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A Study of the Phytochemical Properties and Synergistic Action of Leaf Extracts of Dodonea Viscosa Linn, Annona Comosus (Linn) Merr Peel and Citrus Senensis Peel on Aeromonas hydrophila and Salmonella Species.

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Abstract
The leaf of Dodonea viscosa Linn, peels of Annona comosus (Linn) Merr and Citrus senensis are widely used traditional remedies against various ailments, such as digestive system disorders like: indigestion, ulcers, diarrhoea, constipation, upset stomach and tonic to digestive system. The major chemical constituents reported from the plant parts are alkaloids, flavoniods, saponins, tannins, steroids, triterpenoids and phytosterols which show that these plant parts can be a potential candidate to be use as a therapeutic agent. The aim of the study was to determine the synergism at a concentration ratio of 1:1 between the three extracts using discs diffusion, broth tube dilution and fractional inhibitory concentration techniques against six Salmonella paratyphi B, one Salmonella typhi and three A. hydrophila. In vitro anti-salmonellae and A. hydrophila activities of the extracts were confirmed and no synergism was demonstrated (P = 0.05).
Keywords: Bioactivity, phytochemicals, synergism, Dodonea viscosa, Annona comosus, Citrus senensis, MIC, MBC, FIC.

1. Introduction

The early history of botanical science is to a very large extent concern with man's interest in the use of plant materials for the treatment of human ills [1]. The records of pre Christians, Egyptians, Greeks and Romans discuss several thousands of species of plants commonly used as medicinal agents. Modern chemistry has produced a vast array of synthetic drugs that have replaced many of the old and well established botanicals but others are still important items in our material medica [1].

In the present study, three medicinal plants Viz. *Dodonea viscosa* Linn, *Annona comosus* (Linn) Merr and *Citrus senensis* belonging to the family Sapindaceae, Annonaceae, Rutaceae, were selected to assess their synergistic potential. Various parts of *Dodonea viscosa* plant have been use as common household remedy to treat ailment like fever, sorethroat, cold, malaria, and rheumatism [2,3], itching, swellings, aches [4], painkillers to toothaches and lotion to treat sprains, bruises, burn and wounds [5] indigestion, ulcer, diarrhoea and constipation[2,3]. Also various parts of *Annona comosus* have been use as anti-inflammatory, proteolytic agent, antihelmithic, as treatment to diarrhea, indigestion, pneumonia, bronchitis, arthritis, pain, heart disease, diuretic, to expedite labour, arbortion, intestinal worms, venereal diseases, edema, hemoorrhoids, purgative, emmenogogue and vermifuge [6]. Furthermore, various parts *Citrus senensis* have been use to treat colic, upset stomach[7], phlebitis, cancer, diuretic, cormunative, stomachic, tonic to digestive system and skin [7], in Ayurvedic medicine to tonify liver, strengthening to blood vessels, varicosa, vitamin deficiencies, cold, flu, scurvy [8], the high citric content help to treat heavy-metal poisoning, fight viral and bacterial infections, also use as aromatic, flavour enhancer and immune-enhancing [8].

1.1 Materials And Methods

1.1.2 Collection and identification of plant materials

The leaves of actively growing *Dodonea viscosa* plant were collected from the experimental Garden of the Department of Biological Sciences, Bayero University, Kano, Nigeria, in May, 2010. The plant was first identified at the field using standard keys and description [9,10,11] while for the peel of Annona *comosus* and *Citrus senensis* the fruits were obtained from Yankaba Market in Kano, Nigeria, in May, 2010. Their botanical identity was further confirmed and authenticated at the Botany unit and the voucher specimen numbers (120, 92, and 16) were deposited at the Herbarium section of the Botany unit of Bayero University, Kano, Nigeria for reference.

1.1.3 Preparation of the treatment samples

The leaves and peels were air-dried under the shade and ground to powder using mortar and pestle [12]. The contents were stored in air-dried containers until required for further analysis.

1.1.4 Extraction protocols

This was carried out according to standard method [12].

1.1.5 Phytochemical Screening of Plant Extracts

The extracts were analysed for the presence of alkaloids, flavonoids, saponins, tannins, steroids, glycosides, triterpenoids, phytosterols and amino acids, using standard method as [13,14,15,16].

1.1.6 Preparation of sensitivity discs

What man 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 dry-heat oven 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.

1.1.7 Preparation of extract concentrations

The stock solution of the plant extract was prepared in screw capped bijou bottles containing Dimethly sulphoxide (D.M.S.O). 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^{6}µg/ml) concentration of stock solution. Twelve varied 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.

1.1.8 Preparation of combine ratios of the extracts

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

1.1.9 Test culture

The test organisms were isolated from stool samples of patients presented with diarrhoea attending Aminu Kano teaching Hospital (A.K.T.H) Kano and Murtala Mohammad Specialists Hospital Kano, using standard methods [17]. They include 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.

1.1.10 Preparation of turbidity standard

This was carried out according to standard method [18].

1.1.11 Standardization of innoculum

This was carried out according to standard method [18].

1.1.12 Extracts antibacterial activity testing

Agar diffusion method as modified [19] was employed. The freshly prepared nutrient agar plates were dried in a dryer for about 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 leaf extract of *Dodonea viscosa, Citrus senensis* and *A.comosus* peel at different concentrations were arranged redially and pressed firmly onto the inoculated agar surface to ensure even contact. 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. The zone diameters of the semi-confluent growths were measured with the aid of a meter rule to the nearest millimetre.

1.1.13 Determination of minimum inhibitory and bactericidal concentrations of the extracts

The MIC and MBC were determined in accordance with the standard methods [20]. 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 onto 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.

1.1.14 Determination of fractional inhibitory concentration of plant extracts

The synergistic effect of the combined plant extracts was determined using fractional inhibitory concentration (F.I.C), which is an interaction co efficient indicating whether the combined effect of the plant extracts are: - synergistic (when F.I.C is < 0.5), additive (when F.I.C is = 1) and antagonistic (when F.I.C is > 4) as demonstrated for antibiotics [21,22].

Formula:-

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

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

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

1.1.15 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).

1.1.1 Results and Discussion

Table (1) shows the phytochemical composition of the plant parts screen. Only steroids, glycosides and amino acids were absent in *A. comosus* peel, in *D. viscosa* leaf only glycosides and amino acids were absent and in *C. senensis* peel only glycosides and amino acids were absent . The antibacterial susceptibility pattern of the extract was shown in Table (3-4). The results of the study show that the ethanolic extract of leaf and peels of the plant demonstrate antibacterial potential on all the tested organisms. Furthermore, antibacterial potential was prominent against *S. paratyphi* B_3, which was seen to be more sensitive to the combination at a concentration of 40ug/disc (4000ug/ml) with zone of inhibition of 15mm, in comparison with standard antibiotic Tarivid 10ug which shows a zone of 23mm (Table 2). The lowest MIC (Table 3) was demonstrated at a concentration of 5.00mg/ml and the highest MIC was seen at a concentration of 25.00mg/ml. The lowest MBC (Table 3) was seen at a concentration of 20.00mg/ml and the highest MBC was seen at a concentration of 50.00mg/ml. The observed antibacterial potential of these extracts could be due to the presence of bioactive compounds like alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids and phytosterols, which could be responsible for these note-worthy activities. The FIC (Table 4) demonstrates that the combination was found to be antagonistic. 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 [23]. In a similar study that involve the combination of three different extract [24], reported that the combination of *A. comosus, Aloe barbadensis* and *Allium sativum* was synergistic on *S. typhi*, but in this study the combination of *D. viscosa* leaf, *A. comosus* and *C. senensis* peel was found to be antagonistic on the tested organisms.

Statistical analysis showed that, the potency of the extracts was not greater when the three plants extracts were combined at 1:1 and P = 0.05. Their activities singly was statistically different from each other, for *D. viscosa* leaf extract the F-value (8.10), F-crit value (1.55) at one degree of freedom and at 5% probability level were found to be different from that of *A. comosus* peel and *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 *D. viscosa* leaf and *C. senensis* peel have F-value (11.40), F-crit (1.55) all at one degree of freedom and at 5% probability level are different from each other, these shows no synergistic interaction between the three plant extracts.

Table 1: Phytochemical characteristics of the leaf extracts of *Dodonea viscosa, Annona comosus* and *C. senensis* peel.

Ingredient	Ethanol	Ethanol	Ethanol
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Steroids	+	_	+
Glycosides	_	_	_
Triterpenoids	+	+	+
Phytosterols	+	+	+
Amino acids	_	_	_

Key: + = present; - = absent.

Table 2: Activity of *D. viscosa* leaf, *A. comosus* peel and *C. senensis* peel extracts in ug/disc and the standard antibiotic disc.

	Average Zone of inhibition (in mm)/ Disc Potency												
Test Bacteria Zone	4000	3000	2000	1000	400	300	200	100	40	30	20	10	S.D
Salmonella paratyphi B_1	08	09	08	07	00	09	08	00	00	00	07	07	PN 27
Salmonella paratyphi B_2	10	10	09	10	11	10	10	10	10	08	08	00	SXT 35
Salmonella paratyphi B_3	08	10	10	09	08	11	11	10	15	14	11	13	0FX 23
Salmonella paratyphi B_4	09	00	09	00	00	00	00	00	00	00	00	00	CPX 42
Salmonella paratyphi B_5	14	10	12	00	10	10	13	10	07	07	07	07	PN 21
Salmonella paratyphi B_6	00	00	08	00	07	07	07	07	00	00	00	00	S 12
Salmonella typhi$_1$	08	09	08	09	00	00	00	00	00	00	00	00	PEF 30
Aeromonas hydrophila$_1$	12	10	10	09	07	07	07	07	10	10	07	10	0FX 20
Aeromonas hydrophila$_2$	08	08	08	08	08	07	08	07	08	08	08	08	APX 15
Aeromonas hydrophila$_3$	15	15	15	14	11	00	00	00	00	00	00	00	PEX 30

KEY: CN = GENTAMYCIN 10ug. PEF = PEFLACINE 10ug. CPX = CIPROFLOXACIN 10ug. PN= AMPHICILLIN 30ug.S= STREPTOMYCIN 30ug.AMP = AMPHICILLIN 10ug. OFX = TARIVID 10ug. SXT= SEPTRIN 30ug. PEX=10ug. APX=AMPHICLOXACIN10ug.

Table 3: Minimum Inhibitory and Bactericidal Concentrations of the combined leaf ethanolic extract of *D. viscosa*, *C. senensis* and *A. comosus* peel

Test organism	MIC value (mg/ml)	MBC value (mg/ml)
S. paratyphi B_1	-	-
S. paratyphi B_2	-	-
S. paratyphi B_3	-	-
S. typhi$_1$	12.50	6.25
A. hydrophila$_1$	-	-
A. hydrophila$_2$	-	-
A. hydrophila$_3$	10.00	20.00
S. paratyphi B_4	-	-
S. paratyphi B_5	5.00	10.00
S. paratyphi B_6	25.00	50.00

Key: - No activity

Table 4: Fractional Inhibitory Concentration of *D.viscosa* leaf, *A. comosus*, and *C. senensis* peel

Test organism	MIC(mg/ml)	FIC	Inference
S. paratyphi B_5	5.00	80.0	Antagonistic
S. paratyphi B_6	25.00	26.30	Antagonistic
S. typhi $_1$	12.50	450.0	Antagonistic
A. hydrophila$_3$	10.00	120.0	Antagonistic
S. paratyphi B_3	*	*	*
S. paratyphi B_4	*	*	*
S. paratyphi B_1	*	*	*
S. paratyphi B_2	*	*	*
A. hydrophila$_1$	*	*	*
A. hydrophila$_2$	*	*	*

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

1.1.1.1 Conclusion and Recommendations

The results of the present study, shows that these plants extract possess bioactive constituents of pharmacological significance. Therefore, further studies are recommended for the isolation, purification and characterization of these chemical constituents for the understanding of the synergism mechanism

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