Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(3):18-23

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Characterization and Biological Activities of Fatty Acid Amides Synthesized from Four Underutilized Plant Seed Oils

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ABSTRACT

Four underutilized plant seed oils Thevetia peruviana (TP), Hura crepitans (HC), Sesamum indica (SI) and Mangifera indica (MI) were converted to fatty acid amides by reacting concentrated ammonia with acid chloride. The conversion was monitored and confirmed with sodium fusion test, fourier transform infrared (FTIR) and ^{1}H NMR and ^{13}C NMR (Nuclear Magnetic Resonance). The result of the antifungal test against Fusarium solani, Sclerotium rolfsii, Pyricularia oryzae and Cyrtomium falcatum showed that all amides are potential antifungal agents. In contrary; the results obtained for antibacterial test showed little or no inhibition of these bacterial strains (Staphylococcus aureus, Escherichia coli, Xanthomonas spp., Pseudomonas syringae and Bacillus subtilis) at 15 mg/mL.

Keywords: Underutilized; Amide; Potential; Antifungal; Sodium fusion; Antibacterial

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INTRODUCTION

Fatty amides are significant commercially and they have a wide range of applications, which depends upon the physicochemical properties of the amide or in some instances, of a substance derived from the amide. They are very good raw materials for many industries like textile, plastic, cosmetic, paper etc. Medicinally, they are used as anti-convulsants, anti-hypertensive agents and in the treatment of tuberculosis [5]. It has been established that fatty acyl amides modulate several physiological processes [7]. Fatty acyl amides have been synthesized in different ways but the most common are those involving the reaction between ammonia and amines with fatty acids.The ester-ammonia route is not widely applied because it does not have much practical value; apart from yielding products in trace amounts, the method requires harsh reaction conditions such as high temperatures, long reaction times and the use of catalysts [5]. Various drugs like *Penicillin* and *pyrazineamide* possess their specific activities due to the amide linkage in their structures [2]. The carboxylic acid is first activated by converting into acid chloride and latter reacted with amine.

Acid halides react vigorously with moisture therefore they are immediately converted to more stable and inert organic compounds amides [6]. Thionyl chloride is the most popular reagent of preparation of acid chlorides with many advantages. Olah successfully converted acid to acid chloride by refluxing with thionyl chloride [4].

In this research work, four fatty acid amides were synthesized from four plant seed oils using thionyl chloride as the chlorinating agent and the potential activities of the amides were evaluated using four fungi and bacteria isolates.

MATERIALS AND METHODS

Materials

Thevetia peruviana seed was collected from various locations at High School area in Akure, Ondo State in September, 2012. The good quality seeds were hand-picked to separate them from bad ones, *H. crepitans* seeds were obtained from a tree in front of Faculty of Agriculture, Federal University of Technology, Akure, Ondo State in October, 2012, S. *indica* was obtained from 'Oja Oba' in Akure and *M. indica* was obtained behind

Great Hall, Obakekere, Federal University of Technology, Akure as well. They were sundried, ground into powder with Marlex electroline Emerald blender and preserved in different air tight container for further processing. Thionyl chloride ($SOCl_2$), Chloroform and concentrated ammonia solution and *n*-hexane were procured from Aldrich Chemical Co. (USA).

Methods

Extraction of seed oils:

The powdered seeds (50 g) were placed in a stoppered container with 1 L *n*-hexane and allowed to stand at room temperature for a period of 24 h with frequent agitation. The mixture was filtered, the damp solid material pressed, and returned into the container. The process was repeated for 7 days to ensure complete extraction (batch extraction). The oil was concentrated using rotary evaporator.

Fatty acid composition of the oils:

Fatty acid methyl esters of the oils were prepared by trans-esterification. 50 mg of the fat contents was mixed for 5 min at 95 ^oC with 3.4 mL of the 0.5 M KOH in dry methanol. The mixture was neutralized by using 0.7 M HCl. 3 mL of the 14 % BF_3 in methanol was added. The mixture was heated for 5 min at 90 ^{o} C to achieve complete methylation process. The fatty acid methyl esters were extracted three times from the mixture with redistilled *n*-hexane. The content was concentrated to 1 mL for gas chromatography analysis, 1 µl of this was injected into the injection port of GC which was oven programmed with Model HP 6890 powered with HP Chemstation Rev. A 09.01 [1206] software equipped with flame ionization detector and a capillary column HP INNOWax (30 m x 0.25 mm x 0.25 µm) to obtain individual peaks of fatty acid methyl esters. The inlet and detector temperatures were set at 250 and 320 ^oC, respectively. Nitrogen gas was used and the split ratio was 20:1 with hydrogen pressure at 22 psi. The fatty acid methyl esters peaks were identified by retention times in comparison with calibration curve of the standards analyzed under the same conditions.

Syntheses of the amides:

Fatty acid chloride was first prepared by reacting fatty acid in 100 mL round bottom flask with 5 mL of thionyl chloride ($SOCl_2$); the mixture was refluxed in dichloromethane for 30 min to afford fatty acid chloride (pale yellow). Excess of $SOCl_2$ and solvent were removed by distillation. The pale yellow semi-solid obtained was employed in the next step without further purification. Excess concentrated ammonia solution was then added to the fatty acid chloride. The reaction product was dissolved in 300 mL petroleum ether (*n*-hexane), poured in a separatory funnel to isolate the lower layer, a mixture of excess ammonia, and ammonia chloride, from the fatty amide. The amide, obtained from the hexane layer by crystallization at 10 ^oC, was recrystallized from acetone [6].

$$RCOOH \xrightarrow[CHCl_3\ Reflux]{SOCl_2} RCOCl + SO_2 + HCl$$

$$RCOCl + NH_3 \xrightarrow[Continuous\ stirring]{} RCONH_2 + HCl$$

Scheme 2: Synthesis of amide using thionyl chloride

Spectroscopic analysis:

Fourier Transform Infrared (FTIR): The FTIR spectra were obtained with a Perkin-Elmer grating spectrophotometer at a resolution of 4 cm^{-1} between wave numbers 4000-400 cm^{-1}, using potassium bromide disc method.

Nuclear Magnetic Resonance (NMR) Spectroscopy: ^{1}HNMR and ^{13}CNMR spectra of these samples were obtained with Agilent-NMR- vnmrs 400 with pulse sequence: Proton (s2pul), Temp. 26.0 C/299.1 K, Relax. Delay 1.000 sec, Pulse 45.0 degrees, Acq. Time 2.556 sec and Width 6410.3 Hz spectrometer using deuterated chloroform ($CDCl_3$) as the solvent.

Determination of fungicidal activities:

The fungi of choice used for this experiment are *Fusarium solani, Sclerotium rolfsii, Rhizoctonia solani, Pyricularia oryzae, Drechslera oryzae*. 0.015 g of the sample was dissolved in 1 mL of 5 % DMSO. 5 mL of each concentration was aseptically mixed with 15 mL of sterile molten potato Dextrose Agar (P.D.A). They were allowed to cool to 45 ^oC before pour plated and allowed to solidify at ambient temperature. The pour plates were inoculated aseptically with a 5 mm fungal disc. For the negative control test, 5 mL of 5 % DMSO was used while Bentlate, (a standard antifungal agent) was used as a positive control at 15 mg/mL. All the plates were incubated at 27 ^oC for 7 days and the percentage mycelial inhibition was calculated by the following formula (Albuquerque *et al.* 2006)[].

$$\text{percentage of mycelial inhibition } = \frac{\text{dc} - \text{dt}}{\text{dc}} \times 100$$

Where, dc= average diameter of fungal colony in control sets, and dt= average diameter of fungal colony in treated sets.

Determination of antibacterial activities:

The compound was subjected to antimicrobial assay using the agar-well diffusion method of Murray *et al.* (2004) modified by Olurinola (2004). Nutrient agar (20 mL) was dispensed into sterile universal bottles according to [10]. Then, the nutrient was inoculated with 0.2 mL of cultures, mixed gently and poured into sterile petri dishes. After setting a number 3-cup borer (6 mm diameter) was properly sterilized by flaming and used to make five uniform cups/wells in each petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 0.5 mg/mL of the compound and allowed to diffuse for 45 min. The plates were incubated at 37 ^{o}C for 24 h. The zones of inhibition were measured with antibiotic zone scale in millimetre (mm) and the experiment was carried out in triplicate.

RESULT AND DISCUSSION

Syntheses of amides

These have been synthesized following the method reported under methodology and the percentage yield for each product is presented in table 1. M.*indica* L. has the highest yield (95%), followed by T. *peruviana* (90%), H.*crepitans* and S.*indica* L. have (60%) each. These results showed that the formation of fatty acid amide using thionyl chloride as the chlorinating agent is a promising route of synthesizing amide.

Table 1: Percentage yield of the amides

S/N	Samples	% yield
1	*Thevetia peruviana*	90
2	*Hura crepitans*	60
3	*Sesame indica*	60
4	*Mangifera indica*	95

Characterization

Both asymmetric and symmetric bonds of the four amides appeared between 2929 and 2851 cm^{-1} which falls within the characteristic frequencies of amide. The NH stretches of the amides appeared at 3436, 3428, 3437 and 3431 cm^{-1} respectively. The carbonyl (C=O) stretches appeared at 1637 cm^{-1} except that of HC amide which appeared at 1718 cm^{-1}. The shift of carbonyl absorption frequencies from 1700 to 1637 cm^{-1} was an indication that the fatty acids were converted into amides because amides show a very strong C=O peak between 1680 and 1630 cm^{-1}.Table (2)

Table 2: Interpretation of IR spectra of fatty acid amides

Samples	Amide C=O stretch (1690-1630) S	Amide NH stretch (3700-3500) M	Carboxylic acid for OH stretch (3000-2500) and C-H bond
TP amide	1637.63	3436	
HC amide	1718	3428.57	2868.57
			2929
SI amide	1637.62	3437	2851.64
			2928.57
MI amide	1637.77	3431	2857.14
			2928.57

Legend: TP is *Thevetia peruviana*, HC is *Hura crepitans*, SI is *Sesame indica*, MI is *Mangifera indica.*

Table 3 summarizes the chemical shifts of ^{1}H NMR spectra of fatty acid and fatty acid amide. The peaks at δ 0.81 and 0.80, 1.24 and 1.18, 1.55 and 1.52, 2.27 and 2.23 ppm were attributable to the terminal methyl protons, protons of the repeating methylene units, and protons of the methylene group β and α to the carbonyl groups of the fatty acid and amide respectively [8,9]. The slight changes in the absorption frequencies of fatty acid as explained above and that of fatty acid amide were as a result of change in the functional group. The signals at δ 1.99 and 1.71 ppm were assigned to allylic methylene protons while the peaks at δ 4.26 and 4.21 ppm were considered to be from the olefinic protons, confirming that the olefinic functional was not affected but the reaction only took place at carboxylic acid functional group (COOH). The functional group protons of amide ($CONH_2$) appeared at 6.465 ppm as against 5.31 ppm in fatty acid (COOH) spectrum. Figure (2). The shift even though small is indicative of the amide production. Figure 1, shows the proton absorption peaks of oleic amide in different chemical environments.

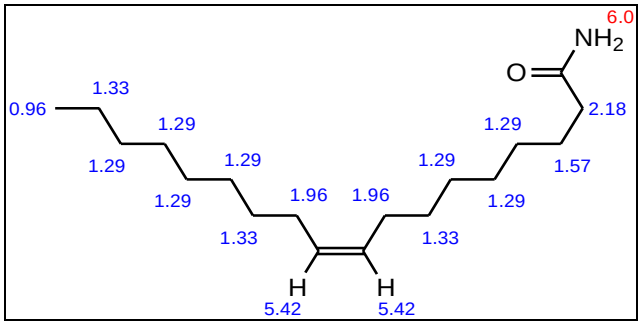

Figure 1: ^{1}H NMR Chemical shift

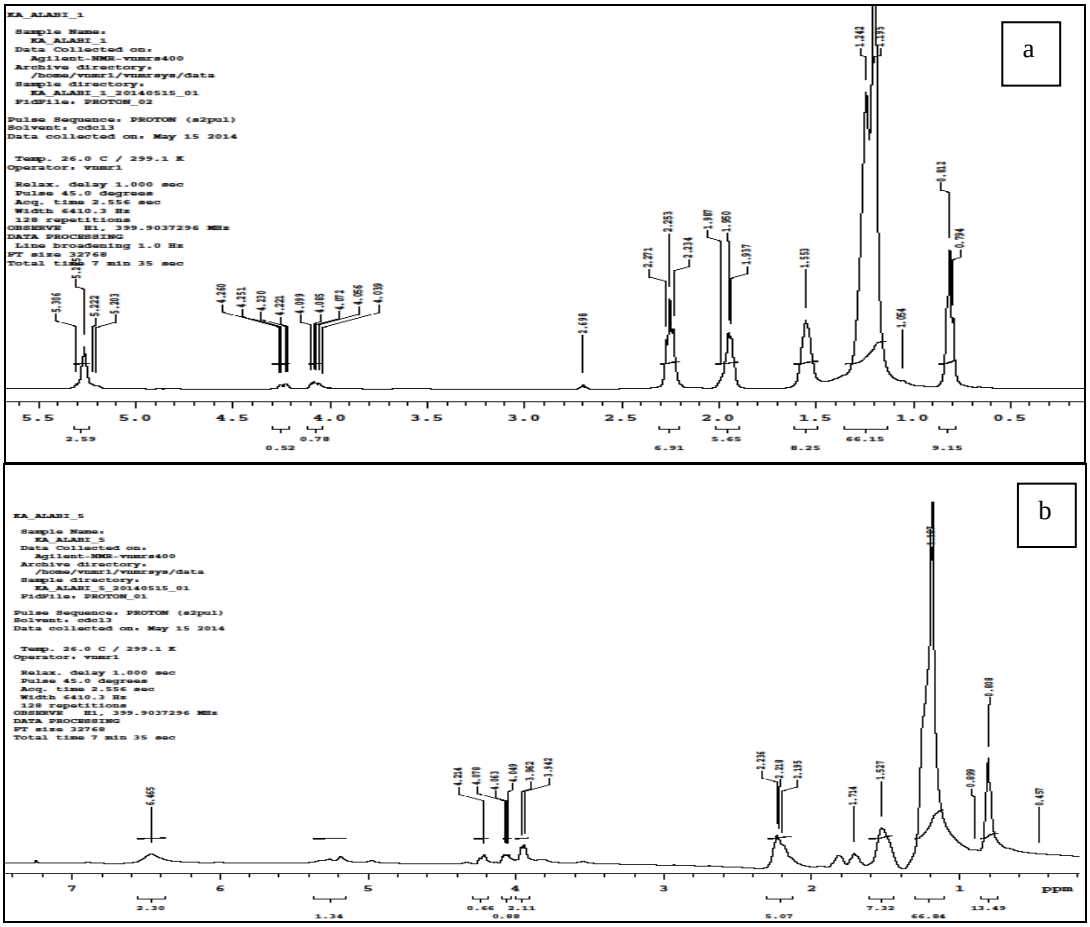

Figure 2: ^{1}H NMR Spectra of fatty acid (a) and fatty acid amide (b)

Table 3: Interpretation of ^{1}H NMR spectra of fatty acid amides

s/n	^{1}H NMR chemical shifts	Fatty acid	Amide
1	Terminal methyl proton	0.81	0.8
2	Repeating methylene units	1.24	1.18
3	β methylene proton	1.55	1.52
4	Allylic proton	1.99	1.71
5	α-Methylene proton	2.27	2.23
6	Functional group proton	5.31	6.46
7	Olefinic proton	4.26	4.21

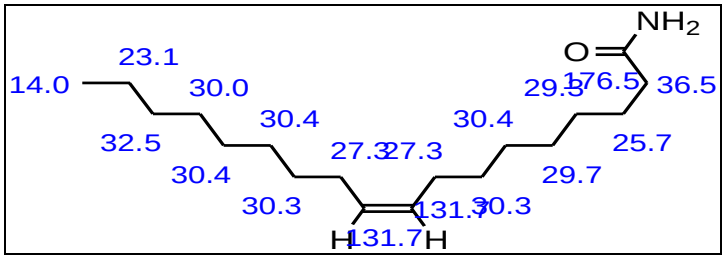

Figure 3: ^{13}C NMR chemical shift

Table 4 summarizes the ^{13}C NMR spectra of the fatty acid and fatty acid amides as shown in figure 4a and 4b respectively. The signals between δ 13-33 and 13-34 ppm represent the carbon of repeating methylene groups respectively, the signals between δ 61 and 77 ppm are characteristic absorption regions for C-O group. The signal at δ 129 and 130 ppm indicate the presence of olefinic carbon (C=C) as evident in the proton NMR spectrum above; and the spectra showed that the absorption peaks of carbonyl group in fatty acid was de-shielded down the field than that of amide which appeared at δ 179 ppm while amide appeared at δ 173 ppm. This shift was as a result of change in chemical environment of the carbonyl carbon of the two compounds.

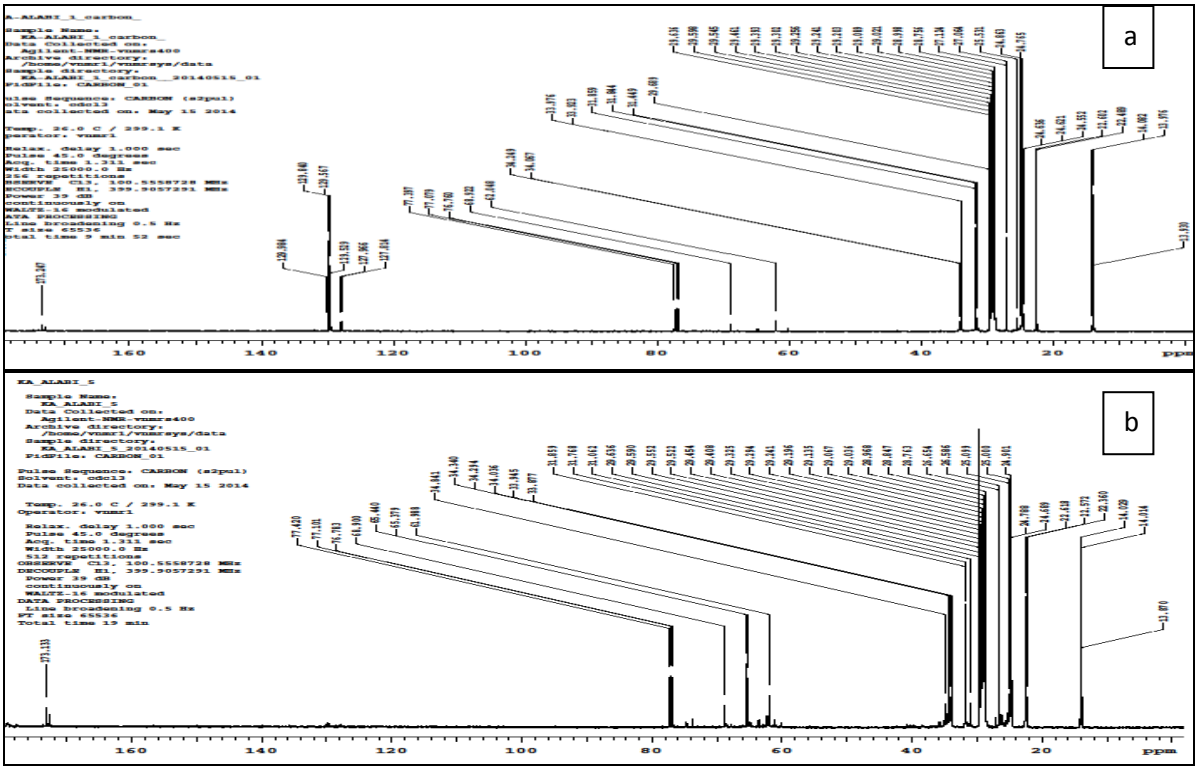

Figure 4: ^{13}C NMR spectra of fatty acid (a) and fatty acid amide (b)

Table 4: Interpretation of ^{13}C NMR spectra of fatty acid amides

s/n	^{13}CNMR chemical shifts	Fatty acid	Amide
1	C-C	13-33	13-34
2	C-O	62-77	61-77
3	C=C (vinyl) group	129	130
4	C=O (carbonyl)group	179	173

The results of antifungal activities of synthetic amide are presented in table (5). All the samples inhibited the isolates at varied degrees (*Fusarium solani*, *Sclerotium rolfsii*, *Pyricularia oryzae* and *Cyrtomium falcatum*). MI amide was the best for *Fusarium solani*, with 58.82 % inhibition followed by SI amide, HC amide and TP amide. For *Sclerotium rolfsii*, HC amide inhibited it at 67.14 %, followed by MI amide, TP and SI amides (51 %). HC amide inhibited *Pyricularia oryzae* at (82 %), followed by MI amide (62 %), TP and SI amides at (52 %). *Cyrtomium falcatum* was inhibited by MI amide at 54 % followed by SI amide (45 %), HC amide (34 %) and TP amide (22 %). Generally, if compared with benlate (standard) which percentage inhibition ranged from 86 to 100 % against all the isolates; the amides could be considered as potential fungitoxic agents.

Table 5: Anti-fungi result

S No	Isolates (%)	TP Amide	HC Amide	SI Amide	MI Amide	Benlate
1	*Fusarium solani*	26.68±0.05	32.86±0.04	48.75±0.05	58.82±0.04	86±0.02
2	*Sclerotium rolfsii*	51.68±0.02	67.14±0.05	51.73±0.04	56.86±0.06	90±0.03
3	*Pyricularia oryzae*	52.24±0.09	82.14±0.04	52.16±0.07	62.75±0.04	100
4	*Cyrtomium falcatum*	22.37±0.08	34.28±0.05	45.14±0.04	54.90±0.04	89±0.04

Mean of three values with their standard deviations.
Legend: TP is *Thevetia peruviana*, HC is *Hura crepitans*, SI is *Sesamum indica*, MI is *Mangifera indica*

Antibateria properties of the samples

All the samples are not good inhibitors of the bacteria isolates used (S. *aureus*, E. *coli*, *Xanthonmouns* sp., P. *syringae* and B. *subtilis*) at 15 mg/mL compared with the standards at the same concentration. The solvent used (5 % DMSO) did not inhibit at all while the positive control showed inhibition at various degrees ranging from 0 to 26 mm. Therefore at this concentration the samples could not inhibit the bacterial used.

Table 6: Antibacterial properties of the samples at 24 hr of incubation (37 ºC)

Samples	S. *aureus* (mm)	E.*coli (mm)*	*Xanthonmouns* sp.(mm)	P. *syringine (mm)*	B.*subtilis (mm)*
TP amide	1.5	-	3	-	3.5
HC amide	3.5	3	3	-	-
SI amide	2	-	-	-	1.2
MI amide	1.5	-	4	2.5	1.5
5% DMSO	-	-	-	-	-
A. Ampicilin	13	7	9	8	11
B. ciprotab	26	18	21	15	23
C. streptomu	15	12	11.5	12	13
D. tetra	-	9	14	11.5	17
E. ethanol	3	-	8	4	8

Legend: *Staphylococcus aureus*, *Escherichia coli*, *Xanthonmouns* species, *Pseudomonas syringae*, *Bacillus subtilis*, TP is *Thevetia peruviana*, HC is *Hura crepitans*, SI is *Sesamum indica*, MI is *Mangifera indica*

CONCLUSION

Thionyl chloride converted the fatty acids to acyl chloride which was later converted to fatty acid amides. The formation of the products was confirmed with instrumental analyses (IR, ^{1}H NMR and ^{13}C NMR). The shift in the absorption peaks of fatty acids and the amide confirm the modification. The products are potential fungicides but the results obtained for the antibacterial assay indicated that they are not active against bacteria.

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