaminopropionyloxy)-benzoic acid and 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one, their toxicity, ionization constant, antimicrobial and antioxidant activities

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ABSTRACT

Mannich bases 2-(3-Phenylaminopropionyloxy)-benzoic acid (A) and 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one (B) were synthesized. Structures were confirmed by ultraviolet/visible and infra-red spectroscopies. The ionization constant (pKa) values at 8.3 and 8.0 reported for compounds A and B, respectively, indicated that protonation might occur at physiological pH. The LC_{50} values of 145595 µg/ml (A) and 82526 µg/ml (B) obtained from Brine shrimp lethality testing showed that both compounds were non-toxic. The two compounds possessed significant antimicrobial activity against bacterial and fungal strains; *Escherichia coli*, *Staphylococcus aereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiellae pneumoniae*, *Salmonellae typhi*, *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillum notatum* when compared with standards, gentamicin and tioconazole for bacteria and fungi, respectively. *In vitro* antioxidant screening by the DPPH free radical scavenging method and the scavenging effect on hydrogen peroxide showed that the compounds possessed significant antioxidant activity when compared with antioxidant standards ascorbic acid, butylatedhydroxylanisole and α-tocopherol.

Keywords: Mannich bases; 2-(3-Phenylaminopropionyloxy)-benzoic acid; 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one; toxicity; ionization constant; antimicrobial; antioxidant

1. Introduction

Organic synthesis, of which the Mannich reaction is one part, has provided the chemistry community with detailed, reliable and carefully checked procedures for the synthesis of organic compounds. Mannich reactions occur when a compound containing at least one active hydrogen atom (ketones, nitroalkanes, β-ketoesters and β-cyanoacids) condenses with formaldehyde, a primary or secondary amine or ammonia (in the form of its hydrochloride) to give a product known as a Mannich base, which is a β-amino-carbonyl compound (Nicolaou et al., 2007; Abdul Rahiman & Balakrishina, 2008). Spectroscopic methods used to determine structures include nuclear magnetic resonance (NMR), infrared (IR) absorption, ultraviolet/visible absorption and mass spectroscopies. Crystallography has also been employed (Johnstone & Rose 1996; Pavia et al., 2001). Mannich bases have a broad range of medicinal activities as diuretic, antipsychotic, oxytocic, anticonvulsant, anti-filaricidal, centrally acting muscle relaxant, antimalaria, anthelminthic, antiviral, anticancer, antihypertensive, antimicrobial, vaso-relaxing, anti-inflammatory, antioxidants, antiplatelet and antitumor compounds. It is believed that the Mannich base functional group can increase the lipophilicity of parent amines and amides, which results in the enhancement of absorption through bio-membranes. The lipophilicity of Mannich bases also enables them to cross bacterial and fungal membranes. They are also known for their use in the production of polymers, resins, surface active agents, detergents and additives (Chhonke 2009; Manikpuri et al., 2010; Saraswathi et al., 2010; Oloyede et al., 2011; Valarmathi et al., 2011).

The use of chemical additives, for example vitamins and minerals in food, especially those with antioxidant activity, has also recently been encouraged. The aim of this research work therefore is to synthesize Mannich bases 2-(3-Phenylaminopropionyloxy)-benzoic acid and 3-

Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one, determine the purity of the synthesized Mannich bases by analytical procedures, such as melting point determination and thin layer chromatography, elucidate and confirm the structures of the synthesized compounds by using spectroscopic methods, infrared and ultraviolet/visible spectroscopy, 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 will be determined by the Agar well diffusion method, while the antioxidant screening will be assayed using two free radical scavenging methods; scavenging activity on 2,2-diphenyl picryl hydrazyl radical (DPPH) and scavenging effect on hydroxyl radicals generated from hydrogen peroxide. It is hoped that the synthesized compounds can be used as an antioxidant or antimicrobial agent in drugs or as food additives.

2. Materials and methods

2.1. Chemicals and reagents

The following are the major chemicals and reagents used; methanol, chloroform, 2-acetoxybenzoic acid (aspirin), formaldehyde, aniline, glacial acetic acid, diethylamine, dimethylsulfoxide (DMSO), 2,2-dipenyl-1-picrylhydrazyl (DPPH), vitamin C, hydrogen peroxide, α-tocopherol, conc. hydrochloric acid, butanol, tetrahydrofuran, isopropanol, diethylether, ethylacetate, acetonitrile 1,4-dioxan, ammonium thiocyanate, perchloric acid, potassium hydrogen phthalate, sodium tetraborate, ethanol, benzene, hexane, butylated hydroxy anisole (BHA), acetone, acetophenone, ferrous chloride, linoleic acid, dimethylformamide, 2-propanol and ammonia solution. All chemicals and reagents were

BDH analytical grade except 2,2-dipenyl-1-picrylhydrazyl (DPPH), which was purchased from Sigma Aldrich, Germany.

2.2. Equipment/apparatus and general experimental procedure

All measurements were done on a Mettler H18 weighing balance and solvents used were BDH analytical grade. Assessments of the degree of purity of the final products obtained were achieved by determination of melting point using a Gallemkamp melting point apparatus model MFB 595 and also analytical thin layer chromatography. Thin layer chromatography was carried out using silica gel F_{254}, and mobile phase chloroform: methanol (3:1), methanol: ethylacetate (3:1) and benzene: methanol (3:1) for 2-(3-Phenylaminopropionyloxy)-benzoic acid (Compound A) and ethyl acetate: hexane (2:1), ethyl acetate: hexane (3:1) and chloroform: hexane (3:1) for 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one (Compound B), respectively. These compounds were further characterized by spectroscopic analysis, UV-visible and infra-red. The UV/visible spectra of 0.01% w/v of the samples were determined with the aid of a 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 a 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). Ionization constant calculations by pH and pKa determination were also carried out via non-aqueous titration using a pH meter 7020 (Electronic Instrument Ltd London).

2.2. Test organisms

Escherichia coli, *Staphylococcus aereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiellae pneumoniae*, *Salmonellae typhi*, *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillum notatum* (Micro organisms were collected from the stock of the Department 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 ^{o}C. 100 ml aliquots of nutrient broth were inoculated with the culture of test micro-organisms using a loop and then incubated at 37 ^{o}C for 24 hrs.

2.3. 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.

The preparation of 2-(3-phenylaminopropionyloxy)-benzoic acid (A) and 3-phenylamino-1-(2, 4, 6-trimethoxyphenyl)-propan-1-one (B) Mannich bases were carried out based on the procedure used for the synthesis of Mannich bases from substituted benzenes as reported in literature but with minor modifications. The lead compounds in both preparations are medicinally active and non-toxic (Josephsohn et al, 2004; Zhao et al, 2009; Hatano et al, 2010; Muthumani et al, 2010).

2.3.1. Preparation of 2-(3-phenylaminopropionyloxy)-benzoic acid (Compound A)

A mixture of 0.02 mole aspirin, 0.02 mole formaldehyde and 0.02 mole aniline in 40 ml of absolute methanol were refluxed in a basic medium for one hour; the reaction was monitored using thin layer chromatography. The reaction mixture was kept overnight in the refrigerator at – 4 ^{o}C. Crystals obtained were washed with chloroform, filtered under pressure using a suction pump and recrystallized from ethanol.

The equation of the reaction is shown in Scheme 1.

2.3.2. Preparation of 3-phenylamino-1-(2,4,6-trimethoxyphenyl)-propan-1-one (Compound B)

A mixture of 0.01 mole 2, 4, 6-trimethoxyacetophenone, 0.01 mole formaldehyde and 0.01 mole aniline in 20 ml of absolute methanol were refluxed in a basic medium for 10 hours, the reaction was monitored using thin layer chromatography. The reaction mixture was kept overnight in the refrigerator. The crystals obtained were washed with chloroform, filtered under pressure using a suction pump and recrystallized from warm ethanol. The equation of the reaction is shown in Scheme 2.

2.4. Analysis of the synthesized compounds

Thin layer chromatography and melting point determination of the synthesized compounds (A and B) were carried out to assess the degree of purity of the compounds. Spectroscopic analysis (ultra/violet and infra-red spectrometry) of the synthesized compounds were carried out to ascertain the structures. Determination of the ionization constant, toxicity, antimicrobial and antioxidant activities of the synthesized compounds were carried out to investigate their pharmacological importance.

2.5. Thin layer chromatography (TLC)

The solvent systems used were; chloroform: methanol (3:1), methanol: ethylacetate (3:1) and benzene: methanol (3:1) (Compound A) and ethyl acetate: hexane (2:1), ethyl acetates: hexane (3:1) and chloroform: hexane (3:1) (Compound B). Silica gel F_{254} precoated plate (Merck, Germany) was used as adsorbent. Visualisation was aided by the use of iodine vapour and the retardation factor (R_f) was calculated for each of the synthesized compounds.

2.6. Melting point determination

A Gallenkamp melting point apparatus was used to determine the melting point of the dried crystals.

2.7. Spectroscopic analysis

The IR spectra of the synthesized compounds were recorded as KBr discs on a Perkin-Elmer FT - IR spectrophotometer in the range of 4000 – 400 cm^{-1}. The relevant vibrational frequencies are given. The ultra-violet/visible absorption of the two samples was measured with the aid of Spectro UVD-2960 and the absorbance/extinction coefficient was recorded.

2.8. Determination of Ionization constant (pKa) of Compounds A and B via potentiometric titration

2.8.1. General

Drug absorption is determined by the drug's physicochemical properties, formulation and route of administration. Absorption and passage of the drug through cell membranes are influenced by factors such as biological availability, metabolism and excretion of drugs. 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 un-dissociated state either directly or by ion pair or complex formation and that most drugs are weak organic acids and bases, existing in un-ionized and ionized forms in an aqueous environment.

2.8.2. Procedure

The pH was standardised using a standard buffer solution of pH 4 (0.05 M potassium hydrogenphthalate) and pH 9 (0.01 M borax solution). Standardized perchloric acid was used to titrate 2.5 ml of solution of Compounds A and B 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 pKa results represent mean values of three determinations carried out near pH equivalence at 30^{o}C. The results were statistically analysed and the limit of experimental error found to be ±0.001.

2.9. Toxicity analysis

2.9.1. Brine shrimp lethality test

The toxicity level of the Mannich base crystals (Compounds A and B) was conducted according to Falope et al, (1993) and Oloyede et al, (2010). The brine shrimp lethality test (BST) was used to predict the toxicity of the crystals, 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 and 100 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 hours the vials were examined against a lit 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.

2.10. Antimicrobial Screening of Compounds A and B

2.10.1. Preparation of samples for antimicrobial analysis

Each sample A and B was weighed separately (0.25 g) and dissolved in 5 ml of solvent (DMSO) to give 50 mg/ml. Five other test tubes containing 2.5 ml of the same solvent were serially diluted until a concentration of 1.56 mg/ml 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.

2.10.2. Agar diffusion: Pour plate method for bacteria

A culture of each of the following organisms *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiellae pneumonae* and *Salmonellae typhi* was 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 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 and cooling the wire loop (air cooling). The discs were prepared using a Grade No. 1 Whatman filter paper. 100 discs were obtained by punching, putting in vials or 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. The sterile cork-borer was used to create wells (or holes) inside the set plates. The test solutions (50 µl) 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 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 hours 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 millimetres (mm) using a transparent well-calibrated ruler. The positive control for bacteria was gentamicin at a concentration of 5 mg/ml. The

analyses were done in triplicate and the average readings were calculated (Cushine & Lamb, 2005; Duraipindiyan et al, 2006).

2.10.3. Agar diffusion: surface plate method for fungi

Candida albicans, Rhizopus stolonifer, Aspergillus niger and *Penicillum notatum* were used in this study. Molten sterile sabouraud dextrose agar (SDA) was poured aseptically into the sterile plates and allowed to cool for 45 minutes. 0.2 ml of 1: 100 dilutions of the organisms were spread on the surface using a sterile spreader, after which a sterile cork-borer was used to create a hole inside the plates. The same procedure described for antibacterial activity above was followed from this point. The positive control for fungi was 70 % tioconazole. Plates were incubated at 28 ^{o}C for 48 hours. Clear zones of inhibition was observed and recorded using the same method as described in the case of bacteria (Bayer et al, 1986; Hadecek & Greger 2000).

2.11. Antioxidant activities of the synthesized Compounds

2.11.1. DPPH analysis

The antioxidant activity of the synthesized compounds or the capacity to scavenge the "stable" free radical 2, 2 – diphenyl-picrylhydrazyl (DPPH) was determined using the DPPH free-radical scavenging activity (Lugasi et al, 1999). A 3.94 mg of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) was dissolved in 100 ml methanol to give a 100 μM solution. To 3.0 ml of the methanol solution of DPPH was added 0.5 ml of the crystals dissolved in methanol taken from the stock solution. The stock solution was prepared by dissolving 3.0 mg of the crystals in 3 ml methanol. The mixture was shaken vigorously and left to stand for 10 minutes, after which the absorption at 517 nm of the 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 analysed the same way as the stock solution (Gulcin et al, 2002; Mutee et al, 2010). All tests and analyses were carried out in triplicate and the results obtained were averaged. The analysis was carried out for the synthesized compounds with doses ranging from 1.0 mg/ml to 0.0625 mg/ml. The same experiment was carried out using the following antioxidant standards, butylated hydroxyl anisole (BHA), Vitamin C and α- tocopherol.

2.11.2. Hydroxyl radical scavenging effect of Compounds A and B using Hydrogen peroxide

Hydroxyl radicals were generated from hydrogen peroxide (H_2O_2). Excess hydroxyl radicals are known to participate in free radical chain reactions which often lead to oxidative stress. The hydroxyl radical scavenging ability of the synthesized compounds (A and B) was determined according to the method of Oloyede & Farombi (2010) using a UV/visible spectrophotometer. A solution of 2 μM hydrogen peroxide was prepared in phosphate-buffered saline (PBS) at pH 7.4. Each of the compounds (A and B) at the following concentrations (1.0, 0.5, 0.25, 0.0125 and 0.00625 mg/ml) was added to the hydrogen peroxide solution. Decrease in absorbance of hydrogen peroxide at 285 nm was determined, 10 minutes later against a blank solution containing the test drug in PBS without hydrogen peroxide. All tests were run in triplicate.

2.12. 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 hours was determined by probit analysis tested using the Finney computer programme.

3. Results

3.1. Mannich Base 2-(3-Phenylaminopropionyloxy)-benzoic acid (Compound A)

Yellow crystals; yield: 73 % (on dry weight basis); m.pt: 159 - 160 ºC. Soluble in methanol, ethanol, acetone, n-butanol, ethylacetate, hydrochloric acid, glacial acetic acid, 2-propanol, dimethyl sulphoxide, 1, 4-dioxan and dimethylsulfoxide, sparingly soluble in chloroform, tetrahydrofuran, hexane, insoluble in water, acetonitrile, and diethyl ether. R_f 0.52, 0.46 and 0.50 (Silica gel $F_{254,}$ chloroform: methanol (3:1), methanol: ethyl acetate (3:1) and benzene: methanol (3:1). UV nm (EtOH, λ_{max}nm): 210.00 (0.167), 215.00 (9.999), 299.00 (0.068), 484.00 (0.013). IR (KBr) V_{max} cm^{-1}: 3422 (O-Hstr.(Hydrogen bonded)), 3242 (N-H stretch), 3000 (C-H aromatic stretch), 2857 (C-H aliphatic stretch), 1662 (C=O stretch), 1572 (N-H bend), 1296 (C-H aromatic out of plane bend), 1444 (C=C stretch Aromatic). Molecular weight (calc): measured for $C_{16}H_{15}NO_4$, 285 g. pKa= 8.3.

3.2. Mannich Base 3-phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one (Compound B)

Light-yellow crystals; yield: 79.37 % (on dry weight basis); m.pt: 180 - 182 ^{0}C. Soluble in chloroform, dimethyl sulphoxide, tetrahydrofuran, acetone, hexane and 1,4-dioxan, sparingly soluble in methanol, ethanol, n-butanol, ethylacetate and 2-propanol. Insoluble in water, hydrochloric acid, glacial aectic acid, acetonitrile and diethyl ether. R_f 0.50, 0.53 and 0.47 (Silica gel $F_{254,}$ ethyl acetate: hexane (4:1), chloroform: hexane (3:1) and ethyl acetate: hexane (3:1). UV (EtOH, λ_{max}nm): 201.00 (0.000), 229.00 (0.000), 272.00 (0.036), 281.00 (0.049), 330 (0.001), 480 (0.024). IR (KBr) V_{max} cm^{-1}: 3357 (N-H stretch), 3109 (C-H aromatic stretch), 2971, 2857 (C-H aliphatic stretch), 1688 (C=O stretch), 1593 (N-H bend), 1344 (C-N str.Aromatic), 1450 (C-C str.Aromatic). Molecular weight (calc): measured for $C_{18}H_{21}NO_4$ 315g, pKa= 8.0.

3.3. Brine shrimp lethality test

Toxicity to lower organism's living cells was carried out using *Artemia salina* larva. The two synthesized compounds A and B with lethal concentration (LC_{50}) of 145595 and 82526 µg/ml respectively were non-toxic (Table 1).

3.4. Antimicrobial screening of Compounds A and B

The zones of inhibition (mm) obtained when the compounds were screened by the Agar well diffusion method showed that all the tested samples possessed a protective effect against gram positive and gram negative bacteria and fungi which caused them to have broad spectrum activities. The activity was however more pronounced with *Staphylococcus aureus* for compound A and *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* for compound B. Activity was pronounced at 3.125 - 50 mg/ml for the two compounds though lower than the activities of the controls at the same concentration. Little or no activity was observed at 1.5625 mg/ml (Table 2).

3.5. Antioxidant analysis

3.5.1. Free radical scavenging effect on DPPH

A stable free radical 2,2-diphenylpicryl hydrazyl radical (DPPH); which accepts an electron or hydrogen radical to become a stable diamagnetic molecule was used to test for antioxidant activity. The reduction in absorbance of DPPH at 517 nm caused by the samples was measured in triplicate after 10 min. Both tested compounds have moderately high activities as free radical scavengers when compared with controls, ascorbic acid, butylated hydroxylanisole (BHA) and α –tocopherol (Table 3). Compound A had 91.93% inhibition at 0.5 mg/ml while 93.32% inhibition for observed for Compound B at 0.0625 mg/ml. These activities were greater than that of ascorbic acid and α-tocopherol at these concentrations.

3.5.2. Hydroxyl radical scavenging effect of Compounds A and B using H_2O_2

Compounds A and B were screened for hydroxyl radical scavenging activity. Antioxidant agents which can stop or reduce the production of hydroxyl free radicals will terminate free radical chain reactions in biological systems. Highly reactive hydroxyl radicals through the Fenton reaction have been observed to participate in free radical chain reactions, thereby initiating lipid peroxidation and resulting in harmful disorders. Hydroxyl radical scavenging activity using H_2O_2 was measured in triplicate after 10 min of incubation at 285 nm and results showed that absorbance measurements for both compounds decreased (Table 4). Compound A had 53.09 percentage inhibitions at 1.0 mg/ml while Compound B had 94.25 percentage inhibitions at 0.0625 mg/ml. These activities were however greater than that of BHA and α-tocopherol at the same concentration.

4. Discussion

Mannich bases 2-(3-phenylaminopropionyloxy)-benzoic acid and 3-phenylamino-1-(2, 4, 6-trimethoxyphenyl)-propan-1-one were synthesized using the Mannich reaction. The reaction involved an active hydrogen compound, formaldehyde and an amine. The structures of these Mannich bases were confirmed by spectroscopic data obtained from their UV and IR data. IR spectra of Compounds A and B showed peaks at 3241 and 3357 cm^{-1} due to N-H stretch of secondary amines, 3000 and 3109 cm^{-1} (C-H stretch of aromatics), 2857 and 2971 cm^{-1} (C-H stretch of aliphatic), 1662 and 1688 cm^{-1} (C=O stretch), 1572 and 1593 cm^{-1} (N-H bending of secondary amines), 1296 and 1344 cm^{-1} (C-N stretch), 1239 and 1240 cm^{-1} (C-O stretch), 1610 and 1450 cm^{-1} (C=C stretch of aromatics), 886, 756, and 818, 743 cm^{-1} (C-H out-of–plane aromatics) and Compound A in addition showed a broad peak at 3422 cm^{-1} which indicated hydrogen bonded O-H stretch. The characteristic UV bands with λ_{max}: 210, 215 nm for compound A and 201, 229 for compound B were indicative of a π-π* transition (K-band) associated with aromatic and carbonyl compounds. The R - band (n-π* transition) were observed at λ_{max} 299 and 281 nm for compounds A and B, respectively, which were as a

result of a chromophoric group (i.e. carbonyl group). This band occurred due to the excitation of an oxygen lone – pair electron to an anti-bonding π – orbital of the carbonyl group. Therefore, absorption in the near ultraviolet (above 200 nm) is invariably associated with the presence of unsaturated groups or of atoms with unshared pairs of electrons. The various absorptions observed in the visible region showed that the compounds were coloured. Thin layer chromatography (TLC) and melting point determination were also carried out in order to assess the degree of purity of the synthesized compounds. Single spots were obtained from TLC of each of the two compounds and were aided by visualization in iodine vapour. The melting point of compounds A and B were in the range 159 - 160 ^{0}C and 180 - 182 ^{0}C, respectively, which were sharp enough to confirm that the compounds were pure. In order to predict the biological activity of the synthesized compounds, a very important physiochemical property which provides information that can be used to predict biological availability, distribution, metabolism and excretion of drugs by influencing both absorption and passage of the drug through the cell membrane is required. The ionization constant (pKa) is a suitable candidate. The ionization constant (pKa) of each synthesized compounds was obtained from a graph of pH against volume of titrant. It has also been observed that many compounds used for medication are weak acids or bases and knowledge of the pKa values, together with the water – octanol partition coefficient can be used for estimating the extent to which the compound enters the blood stream. Knowledge of the pKa values is also important for the quantitative treatment of systems involving acid – base equilibria in solution. The pKa values of 8.3 and 8.0 were reported for compounds A and B, respectively. At these values, the lone–pair on nitrogen in the compounds may be protonated at physiological pH and then be able to interact with the anionic site (Beckett & Stenlake, 1986, Olaniyi, 1989). The Mannich bases were also screened for their toxicity by the Brine Shrimp lethality test and the result showed that both compounds A and B were non-toxic, having LC_{50} greater than 1000

411 μg/ml. The biological significance of the synthesized compounds was established by
412 screening them against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,*
413 *Pseudomonas aeruginosa, Klebsiellae pneumoniae, Salmonellae typhi, Candida albicans,*
414 *Aspergillus niger, Rhizopus stolonifer* and *Penicillium notatum* and the activities were
415 compared with standard drugs, gentamicin and tioconazole for bacteria and fungi,
416 respectively. The result revealed that both compounds A and B were significantly active
417 against gram positive/negative bacteria and fungi. Compound A showed pronounced activity
418 in inhibiting the growth of *S. aureus* at all concentrations compared to *E. coli, B.subtilis, P.*
419 *aeruginosa, K. pneumoniae* and *S. typhi*. Compound B, on the other hand, showed significant
420 activity in inhibiting the growth of *S. aureus,* and *B. subtilis* compared to *P. aeruginosa, E.*
421 *coli* and *K. pneumoniae*. Many Mannich bases have also been observed to have filaricidal and
422 antimicrobial activities (Pandeya et al, 1999; Oke & Achife, 1999; Bhasin et al, 2005;
423 Saraswathi et al, 2010; Oloyede et al, 2011). The free radical scavenging activity of the
424 synthesized compounds against 2, 2 – diphenyl-1-picrylhydrazyl radical (DPPH) and
425 hydroxyl radicals generated from hydrogen peroxide were determined by a UV Perkin Elmer
426 UV-Vis model Lambda 25 spectrophotometer at 517 and 285 nm respectively. The free
427 radical scavenging activity of the compounds was evaluated based on their ability to
428 scavenge the radicals. A 92 % inhibition was observed for compound A on DPPH radical
429 except for concentrations 1.0 and 0.125 mg/ml which were 90 and 91% respectively. The free
430 radical scavenging activity of the compound was higher than that of the reference standards
431 (ascorbic acid and α - tocopherol). On the other hand, for compound B percentage inhibition
432 of DPPH radical was 93 % at 0.125 and 0.0625 mg/ml and was also higher than that of
433 ascorbic acid and α - tocopherol. The hydrogen peroxide scavenging activity of compound A
434 (% inhibition) showed no significant difference at all concentrations. The activity of the
435 compound was also higher than that of butylated hydroxyl anisole and α- tocopherol.

However, for compound B, it decreased with increasing concentration and showed more inhibitive effect than butylated hydroxyl anisole and α- tocopherol. These results also confirmed the report of antioxidant activities of some synthesized Mannich bases (Shivananda & Shet Prakash, 2011).

5. Conclusion

This research involved a simple approach to the synthesis and determination of toxicity, ionization constant, antimicrobial and antioxidant activities of Mannich bases 2-(3-Phenylaminopropionyloxy)-benzoic acid and 3-Phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one. UV/visible and IR spectrophotometry confirmed the presence of functional groups C=O, O-H, N-H and C-H stretch in their structures. The ionization constant values obtained showed that the compounds will be readily metabolized in cell membranes. A brine shrimp toxicity study showed that they are non-toxic with an LC_{50} greater than 1000 μg/ml. They also possessed significant antioxidant activity when compared to ascorbic acid, butylated hydroxylanisole (BHA) and α-tocopherol. The Mannich bases significantly inhibited gram negative/positive bacteria and fungi growth when compared to standards gentamicin and tioconazole for bacteria and fungi respectively. These compounds can therefore be used as antioxidant food additives or form the basis for the synthesis of pharmacologically important drugs.

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 for the use of spectroscopic equipment. The authors would also like to thank Mr. Festus of the department of Pharmaceutical Microbiology University of Ibadan, Nigeria for assisting in carrying out the antimicrobial analysis.

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Table 1: Brine Shrimp lethality test of Compounds A and B*

CONC.	10000 ppm		1000 ppm		100 ppm		
Sample	Survivor	Dead	Survivor	Dead	Survivor	Dead	LC_{50} µg/ml
A	24	6	28	2	30	0	145594.9
B	23	7	28	2	30	0	82526.0

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

Table 2: Antimicrobial screening of Compounds A and B*

Conc. (mg/ml)	**Zones of inhibition (mm)**																			
	S.a		***E.coli***		***B.sub***		***Ps.a***		***Kleb***		***Sal.***		***A.n.***		***C.a***		***Rh.s***		***Pen.***	
Compound	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	23	30	19	24	19	30	19	18	18	18	19	26	18	18	16	16	17	16	16	14
2	18	24	17	20	16	24	16	14	16	14	16	24	16	16	15	12	15	12	15	12
3	16	18	14	14	14	18	14	12	14	-	13	18	14	12	12	10	12	10	12	10
4	14	14	12	10	12	14	10	-	10	-	10	14	12	10	10	-	10	-	10	-
5	12	10	10	-	10	12	-	-	-	-	-	-	10	-	-	-	-	-	-	-
6	10	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Positive control	38	38	36	36	36	36	36	38	36	36	34	28	26	28	24	26	24	26	26	28

*Integers 1 – 6 represent the concentrations of Compounds A and B at 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/ml respectively. The negative control is Dimethylsulfoxide (DMSO) while the positive control is Gentamicin at 10 mg/ml for bacteria and Tioconazole (70 %) for fungi. "–" represents 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, Rh.s = Rhizopus stolonifer* and *Pen. = Pencillium notatum*

Table 3: Free Radical scavenging activity of Compounds A and B on DPPH (Absorbance at 517 nm) *

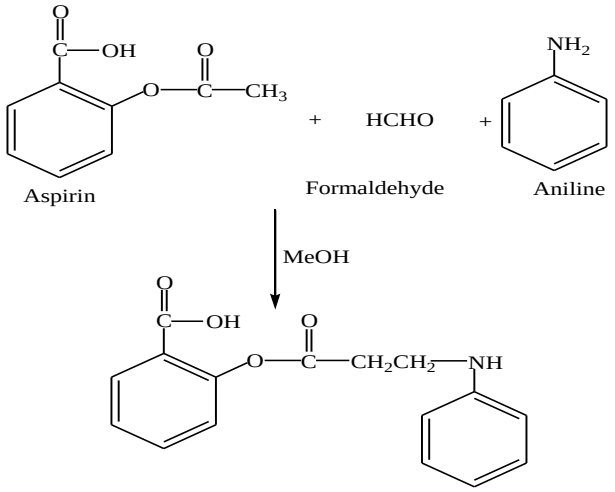
Conc (mg/ml)	Compound A	Compound B	Ascorbic Acid	BHA*	α-Tocopherol
1.0000	0.094±0.003	0.071±0.002	0.0843±0.010	0.0370±0.006	0.6800±0.029
0.5000	0.076±0.012	0.070±0.002	0.2893±0.128	0.0460±0.006	0.7040±0.003
0.2500	0.075±0.013	0.067±0.002	0.2977±0.124	0.0483±0.002	0.7047±0.007
0.1250	0.081±0.007	0.065±0.001	0.3200±0.082	0.0490±0.004	0.7070±0.007
0.0625	0.078±0.001	0.062±0.001	0.5147±0.015	0.0650±0.003	0.7207±0.012

*Absorbance measurement of compounds A and B, Ascorbic Acid, BHA (Butylated hydroxylanisole) and α- Tocopherol at 517nm. Absorbance measurement of DPPH standard is 0.8037 at 517nm and Standard deviation SD for triplicate analysis.

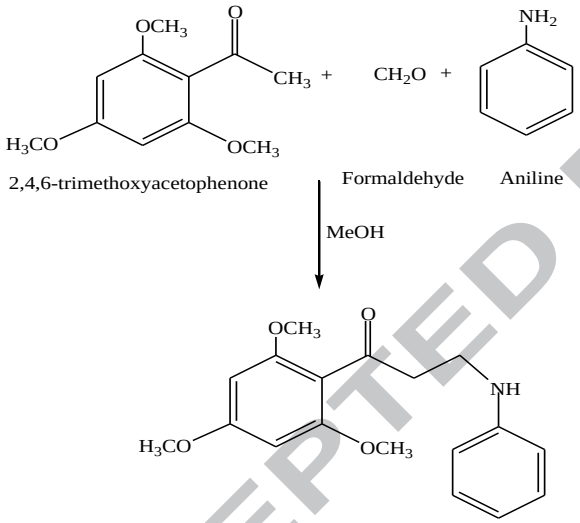
Table 4: Hydroxyl Radical Scavenging activity of Compounds A and B on Hydrogen peroxide (Absorbance at 285 nm) *

Conc (mg/ml)	Compound A	Compound B	Ascorbic Acid	BHA*	α-Tocopherol
1.0000	1.768±0.007	1.655±0.002	0.689±0.002	2.257±0.026	2.951±0.041
0.5000	1.786±0.004	1.430 ±0.004	0.356±0.003	1.975±0.003	2.874±0.064
0.2500	1.774±0.031	0.657 ±0.001	0.138±0.001	1.770±0.017	2.251±0.022
0.1250	1.796±0.005	0.328 ±0.003	0.191±0.001	1.731±0.008	1.781±0.002
0.0625	1.809±0.001	0.217 ±0.002	0.113±0.002	1.699±0.030	0.935±0.002

*Absorbance measurement of Compounds A and B, Ascorbic Acid, BHA and α- Tocopherol at 285nm. Absorbance measurement of Hydrogen peroxide standard is 3.7692 at 285 nm and Standard deviation SD for triplicate analysis.



Scheme 1: Synthesis of 2-(3-phenylaminopropionyloxy)-benzoic acid (Compound A)



Scheme 2: Synthesis of 3-phenylamino-1-(2, 4, 6-trimethoxy-phenyl)-propan-1-one (Compound B)

HIGHLIGHTS

- Mannich bases were non – toxic, LC_{50} values 145594.9 and 82526.0 µg/ml respectively.
- Mannich bases scavenged hydroxyl radical generated from H_2O_2.
- Mannich bases donated proton to 1, 1-Diphenyl-2-Picrylhydrazyl radical.
- Significant activity against bacterial and fungal strains.
- pKa values at 8.3 and 8.0 enable the lone pair of electron on nitrogen to be protonated at physiological pH.