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A STUDY OF THE PHYTOCHEMICAL PROPERTIES AND SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *Annona comosus* (LINN) MERR. PEEL *AND Citrus senensis* PEEL EXTRACTS ON *Aeromonas hydrophila* AND *Salmonella species*.

***[1] Lawal, D., [2]Yunusa, I., and [3]Bala, I.**
[1]Department of Biological Sciences, Federal University Dutse, P.M.B 7156, Dutse, Jigawa State-Nigeria.
[2]Department of Biochemistry, Kano University of Science and Technology, Wudil, Nigeria.
[3]Department of Microbiology, Bayero University, P.M.B. 3011, Kano, Nigeria.
* Correspondence author: lawaldanjuma@yahoo.com. +23439711982

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 0.50-50.00mg/ml. Phytochemical investigation of the extracts revealed the presence secondary metabolites like alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids and phytosterols in C. senensis peels while alkaloids, flavonoids, saponins, tannins, triterpenoids and phytosterols were found in A. comosus peels. In-vitro antibacterial potential was confirmed and no synergism was demonstrated at a combination ratio of 1:1 of the extracts tested (P = 0.05).
Keywords: - Anti-bacterial, Annona comosus, Citrus senensis, synergism, Extracts.

INTRODUCTION

The mass scale productions of synthetic medicines by the western countries in the begging of the current century make the medicines and drugs of vegetables origin lost their significance. They were rejected by the medicinal practitioners all over the world, being referred to as 'old woman's remedy' (Geae, 1986). In view of increasing awareness about adverse side effects of synthetic drugs, a sense of back to nature has been developed and the people in most of the developed countries now prefer raw carrot instead of carotene compound based tablets, instead of vitamin C tablet, they feel happy to use citrus fruits (Chong, 2003). It is mainly due to innumerable side effects being observed following the use of synthetics i.e. resistance to certain antibiotics and immune-suppressive activities of certain drugs. It is felt that there is a sense of revival in medicines from a vegetative source and it is once again gaining recognition' (Geae, 1986).

Annona comosus (L) Peel: Is derived from the fruit of *Annona comosus*, the plant is called pineapple (English) and is locally called Abarba (Hausa). The root and fruit are either eaten or applied topically as an anti – inflammatory and as a proteolytic agent. It is traditionally used as an antihelminthic agent in the Philippine. A root decoction is used to treat diarrhea (Oxford, 2005). This fruit also has anti – inflammatory and digestive properties. The bromelain in *A. comosus* helps fight infections by dissolving layers of slough and bacteria – rich surfaces. This fruit can also be used to aid in digestion. It can clear bronchial passages in those suffering from pneumonia and bronchitis (Oxford, 2005).

The anti – inflammatory properties in this fruit help reduce the symptoms of arthritis, and help reduce pain after surgery and sport injuries. *A. comosus* is currently being studied for its effectiveness in preventing heart disease. *A. comosus* juice is taken as a diuretic and to expediate labour, also as a gargle in cases of sore throat and as an antidote for sea sickness. The flesh of very young (toxic) fruits is deliberately ingested to achieve abortion (a little with honey on 3 successive mornings); also to expel intestinal worms; and as a drastic treatment for venereal diseases. In Africa the dried, powdered root is a remedy for edema. The crushed rind is applied on fractures and the rind decoction with rosemary is applied on hemorrhoids. Indians in panama use the leaf juice as a purgative, emmenogogue and vermifuge (Oxford, 2005).

Previous report shows that the methanolic extract of *A. comosus* peel was inactive to *B. subtilis*, *E. coli* at a concentration of 50mg/ml (50,000µg/ml), but it shows activity on *S. typhi* at a concentration of 100mg/ml (100,000µg/ml) (Ishaii, *et al.*, 1984). The juice has antiviral activity and the undiluted juice was found to have activity on polio virus 1 (Konowalchuk and Speirs, 1978).

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 teanicidal 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, cormunative, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. Also the peels are used in Ayurvedic medicine to tonify liver, strengthening to 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 cm^3) of the extract was added to two cm^3 of HCl. To this acidic medium, one cm^3 of Dragendroff'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 3cm^3 of the extract was added 1cm^3 of NaoH, a yellow colouration indicated a positive test for flavonoids (Odebiyi and Ramstard, 1978; Waterman, 1993).

(c) Test for Saponins (Frothing Test)

A quantity (2 cm^3) of the extract was placed in a test tube and then 2cm^3 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 Ramstard, 1978; Waterman, 1993).

(d) Test for Tannins

1. Two drops of 5% $fecl_3$ was added to 1cm^3 of the extracts. A green precipitate indicated a positive test for the presence of tannins (Odebiyi and Ramstard, 1978; Waterman, 1993).
2. To 5 cm^3 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) Salkwoski's Test for Steroids

To 1cm^3 of the extract 5-drops of conc. H_2S0_4 was added. A red colouration indicated a positive test for steroids (Odebiyi and Ramstard, 1978; Waterman, 1993).

(f) Fehling's Test for Glycosides

A quantity (10 cm^3) of 50% H_2S0_4 was added to 1 cm^3 of the extract in a test tube. The mixture was heated in a boiling water bath for 15 minute. A quantity (10 cm^3) of fehling's solution was added and the mixture was boiled. Formation of brick red precipitate indicated a positive test for glycosides (Odebiyi and Ramstard, 1978; Waterman, 1993).

(g) Test for Triterpenoids

A quantity (10mg) of the extract was dissolve in 1ml of chloroform; 1ml of acetic anhydride was added following the addition 2 ml of conc. H_2S0_4. Formation of reddish violet colour indicated the presence of triterpenoids (Harbone, 1998 and Kokate, 2001).

(h) Test for Phytosterols

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 phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of conc. H_2S0_4. Appearance of bluish green colour showed the presence of phytosterol (Harbone, 1998 and Kokate, 2001).

(i) Test for Amino Acids

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

Preparation of Sensitivity Disc

Whatman No. 1 filter paper was punched using puncher to obtain disc of 6.0mm in diameter. These were placed in a sterile screw-capped Bijou bottles and sterilized in an oven using a dry heat at 140^{o}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 1ml Dimethly sulphoxide (DMSO). One gram of each fraction was weighed on a Metler balance (Model: Scout pro Spu 401, S/N: 7129110037) and dissolved in 1ml of DMSO to arrive at 1000,000 µg/ml (10^6µg/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 (1000,000 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^{o}C until required for use.

Standardization of innoculum

Using inoculating loop, enough material from an overnight culture of the test organisms were transferred into a tube containing 2.0ml 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 15mm from the edge of the plate and 25 mm from disc to disc to prevent overlapping of zones and incubated at 37^{o}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 - 400,000µg/ml) respectively. From the working inoculum 0.1ml was inoculated into fresh nutrient broth tubes at different extract concentrations. The tubes were incubated at 37 ±1^{o}C for 18 – 24hrs. 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 0.01ml of the highest 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^{o}C for 18 – 24hrs 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 co efficient 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)

Formula:-

$$F.I.C = \frac{MIC_X \text{ in combination with Y}}{MIC_X} + \frac{MIC_y \text{ in combination with X}}{MIC_y}$$

$$\text{Example: - } FIC_{C+A} = \frac{MIC_C \text{ in combination with A}}{MIC_C} + \frac{MIC_A \text{ in combination with C}}{MIC_A}$$

Where: C = peel of *C.senensis*, A= peel of *A. comosus*

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*

Plant part	Solvent (ethanol)	Initial weight (g)	Final weight (g)	Colour	Odour	Texture
Peel	*A.comosus*	100.00	19.80	Orange	Fruity	Oily
Peel	*C. senensis*	100.00	31.00	Orange	Fruity	Oily

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

Ingredient	*A. comosus*	*C. senensis*
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Tannins	+	+
Steroids	-	+
Glycosides	-	-
Triterpenoids	+	+
Phytosterols	+	+
Amino acids	-	-

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.

Test Bacteria Zone		Average zone of inhibition (in mm)/Disc Potency in µg/disc 4000	3000	2000	1000	400	300	200	100	40	30	20	10	S.D
Salmonella B_1	*Paratyphi*	07	09	07	11	11	10	10	10	00	00	08	07	CPX	38
Salmonella B_2	*Paratyphi*	10	10	11	10	00	00	00	00	00	00	00	00	SXT	35
Salmonella B_3	*Paratyphi*	00	10	00	08	00	09	00	10	09	10	10	10	AMP	24
Salmonella B_4	*Paratyphi*	10	10	10	10	10	00	00	00	10	09	07	09	CPX	42
Salmonella B_5	*Paratyphi*	10	09	10	09	09	08	11	12	09	08	10	10	OFX	42
Salmonella B_6	*Paratyphi*	00	00	00	00	00	00	00	00	00	00	00	00	SXT	15
Salmonella	*typhi*$_1$	12	12	12	12	12	12	12	11	10	10	10	10	PEF	30
Aeromonas	*hydrophila*$_1$	07	07	07	07	07	07	07	07	00	00	00	00	OFX	20
Aeromonas	*hydrophila*$_2$	07	07	07	07	00	00	00	00	08	00	08	08	APX	15
Aeromonas	*hydrophila*$_3$	00	00	00	00	07	07	07	07	12	10	11	11	PN	35

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

Statistical Relationship

	F-value	F-critical	df	Probability level
A comosus	10.13967	1.553208	1	5%
C senensis	8.880079	1.553208	1	5%
A+C	14.14505	1.553208	1	5%

Key: A= *Annona comosus*, C= Citrus, df = degree of freedom.

Table 4: Minimum Inhibitory and Bactericidal Concentrations of the combined ethanolic extract of *A. comosus* peel and C. senensis peel at 1:1 ratio against the isolates.

Test organism	MIC value (mg/ml)	MBC value (mg/ml)
S. paratyphi B_1	0.25	1.00
S. paratyphi B_2	0.25	0.50
S. paratyphi B_3	0.25	-
S. paratyphi B_4	0.25	0.25
S. typhi$_1$	0.25	0.25
A. hydrophila$_1$	0.25	-
A. hydrophila$_2$	0.25	0.50
A. hydrophila$_3$	10.0	-
S. paratyphi B_5	12.5	50.00
S. paratyphi B_6	*	*

Key: + indicates activity, No activity, * Not tested

Table 5: Fractional Inhibitory Concentration of combined ethanolic extract of *A. comosus* peel and *C. senensis* peel at 1:1 ratio against the isolates.

Test organism	[A.c	C.s	A+C]	MIC(µg/ml)	FIC	Inference
S. paratyphi B_1	250	250	250		2.0	Additive
S. paratyphi B_2	250	250	250		2.0	Additive
S. paratyphi B_3	250	250	250		2.0	Additive
S. paratyphi B_4	250	250	250		2.0	Additive
S. paratyphi B_5	2000	2,500	12,500		11.25	Antagonistic
S. typhi$_1$	62.5	250	250		5.0	Antagonistic
A. hydrophila$_1$	250	250	250		2.0	Additive
A. hydrophila$_2$	250	250	250		2.0	Additive
A. hydrophila$_3$	12,500	2,500	250		4.4	Antagonistic
S. paratyphi B_6	2000	2,500	*		*	*

Key: - F.I.C less than or equal to 0.5 is synergistic, F.I.C greater than or equal to 1.0 is additive
F.I.C greater than 4.0 is antagonistic, * Not tested

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 *S. paratyphi* B_6 that was resistant at all the concentration used. Moreover, anti-bacterial activity was prominent against *S. paratyphi* B_5, 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 500µg/ml and the highest MBC 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 synergistic 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.

In traditional medicine, the dried peel of *A. comosus* is usually boiled with combination of other plant like cloves of garlic and leaves of aloe vera and has been proven to be effective in the treatment of *typhoid* fever and gastro-enteritis (Iwu and Anyanwu, 1982). In a similar study that involve the combination of two different extract (Taura and Oyeyi, 2009) reported the ethanolic extract of the bark of *A. comosus* and *Allium sativum* has antimicrobial activity against *S. typhi* at a concentration of 80, 60 and 40mg/ml. It also inhibited *P. Mirabilis*, *E. coli*, *K. Pneunoniae*, *Pseudomonas aeroginosa*, *S. aureus*, *Streptococcus pneumoniae*, and also synergism was demonstrated on *S. typhi*.

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. comous* 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 that of *C. senensis* peel. Likewise, the activity of each extract was significantly different from their combinations, because for *A. comosus* peel the F-value (10.13), F-crit (1.55) and the combination of *A. comosus* peel and *C. senensis* peel have F-value (14.14), F-crit (1.55) all at one degree of freedom and at 5% probability level are 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.

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