ABSTRACT

In Nigerian traditional medicine, the combination of A. comosus and C. senensis peels has antibacterial effect against typhoid fever and diarrhoea due to Aeromonas hydrophila. A. comosus and C. senensis peels were extracted using percolation method and ethanol solvent. The antibacterial potential of ethanolic extract of A. comosus and C. senensis peels were investigated by disc diffusion and broth dilution techniques. The extracts were subjected to phytochemical screening using standard procedures. This study was aimed at verifying the synergistic effects of the two plant extracts against some clinical isolates: six Salmonella paratyphi B, one S. typhi and three A. hydrophila. The antibacterial activity of the combined extracts was 7-12mm and from 15-42mm for the standard antibiotic disc. The minimum inhibitory concentration (MIC) of the combined extracts was 0.25-12.50mg/ml while the minimum bactericidal concentration (MBC) was 42mm for the standard antibiotic disc. The minimum inhibitory concentration (MIC) of the metabolites like alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids and phytosterols in 0.50-50.00mg/ml. Phytochemical investigation of the extracts revealed the presence secondary. Phytochemical investigation of the extracts revealed the presence secondary.
The aqueous extract was active against *Ascaris lumbricoides* and some microscopic worms (Asenjo, 1940). The ethanolic extract of leaves of *A. comosus* has antifilarial and tanticidal activity (Suresh and Rai, 1990, Feroz, *et al.*, 1990). *Citrus senensis* Peel: is derived from the fruit of *Citrus sinensis* the plant is called sweet orange (English), and is locally called Lemon – zaki (Hausa). Though most people peel the *Citrus senensis* and eat only the fruit, but the peel is used medicinally. *Citrus senensis* peel contains calcium, phosphorus, potassium, ascorbic acid, and vitamin A, as well as volatile oil and hesperidin. In Africa, *Citrus senensis* peel is used to treat colic, and in India, Citrus senensis peel is used to treat upset stomach (Bensalem, 2006). The British pharmacopoeia list *Citrus senensis* peel as an aromatic for use as an aroma and flavor enhancer. The bioflavonoid constituents of this herb are reported to reduce the permeability of blood vessels, especially capillaries, so that extracts from *Citrus senensis* peel are also included in remedies for phlebitis. New studies on a monoterpene found in *C. senensis* peel called “Limonene” show that it very effectively prevents individuals from developing abnormal growths on their skin (Bensalem, 2006).

Limonene also has demonstrated prevention efficacy in preclinical models of breast research, which shows that the herb may help reduce the occurrence of squamous cell skin cancer. The *C. senensis* peel is also used as diuretic, perspiratory, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. Also the peels are used in Ayurvedic medicine to tonify liver, strengthening of blood vessels, help in relieving symptoms and discomfort of varicosa, peripheral circulatory system function. It increased circulation to the extremities. Used to treat and prevent vitamin deficiencies, colds, flu, and scurvy (Monterey, 2005). The high citric acid content in *C. senensis* peel has powerful health benefits in treating heavy-metal poisoning in people and helping fight viral and bacterial infections (Fatope *et al.*, 1993). In spite of all the reported medicinal values of these two plants, attempt to study their synergistic antibacterial potentials especially with regard to ethanol extract was rare. The present study is aimed at establishing the in vitro synergistic potency of extracts of *A. comosus* and *Citrus senensis* peels for the treatment of typhoid fever and diarrhoea due to *Aeromonas hydrophila*.

**MATERIALS AND METHODS**

**Collection and Identification of Plant Materials**

*Annona comosus* and *Citrus senensis* fruits were obtained from Yankaba Market in May, 2010, and the peels were obtained by removing the pericarp. Its botanical identity was further confirmed and authenticated and voucher specimens (92, 16) were deposited at the Herbarium section of the Botany unit of the former Dept. of Biological Sciences, Bayero University, Kano, Nigeria, for future reference.

**Preparation of the Treatment Samples**

The peels were air-dried and ground to powder using mortar and pestle (Fatope *et al.*, 1993). The powdered form were then stored in air-dried containers in the laboratory until required for further analysis.

**Extraction Protocols**

The fine powder of the peels (100g) was weighed and percolated seperately with 1000ml of 95% ethanol (Fatope *et al.*, 1993). It was allowed to stand for two weeks with shaking at regular intervals under room temperature. The percolates were then filtered and solvent (ethanol) evaporated to obtain the ethanolic extract of the peels. The extracts were then kept in a sterile bottle under refrigerated condition until required for further analysis.

**PHYTOCHEMICAL SCREENING OF THE PLANT EXTRACT**

The extract of both peels *A. comosus* and *C. senensis* were analysed separately for the presence of alkaloids, flavonoids, saponins, tannins, steroids, glycosides, triterpenoids, phytosterols and amino acids as follows:  

(a) **Test for Alkaloids**

A quantity (5 cm³) of the extract was added to two cm³ of HCl. To this acidic medium, one cm³ of Dragendorff’s reagent was added. An orange or red precipitate /turbidity produce immediately indicated the presence of alkaloids (Harbone, 1998 and Kokate, 2001).

(b) **Test for Flavonoids**

To 3cm³ of the extract was added 1cm³ of NaoH, a yellow colouration indicated a positive test for flavonoids (Odebiyi and Ramstad, 1978; Waterman, 1993).

(c) **Test for Saponins (Frothing Test)**

A quantity (2 cm³) of the extract was placed in a test tube and then 2cm³ of distilled water was added. The tube was then shaken vigorously. A persistent froth that lasted for at least 15-minutes indicated a positive test for saponins (Odebiyi and Ramstad, 1978; Waterman, 1993).

(d) **Test for Tannins**

1. Two drops of 5% fecl₃ was added to 1cm³ of the extracts. A green precipitate indicated a positive test for the presence of tannins (Odebiyi and Ramstad, 1978; Waterman, 1993).

2. To 5 cm³ of the extract, a few drops of 1% lead acetate were added. Formation of a yellow precipitate indicated the presence of tannins (Harbone, 1998 and Kokate, 2001).

(e) **Salkowski’s Test for Steroids**

To 1cm³ of the extract 5-drops of conc. H₂SO₄ was added. A yellow colouration indicated a positive test for steroids (Odebiyi and Ramstad, 1978; Waterman, 1993).

(f) **Fehling’s Test for Glycosides**

A quantity (10 cm³) of 50% H₂SO₄ was added to 1 cm³ of the extract in a test tube. The mixture was heated in a boiling water bath for 15 minute. A quantity (10 cm³) of fehling’s solution was added and the mixture was boiled. Formation of brick red precipitate indicated a positive test for glycosides (Odebiyi and Ramstad, 1978; Waterman, 1993).
The test organisms were isolated from stools samples. Appropriate quantities of the two extracts to arrive at the stock solution of the plant extract was prepared in chloroform; 1 ml of acetic anhydride was added following the addition of conc. H₂SO₄. Formation of reddish violet colour indicated the presence of triterpenoids (Harborne, 1998 and Kokate, 2001).

**Test for Phytotherapeutics**

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether was evaporated and the residue was tested for the presence of phytotherol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added following few drops of conc. H₂SO₄. Appearance of bluish green colour showed the presence of phytotherol (Harborne, 1998 and Kokate, 2001).

**Preparation of Amino Acids**

A quantity (1 ml) of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour shows the presence of amino acids (Harborne, 1998 and Kokate, 2001).

**Preparation of Sensitivity Disc**

Whatman No. 1 filter paper was punched using puncher to obtain disc of 6.0 mm in diameter. These were placed in a sterile screw-capped Bijou bottles and sterilized in an oven using a dry heat at 140°C for 1 hour. The discs were allowed to cool; twenty five discs were dispensed into each solution with defined concentration by means of sterile forceps. Standard antibiotic (Oxoid, UK) discs were used as positive control.

**Preparation of Extract Concentrations**

The stock solution of the plant extract was prepared in screw capped Bijou bottles containing 1 ml Dimethyl sulfoxide (DMSO). One gram of each fraction was weighed on a Metler balance (Model: Scout pro Spu 401, S/N: 7129110037) and dissolved in 1 ml of DMSO to arrive at 100000 µg/ml (10⁵ µg/ml) concentration of stock solution. Twelve varying extract concentrations (1000 µg/ml - 4000 µg/ml, 10,000 µg/ml - 40,000 µg/ml, 100,000 µg/ml - 400,000 µg/ml), were prepared from the stock solution (100000 µg/ml) using 10-fold serial dilution. One ml of each concentration was poured into Bijou bottle containing 25 discs

**Preparation of Combine Ratios of the Extracts**

Appropriate quantities of the two extracts to arrive at ratio 1:1 were prepared.

**Test Culture**

The test organisms were isolated from stools samples of patients presenting with diarrhoea attending Aminu Kano teaching Hospital (AKTH) Kano and Murtala Mohammad Specialist Hospital Kano, using standard methods as described by Cheesbrough, 2002. They included six *Salmonella paratyphi* B, one *Salmonella typhi* and three *Aeromonas hydrophila*. The isolates were maintained in a freshly prepared nutrient agar slant and kept in a refrigerator at 4°C until required for use.

**Standardization of inoculum**

Using inoculating loop, enough material from an overnight culture of the test organisms were transferred into a tube containing 2.0 ml normal saline, until the turbidity of the suspension matched the turbidity of 0.5 standard (Cheesbrough, 2000).

**Bioassay**

Agar diffusion method (Khan and Saeed, 2000) was employed. The freshly prepared nutrient agar plates were dried in a dryer for 15-minutes to remove surface moisture. The plates were aseptically inoculated uniformly with test organism by streaking method. With the aid of a sterile forceps, impregnated paper discs containing the peel extract of *A. comosus* and *C. senensis* at varying concentrations were arranged radially and pressed firmly onto the inoculated agar surface. Each disc was sufficiently spaced out and kept at least 15 mm from the edge of the plate and 25 mm from disc to disc to prevent overlapping of zones and incubated at 37°C for 24 hours. The zone diameters of the semi-confluent growths were measured with the aid of a meter rule to the nearest millimetre.

**Determination of Minimum Inhibitory and Bactericidal Concentrations of the Extracts**

The MIC and MBC were determined (Ochei and Kolhatkar, 2008). The following concentrations were prepared; (1000 µg/ml - 4000 µg/ml, 10,000 µg/ml - 40,000 µg/ml, 100,000 - 400,000 µg/ml) respectively. From the working inoculum 0.1 ml was inoculated into fresh nutrient broth tubes at different extract concentrations. The tubes were incubated at 37 ±1°C for 18 – 24 hrs. The lowest concentration of the extract that inhibited the growth of the test bacterium was noted and recorded as the MIC while the MBC was determined by sub-culturing the lowest concentration of the agent which shows no visible signs of growth in the MIC tube dilution test to fresh antibiotic free nutrient agar (oxoid). The plates were incubated at 37 ±1°C for 18 – 24 hrs after which they were observed for growth or otherwise of the test organism.

**Determination of Fractional Inhibitory Concentration of Plant Extracts**

The synergistic effect of the combined plant extracts was determined using fractional inhibitory concentration (FIC), which is an interaction coefficient indicating whether the combined effect of the plant extracts are: - synergistic (when FIC is < 0.5), additive (when FIC is = 1) and antagonistic (when FIC is > 4) (Amsterdam, 1989; Edberg, 1988).
**Statistical Analysis**

Statistical Package for Social Sciences (SPSS), statistical software for Windows (Version 12.0; Standard Licensed Incorporated, 2003 and Microsoft office excel 2007 were used for calculation of Mean, Standard deviation, Standard error of mean and analysis of variance was conducted to find whether there was variation in the activity of the extracts singly and in combination ($P = 0.05$).

**RESULTS**

A total yield of 19.80g and 31.00g of the ethanolic extracts from the original weight of 100g were recovered from the peel of *A. comosus* and *C. senensis* respectively. The physical characteristics were indicated in the Table 1. Table 2 shows the phytochemical composition of the plant parts screen. Only steroids, glycosides and amino acids were absent in *A. comosus* peel but in *C. senensis* peel only glycosides and amino acids were absent. The antibacterial susceptibility pattern of the extract was shown in Table 3-5.

**Table 1: Physical characteristics of the peel extract of Annona comosus and C. senensis**

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Solvent (ethanol)</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Colour</th>
<th>Odour</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peel</td>
<td><em>A. comosus</em></td>
<td>100.00</td>
<td>19.80</td>
<td>Orange</td>
<td>Fruity</td>
<td>Oily</td>
</tr>
<tr>
<td>Peel</td>
<td><em>C. senensis</em></td>
<td>100.00</td>
<td>31.00</td>
<td>Orange</td>
<td>Fruity</td>
<td>Oily</td>
</tr>
</tbody>
</table>

**Table 2: Phytochemical characteristics of the peel extracts of Annona comosus and C. senensis**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th><em>A. comosus</em></th>
<th><em>C. senensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key:** + = present; - = absent.

**Table 3: Antibacterial activity of combined extracts of Annona comosus peel and Citrus senensis peel at 1:1 ratio against the isolates.**

<table>
<thead>
<tr>
<th>Test Bacteria Zone</th>
<th>Average zone of inhibition (in mm)/Disc Potency in µg/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella B$_7$</td>
<td>07 09 07 11 11 10 10 00 08 00 09 10 09 10 10 10 10 08 07 CPX 38</td>
</tr>
<tr>
<td>Salmonella B$_7$</td>
<td>10 10 11 10 00 00 00 00 00 00 00 00 00 00 00 00 00 00 SXT 35</td>
</tr>
<tr>
<td>Salmonella B$_8$</td>
<td>00 10 00 08 00 09 00 10 09 10 10 10 10 10 10 10 10 CPX 24</td>
</tr>
<tr>
<td>Salmonella B$_8$</td>
<td>10 09 10 09 09 08 11 12 09 08 10 10 10 10 10 10 10 10 10 SXT 42</td>
</tr>
<tr>
<td>Salmonella B$_8$</td>
<td>00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 SXT 15</td>
</tr>
<tr>
<td>Salmonella typhi$_2$</td>
<td>12 12 12 12 12 12 12 12 11 10 10 10 10 10 10 10 10 10 10 OFX 42</td>
</tr>
<tr>
<td>Aeromonas hydrophila$_1$</td>
<td>07 07 07 07 07 07 07 07 07 07 00 00 00 00 00 00 00 00 00 APX 15</td>
</tr>
<tr>
<td>Aeromonas hydrophila$_2$</td>
<td>00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 APX 15</td>
</tr>
<tr>
<td>Aeromonas hydrophila$_3$</td>
<td>07 07 07 07 07 00 00 00 00 00 08 00 08 08 08 08 08 APX 15</td>
</tr>
<tr>
<td>Aeromonas hydrophila$_4$</td>
<td>00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 00 APX 15</td>
</tr>
</tbody>
</table>

**Key:** CN = Gentamycin 10µg. PEF = Peflacine 10µg. CPX = Ciprofloxacin 10µg. PN= Ampicillin 30µg.S= Streptomycin 30µg.AMP = Ampicillin 10µg. OFX = Tarivid 10µg. SXT= Septrin 30µg. PEX=10µg. APX=Amphicloxacin10µg.

**Statistical Relationship**

<table>
<thead>
<tr>
<th></th>
<th>F-value</th>
<th>F-critical</th>
<th>df</th>
<th>Probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. comosus</em></td>
<td>10.13967</td>
<td>1.553208</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td><em>C. senensis</em></td>
<td>8.880079</td>
<td>1.553208</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td><em>A+C</em></td>
<td>14.14505</td>
<td>1.553208</td>
<td>1</td>
<td>5%</td>
</tr>
</tbody>
</table>

**Key:** A = *Annona comosus*, C = Citrus, df = degree of freedom.
The results of the study show that the ethanolic extract of A. hydrophila was demonstrated at a concentration of 100µg/disc (10,000µg/ml) with zone of inhibition that was resistant at all the concentration used. Moreover, anti-bacterial activity was prominent against A. hydrophila, which was seen to be more sensitive to the combination at a concentration of 100µg/disc (10,000µg/ml) with zone of inhibition of 12mm, in comparison with standard antibiotic Tarivid 10µg which shows a zone of 40mm.

### DISCUSSIONS

The results of the study show that the ethanolic extract of both peels of the plants demonstrated antibacterial activity on all the tested organisms, except a species of A. comosus that was resistant at all the concentration used. Moreover, anti-bacterial activity was prominent against S. paratyphi B, which was seen to be more sensitive to the combination at a concentration of 100µg/disc (10,000µg/ml) with zone of inhibition of 12mm, in comparison with standard antibiotic Tarivid 10µg which shows a zone of 40mm. The lowest MIC was demonstrated at a concentration of 250µg/ml and the highest MIC was seen at a concentration of 12,500µg/ml. The lowest MBC was seen at a concentration of 50,000µg/ml. The latest MIC was seen at a concentration of 50,000µg/ml. The observed bioactivity of the combination of this extracts is probably due to the presence of bioactive compounds like alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids and phytosterols. The FIC demonstrated that the combination was found to be additive and antagonistic. The combined ethanolic extract of D. viscosa leaf and A. comosus peel was found to be synergetic on two A. hydrophila strains (Lawal et al., 2011), but in this study the combination of A. comosus and C. senensis peel was found to be additive and antagonistic on the tested organism respectively. The result of this finding shows a clear relationship that existed in the utilization of the concoction of the plants used in the traditional medicine and as observed in the bioactivity, MIC, MBC and FIC of the extracts.

### CONCLUSION AND RECOMMENDATIONS

Statistical analysis showed that, the potency of the extracts was not greater when the two plants extracts were combined at 1:1 and P = 0.05. Their activities singly was statistically different from each other, for A. comosus peel extract the F-value (10.13), F-crit value (1.55) at one degree of freedom and at 5% probability level were found to be different from each other. Statistical analysis showed that, the potency of the extracts was not greater when the two plants extracts were combined at 1:1 and P = 0.05. Their activities singly was statistically different from each other, for A. comosus peel extract the F-value (10.13), F-crit value (1.55) at one degree of freedom and at 5% probability level were found to be different from each other.
The ability of these extracts to inhibit the growth of the test organisms indicated the presence of chemical constituents of pharmacological importance. Therefore the understanding of synergistic mechanism is fundamental to development of pharmacological agents to treat diseases by *Salmonella sp* and *A. hydrophila* using medicinal plant.

**REFERENCES**


