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The antibacterial and antidiarreal activities of the crude methanolic Syzygium cordatum [S.Ncik, 48 (UZ)] fruit pulp and seed extracts

Maliehe Tsolanku Sidney* Shandu Jabulani Siyabonga Basson Albertus Kotze Article Number - CEFF46755111 | Vol.9(33), pp. 884-891, September 2015 | https://doi.org/10.5897/JMPR2015.5789

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ABSTRACT

Diarrheal infections are the major cause of high morbidity and mortality rates, especially in the developing countries. Different parts (roots, bark and leaves) of the species Syzygium cordatum have been used as antidiarrheal extracts with the exception of its fruit-pulps and seeds. This study aimed at evaluating the antibacterial and antidiarrheal activity of S. cordatum pulp and seed extracts so to find newer and more cost-effective means to prevent diarrhoea. The harvested fruits were separated into pulp and seeds, dried and extracted with methanol using Soxhlet extraction. The extracts were screened for phytochemicals. The antibacterial and invivo antidiarrheal activities were determined using the microdillution method and castor oilinduced rat model, respectively. The percentage yields of 10 for fruit-pulp extract and 6 for seed extract were obtained. The detected phytochemicals were phenolics, alkaloids, cardiac glycosides, phytosterols, flavonoids, saponins, terpenoids and betulinic acid with the total phenolic content of 16.4±1.8 and 21.4±1.4 µg/mg for pulp and seed extracts, respectively. The pulp extract exhibited the lowest minimum inhibitory concentration (MIC) value of 3.13 mg/ml against some gram-positive and gram-negative bacteria while the seed extract had lowest MIC on. The in vivo antidiarrheal activity showed the percentage inhibition of 41 for the seed extract and 49 for pulp extract. The antibacterial and antidiarrheal activities were owed to the detected phytochemicals, and thus promoting S. cordatum fruit-pulps and seeds as potential sources of therapeutic compounds against diarrheal infections.

Key words: Antibacterial, antidiarrheal, antimotility, phytochemicals.

INTRODUCTION

Diarrheal infections are major causes of morbidity and mortality worldwide, especially in developing countries among infants and children. There are approximately 1.5 billion episodes of diarrheal infections per year. More than one in ten deaths of children under the age of 5 years are due to diarrhoeal infections (WHO and UNICEF, 2009). Diarrhoea is gastrointestinal disorder that is characterized by a decrease in the stool consistency and an increase in frequency, fluidity, or volume of the faeces during defection for a period of days or weeks (Mazzolin et al., 2013). The most common symptoms of diarrhoeal infections range from mild and self-limiting symptoms (Mims et al., 2004).

However, severe diarrhoea may lead to the disordered gastrointestinal tract (GIT) motility, dehydration, electrolyte imbalance, acidosis and malnutrition (Dyer and Gould, 2011). Diarrhoea often occurs due to the damage of the intestinal mucosal cells by exotoxins and endotoxins of microbial origin in contaminated food and water and metabolic disorder in gastrointestinal tract (Kumar et al., 2001).

Several studies have reported the beneficial effects of fruit pulps and seed extracts in the treatment of diarrhoeal infections (Ashorobi and Umukoro, 2005; Maha et al., 2013). The antibacterial and antidiarrheal activitiv of fruit pulp and seed extracts depend on the presence and concentrations of phytochemicals (Arup et al., 2012). According to Neethiriajan et al. (2012), phytochemicals have strong antibacterial and antidiarrheal properties. Although fruits and seeds are excellent sources of therapeutic phytochemicals, pulps and seeds have been rarely used as medicine (van Wyk et al., 2009; Kossah et al., 2011; Srividhya et al., 2013).

However, the prohibitive costs and the negative side effects of allopathic medicine used against diarrheal infections have recently elevated fruits and seeds as sources for novel antidiarrheal agents. *S. cordatum* are edible fruit-trees native to the Republic of South Africa (RSA). They are widely distributed in the Eastern Cape, KwaZulu-Natal, across northern part of the RSA and in areas with high rainfall (Orwa et al., 2009). The bark and leaves of *Syzygium cordatum* trees have been proven to possess antidiarrhoeal properties (Sibandze et al., 2010; Amabeoku and Deliwe, 2013).

The fruits of *S. cordatum* have only been used for consumption and not for pharmacological purposes such as treatment of diarrheal infections. The fruits are purple, ovoid and fleshy up to 2 cm fruits with 2.8 cm thick seeds (Downs and Wilson, 2012). The fruiting season is usually from October to June in Republic of South Africa (RSA) (Drummond and Moll, 2002).

The study was undertaken to evaluate the antibacterial and antidiarrheal activities of *S. cordatum* pulp and seed extracts as to find novel sources that can be developed for treatment of diarrhoeal infections.

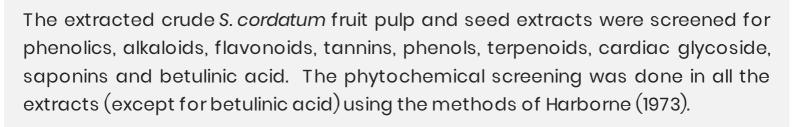
MATERIALS AND METHODS

Fruits of *S. cordatum* were randomly harvested in summer (February, 2014) from the trees at the main campus of the University of Zululand (UZ), KwaZulu-Natal, RSA. The fruits were washed with distilled water, seeds and pulps were manually separated. The fruit pulps and seeds were air-dried at room temperature. The dried *S. cordatum* fruit-pulps and seeds were separately ground to a coarse powder form using an electric grinder and filtered with a filter of mesh size 1.0 mm to increase the surface area for solvents during the extraction process. The grounded samples were stored at 4°C until required for use.

Extraction

A Soxhlet extraction was done according to Bii et al. (2009) with some modifications. The ground S. cordatum fruit-pulp sample (100 g) was subjected to Soxhlet extraction using 400 ml of methanol (Univ.AR). The sample was put on a mechanical shaker at a speed of 200 rpm at 37°C for 12 h. The extract obtained was filtered through Whatman filter paper and concentrated using a B?chi rotary evaporator at 45°C. The yield of the extract was weighed and re-dissolved in 100 ml of 10% dimethyl sulfoxide (DMSO) to the volume concentration of 100 mg/ml. The extracts were stored at 4°C until they were to be used. The percentage yield from S. cordatum fruit-pulp extract was calculated using the formula below that was used by Shahid (2012).

Phytochemical compounds



Betulinic acid -thin-layer chromatography

An original line of 2 cm from the edge, across the plate was drawn. Betulinic acid was loaded on thin-layer chromatography plate as standard indicator followed by loading of methanol extract of *S. cordatum* fruit-pulp. The thin-layer chromatography plate was placed in a chromatography tank containing mixture of hexane and ethyl acetate in the ratio of 7:3, respectively, covering about 1 cm of the plate. The chromatography was allowed to proceed until the hexane-ethyl acetate reaches the top of the plate. At that point, the chromatogram was removed from the tank and dried using hot air dryer. The plate was viewed under ultra violet light at 354 nm. It was then sprayed with 5% sulphuric acid-methanol solution. The appearance of a pink colour indicated the presence of betulinic acid (Walker, 1984).

Quantification analysis of total phenolic content

The total phenolic contents were determined by the Folin-Ciocalteau method according to Makkar et al. (1993). An aliquot (0.2 ml) of $500 \, ^{\square} \, \text{g/ml}$ methanolic fruit-pulp and seed extracts were made up to 1.0 ml with distilled water, respectively. 0.5 ml of Folin-Ciocalteau reagent (1N) was added, followed by 2.5 ml of sodium carbonate solution (20%). The mixtures were mixed properly, and then incubated at room temperature for 40 min. The absorbance of the blue-colored complex formed was measured at 725 nm against the appropriate blank. The total phenollic content was determined from the standard curve of tannic acid and expressed as equivalents of tannic acid ($\mu g/mg$).

Antimicrobial activity

The bacterial strains known to cause GIT infections used in this study included; Bacillus cereus (ATCC 10102), Staphylococcus aureus (ATCC 25925), Enterococcus hirae (ATCC 8043), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 7700) and Vibrio vulnificus (AL 042).

Revival of the selected bacterial strains

The selected bacteria were inoculated into nutrient broth and incubated at 37°C for overnight. Afterwards, 1 ml from each of the bacteria species was pipetted into 9 ml of fresh prepared nutrient broth in separate test tubes labelled with corresponding microorganism. The test tubes were then incubated at 37°C for overnight .After overnight incubation, absorbance of the selected bacterial

strains was read in the spectrophotometer (600 nm) for determination of their turbidity. The turbidity of the resulting suspensions was diluted with nutrient broth to obtain an absorbance of 0.132. This absorbance was taken as comparable to 0.5 McFarland turbidity standard. The turbidity was estimated to be equivalent to 1.5 x colony forming unit (CFU)/ml (Qaralleh et al., 2012).

Minimum inhibitory concentration (MIC)

A serial microdilution method was adapted as described by Eloff (1998) and Qaralleh et al. (2012) to measure the MIC of the fruit-pulp extract. The MIC is the lowest concentration of the extract required to inhibit microbial growth. 96-well microplate was used to quantitatively determine the MIC of the extract. The sterile nutrient broth (50 l) was added to all the wells of the 96-well microplate and 50 I of the extract (50 mg/ml, in 10% DMSO) was poured in the wells in the first rows and mixed well. The extract mixture (50 l) were removed from all the wells in the row A to perform a 3-fold serial dilution down the columns. The last 50 🗖 I, in the last column was discarded so that the total volume solution of each well was 50 ■I. The selected bacterial strains (50 ■I) were transferred into the corresponding wells. 10% DMSO was used as negative control while ciprofloxacin (20 ■g/ml) was used as a positive control. The plate was covered and incubated at 37°C for overnight. 0.2 mg/ml of P-iodonitrotetrazodium violet (INT) solution was used after the incubation period. 40 I of 0.2 mg/ml INT solution was added to each well and incubated at 37°C for 30 min. A reddish colour which was the result of INT being reduced by the metabolic activity of microorganism to formazan indicated microbial activity. The clear colour was to be the indication of the absence of bacterial activity since the INT was not broken-down to form formazan. The test was replicated three times and the mean value was reported.

Minimum bactericidal concentration (MBC)

For the determination of MBC, the agar dilution method was used. The MBC of the extract was determined by removing a loop full of each culture medium from the wells that had no bacterial growths. They were streaked on different sterile nutrient agar plates. The agar plates were incubated at 37°C for 12 h. The lowest concentration of the *S. cordatum* fruit-pulp extract that exhibited the complete killing of test microorganisms was considered as the MBC (Qaralleh et al., 2012).

Antidiarrheal and antimotility activities

Animals

Ethical clearance for the use of animals was collected from the Research Animal Ethics Committee (RAEC) of the UZ and the twelve white Sprague-Dawley rats (150 to 260 g) were collected from the animal house in the Department of Biochemistry and Microbiology at the same institution. Prior to the determination of the antidiarrhoeal and antimotility activities, rats were fed with standard food and given free access to water for one week to adapt to the laboratory conditions (temperature 23±2°C and 12 h light dark cycle). The rats were then fasted for 18 h before the start of the experiment to empty the GIT and to increase their responsiveness to the extracts and drugs used, but allowed free access to water (Orhan et al., 2013).

Antidiarrheal activity

The method used in for determination of antidiarrheal activity was adopted from Teke et al. (2007), with some modifications. The rats were divided into four groups of four rats each namely: Group A, Group B, Group C and Group D. Group A served as a negative control. It received vehicle distilled water (2 ml /kg) orally. Group D served as a positive control. It received atropine at the dose of 5 mg/kg orally by gavage. Group B and Group C received the seed and fruit-pulp extracts (400 mg/kg), respectively. Each rat was put in its own cage. Diarrhoea was introduced to each rat by orally administering 0.2 ml of castor oil. After 30 min of administration of castor-oil, observation of the defecation was done for 5 h. The onset time of faeces and number of normal and wet faeces were the determined parameters. A score based on stool consistency was assigned as follows; normal-stool = A and wet-stools = B. The presence of normal stools was recorded as a positive result, indicating protection offered by the controls and the extracts from diarrhea while the presence of watery stools was recorded as negative results.

Antimotility activity

The method used in the antimotility test was adopted from Teke et al. (2007), with some modifications. The animals were divided into four groups of four rats each namely: Group A, Group B, Group C and Group D. Diarrhoea was introduced to each rat in all groups by orally administering 0.2 ml of castor oil. After 30 min of administration of castor-oil, all rats received different treatments. Group A served as a negative control and received distilled water (2 ml/kg) orally. Group D served as a positive control. It received atropine at the dose of 5 mg/kg orally by gavage. Group B and C received the seed extract and fruit-pulp extract of 400

mg/kg, respectively. Thereafter, each rat was put in its own cage after the administration of 2 ml of charcoal meal (3 % deactivated charcoal in distilled water) orally. The rats were sacrificed 30 min thereafter for determination of gastrointestinal motility. The intestinal distance moved by the charcoal meal from pylorus to caecum was measured and expressed as a percentage of distance travelled from pylorus to caecum. The mean movement of charcoal meal in ratio to the intestinal length and percentage of inhibition were arithmetically measured. The following formulas were used:

RESULTS

The use of methanol as an extracting solvent resulted in a good percentage yield of 10 for fruit-pulp extract and 6 for seed extract. Phytochemicals are non-nutritional bioactive chemicals from plants that help plants to survive biotic and abiotic environmental changes and have therapeutic properties in humans. The total phenolic content of 16.4±1.8 and 21.4±1.4 µg/mg were obtained in pulp and seed extracts, respectively. The qualitative and quantitative analysis of phytochemicals from *S. cordatum* fruit-pulp and seed samples and extracts are presented in Table 1.

The antibacterial results are as presented in Table 3. Pulp extract showed the lowest MIC value of 3.13 mg/ml on S. aureus (ATCC 25925), B. cereus (ATCC 10102), E. hirae (ATCC 8043) and P. auriginosa (ATCC 7700) isolates while the seed extract had the lowest MIC value (6.25 mg/ml) on all gram-positive bacteria. S. cordatum fruit-pulp and seed extracts exhibited different percentage of inhibition against the diarrheal activity in castor oil induced-rats. S. cordatum fruit-pulp and seed extracts both reduced the number of wet stools, total stools and onset time generally in comparison to the negative control (distilled water). S. cordatum fruit-pulp and seed extracts, in a dose-related manner (400 mg/kg of rat), exerted the antidiarrhoeal properties by reducing intestinal motility. The results are tabled in Tables 4 and 5 below.

DISCUSSION

The use of methanol as an extracting solvent resulted in a good percentage yield of 10 for fruit-pulp extract and 6 for seed extract. The good percentage yields

implied that methanol is an important solvent to be used when determining the biological activities of the extracts. The ability of methanol solvent to extract good yields is owed to its polarity.

Phytochemicals have been reported to possess strong antibacterial, antidiarrheal and gastroprotective properties (Neethirajan et al., 2012). The phytochemicals detected in both extracts were phenolic compounds, alkaloids, cardiac glycosides, phytosterols, flavonoids, saponins, terpenoids and betulinic acid (Table 1). The quantitative analysis showed the significant amount of the total phenolic compounds (16.4±1 g/mg) in pulp extract and (21.4±1.4 µg/mg) in seeds extract (Table 2). The detected phytoconstituent implied that *S. cordatum* pulps and seeds can be potential sources for novel lead substances with therapeutic and preventive applications against bacteria that may cause diarrheal infections.

Ciprofloxacin is a broad-spectrum antibiotic which is effective against gramnegative and gram-positive bacteria (Volans and Wiseman, 2010). Ciprofloxacin has bactericidal effect against *E. coli, Salmonella spp.*, *Pseudomonas aeruginosa*, *Staphylococcus spp*, *Streptococcis pp* and *Klebsiella spp.*strains (Paw and Shulman, 2010). Ciprofloxacin is widely used to treat urinary and respiratory infections as well as gastroenterities. Ciprofloxacin (20 ■ g/ml) was used as a positive control on the tested bacteria in this study. Ciprofloxacin had the inhibitory effects on all the selected bacteria with the lowest MIC values of (1.56 mg/ml) on *V. vulnificus* (AL 042), *V. fluvialis* (AL 019) and *S. typhimurium* (ATCC 700030). The highest MIC value (3.13 mg/ml) of ciprofloxacin was recoded on all other selected bacterial strains.

Many naturally occurring compounds found in pulp extract have been reported to possess antibacterial activity. *S. cordatum* pulp extract showed broadspectrum antibacterial action with the lowest MIC value of 3.13 mg/ml on *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043) and *P. aeruginosa* (ATCC 7700). Even though the antibacterial action of *S. cordatum* pulp extract was more pronounced on all gram-positive bacterial strains, the extract also show remarkable antibacterial activity against gram-negative bacteria (*P. aeruginosa* (ATCC 7700) as well with the same MIC value of 3.13 mg/ml. Gram-negative bacteria, in addition to a thin peptidoglycan layer (2 to 7 nm), possess about 7 to 8 nm of the outer membrane. This outer membrane composes of additional protective lipopolyssachride layer that exhibits toxicity and antigenicity

against antimicrobials or chemotherapeutic agents (Martinko and Madigan, 2006). It was concluded that the high resistance shown by some gram-negative bacteria as compared to gram-positive bacteria to both *S. cordatum* pulp extract was due to the mechanism of action of this layer. Gram-positive bacteria do not possess this layer and therefore, they were generally sensitive to the action of the antibacterial action of the detected phytochemicals. Gram-positive bacteria allow the direct contact of the extract constituents with the phospholipid bilayer of the cell membrane, enabling the antibacterial compounds to inhibit bacterial growth easily.

The low MIC values displayed by the fruit-pulp extract indicated its higher efficacy against bacteria causing GIT infections than the seed extract. According to Jayashree et al. (2014), the good and promising potency of methanolic fruit extract has the MIC value ranging between 3.125 to 12.5 mg/ml. This implied that *S. cordatum* pulp extract has a potential to be used as sources of novel antibacterial agent. Antimicrobial substances are considered as bactericidal agents when the ratio is MBC/MIC \$\text{MIC}\$ 4 and bacteriostatic agents when the ratio is MBC/MIC > 4 (Erhabor et al., 2013). *S. cordatum* fruit-pulp extract exhibited bactericidal effect on all selected bacterial species. However, the standard drug-ciprofloxacin showed bactericidal effect on all selected bacterial species with the exception on *V. vulnificus* (AL 042) where it showed the bacteriostatic effect.

Castor oil is an effective emollient laxative agent. Castor oil causes a decrease in fluid and nutrient absorption, increase in the electrolyte secretion and

water and produces alterations in intestinal motility (Priff and Harold, 2005). The diarrheal activity of castor oil is attributed to its active cathartic glyceride known as ricinoleic acid (Chambers et al., 2015). Thus, castor oil-induced diarrhoea is as a result of the action of ricinoleic acid formed from the hydrolysis of its triglyceride in the duodenum by pancreatic lipase. The ricinoleic acid stimulates intestinal hypersecretion, hypermotility and decreases gastrointestinal transit time (Schellack, 2004).

In this study, castor oil was used to induce diarrhoea in the test rats. Atropine was used in this study as a positive control in *in-vivo* antidiarrheal activity. Atropine is a tertiary amine belladonna alkaloid (Chambers et al., 2015). Atropine is a racemic hyoscyamine tropic acid ester of the base tropine. Atropine has high affinity for

muscarinic receptors (Hollinger, 2008; Champe and Harvey, 2009). Atropine exerts its pharmacodynamic effect by binding competitively at the muscarinic receptors to prevent acetylcholine to bind, and thus reversing excessive secretions of fluids and electrolytes. Atropine actions reduce the interstinal hypertonicity and hypermotility of the GIT and thus act as an antidiarrheal agent (Lehne, 2004)

Group C was fed S. cordatum pulp extract at a dose of 400 mg/kg of a rat weight. Group C had stool onset time of 68 minutes, total normal stools of 8.25±0.17 and the total wet stools of 2.75±0.3 while Group B (seed extract) at the same dose as Group C and had the stool onset time of 98 minutes, total normal stool of 5.25±0.20 and the total wet stools of 1.5±0.41 in comparison to the Group A (distilled water) which had stool onset time of 51 min, total normal stools of 13±0.15 and the total wet stools of 10.3±0.2. Group D had the longest onset time (127 min) and the least total number of normal stools (1.25±0.19) with wet stools not being observed. S. cordatum fruit-pulp and seed extracts exhibited the antidiarrheal activity by reducing the number of wet stools, total stools and onset time generally. Phytochemicals mediate antidiarrheal activity through antisecretory and antimotility mechanisms (Cowan, 2015). It was therefore esteemed that the antidiarrheal activity observed in Group B and C was due to the presence of these phytochemicals in S. cordatum pulp and seed extracts. The extracts mechanism of action in antidiarrheal activity was esteemed to mimic that of an atropine (control).

Group D was given an antimuscarinic drug (atropine) and had the highest inhibitory percentage of 64 followed by Group C with 49 of inhibition. Group B (seed extract) had the inhibitory percentage of 41. There was no inhibition observed in Group A (distilled water). The reduction in the distance travelled by charcoal in the S. cordatum fruit-pulp and seed extracts treated groups indicated that S. cordatum pulp and seed extracts exerted antidiarrheal activity by decreasing the GIT motility. The reduction of GIT motility of extracts in comparison to the negative control (distilled water) was attributed to the presence of the detected phytochemicals (saponins, alkaloids, triterpenoids, flavonoids, tannins and betulinic acid). Phytochemicals exert similar mode of action as antimotility agents (Ahmad et al., 2006; Saleem et al., 2010; Chollet and Gleason, 2012).

Thus, S. cordatum pulp and seed extracts might have exhibited the antimotility

action through the same mechanism of action exerted by the drug-atropine. The results scientifically support *S. cordatum* pulp and seed extracts as potential sources for effective, novel antibacterial and antidiarrheal agents.

CONCLUSION

S. cordatum pulp and seed extracts demonstrated the therapeutic and biological efficacy (antibacterial, antidiarrheal and antimotility activities). Due to the pharmacodynamic effects revealed by S. cordatum pulp and seed extracts, S. cordatum pulps and seeds can be viewed as satisfactorily beneficial sources of therapeutic compounds against diarrheal infections. Further studies will focus on the purification and identification of some of the bioactive compounds that are responsible for the antibacterial and antidiarrheal activities.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

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