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EVALUATION OF THE ANTIOXIDANT, ANTIBACTERIAL, AND ANTIPROLIFERATIVE ACTIVITIES OF THE ACETONE EXTRACT OF THE ROOTS OF *SENNA ITALICA* (FABACEAE)

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Abstract

Senna italica, a member of the Fabaceae family (subfamily Caesalpinaceae), is widely used traditionally to treat a number of disease conditions, such as sexually transmitted diseases and some forms of intestinal complications. The roots of *Senna italica* were collected from Zebediela subregion, Limpopo province (S.A), powdered and extracted with acetone by cold/shaking extraction method. The phytochemical composition of the extract was determined by thin layer chromatography (TLC). The chromatograms were visualised with vanillin-sulphuric acid and *p*-anisaldehyde reagents. The total phenolic content of the extract was determined by Folin-Ciocalteu method and expressed as TAE/g dry weight. The extract was assayed for the *in vitro* anticancer activity using Jurkat T cells, antioxidant activity using DPPH assay and antibacterial activity by bioautographic method and the microtitre plate method. The acetone extract of the roots of *Senna italica* inhibited the growth of Jurkat T cells in a dose- and time-dependent manner. The extract also had free radical scavenging activity as well as reasonable antibacterial activity against *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus* with MICs ranging from 0,08 to 0.16 mg/ml in the same order as ampicillin the positive control. The biological activities observed in the acetone extract validated the ethnomedicinal use of *Senna italica*.

Key words: *Senna italica*; Antibacterial; Antioxidant; Antiproliferative

Introduction

In South Africa, as in many other developing countries, the rich cultural diversity is reflected in the use of plants as medicines, and it has been estimated that up to 60% of South Africans consult traditional healers, usually in addition to making use of orthodox medical services (van Wyk et al., 1997). Plants produce a diverse array of secondary metabolites which among others are defences against herbivores and plant diseases caused by virus, bacteria, molds and parasites (Pieters and Vlietinck, 2005). These secondary metabolites may possess complex chemical structures which are not available in synthetic compound libraries (McGaw and Eloff, 2008). The need for ethnobotanical research and the importance of existing accounts of utility of the flora of southern Africa have grown as a result.

In the current study we present an analysis of uses of *Senna* species (Fabaceae) for medicinal and other health benefits in southern Africa. Some *Senna* species from Venda in South Africa have antibacterial activities and are used for treatment of sexually transmitted diseases (Tshikalange et al., 2005). *Senna* is also a stimulant laxative used for the treatment of constipation and for bowel evacuation (Franz, 1993). It is usually effective within 6 to 12 hrs. Adverse effects include gastrointestinal disturbances. It stimulates the muscular coat of the intestine and produces purgation that is not followed as is commonly the case, by constipation. It is therefore one of the most useful of purgatives, especially in cases of habitual constipation (Franz, 1993).

Only a few bioactivity studies have been reported for a variety of *Senna* species. In an attempt to contribute towards the latter, we examined the effects of the root extracts of *Senna italica* subsp. *arachoides* (Caesalpinaceae), commonly known as *sebeta* amongst the Batswana people and as *morotwa ditšhoši* (Sepedi) of South Africa and Botswana, against pathogenic bacteria. Traditional claims by the Batswana people are that

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the root extracts of *S. italica* subsp. *arachoides* have among others, antibacterial properties. *Senna italica* is widely used traditionally to treat sexually transmitted diseases (Gololo, personal communication).

Other researchers have reported that the *Senna italica* extracts of leaves and pods stimulate intestinal contractions on a dose-dependent basis and this supports the purported purgative activity of the plant. The ethanolic extract of the whole plant has antiinflammatory, antipyretic, analgesic, antineoplastic and antiviral activities and causes prostaglandin (PG) release by rat peritoneal leukocytes (Ali Bu-Bashir and Tamira, 1997). The ethanolic extract also has central nervous system (CNS) depressant properties, manifested as antinociception and sedation. It has a weak effect on writhing induced by acetic acid (Ali Bu-Bashir and Tamira, 1997).

A literature survey on the chemical constituents of the genus *Senna* revealed the presence of alkaloids, quinines and anthraquinones (Barbosa *et al.*, 2004). These types of compounds have been isolated from heartwood, seeds, root bark, roots and leaves of the genus *Senna* (Barbosa *et al.*, 2004). Ten flavonoids were isolated from the aerial parts of *S. italica* including tamarixetin (3-rutinoside-7-rhamnoside). Moreover, beta-sitosterol, stigmasterol, alpha-amyrin, 1, 5-dihydroxy-3-methyl anthraquinone and anthraquinone were also isolated by Elsayed *et al.* (1992).

Over the past few decades, a number of publications reported on the biological activities of extracts from different *Senna* species. However, no work has been performed on the biological activities of *Senna italica*, especially in light of the recent focus on the use of its bioproducts in a sustainable world economy. Thus, the current work, critically analyses the biological activity of extracts from *Senna italica*. This study may also help in validating the widely claimed ethnobotanical usage of the plant in traditional and folk medicine.

Materials and methods

The roots of *Senna italica* Mill subsp. *arachoides* (Burch.) Lock (UNIN 11129) were collected from Bolahlakgomo village (Zebediela subregion, Limpopo Province, RSA). The plant was identified by Mr Gololo (traditional healer) and the identity was confirmed by the University of Limpopo Herbarium.

Extraction

The roots were dried at room temperature and ground to a fine powder using a grinder (ML 90L4, Monitoring and Control Laboratories (Pty) Ltd, RSA). The ground powder (150 g) was extracted with absolute acetone using the cold extraction method. The acetone extract was filtered and concentrated using a rotary evaporator (Büchi Labotec rotavapor model R-205, Germany) at 40°C. The concentrated extract was transferred into pre-weighed beakers and dried under a stream of air and weighed.

Total phenolic content

Total phenolic content of the acetone extract of the roots of *Senna italica* was determined using Folin-Ciocalteu method as described by Abdille *et al.* (2005). Two hundred microlitre of 1:10 diluted sample was added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 mins of incubation, 800 µl of sodium carbonate (75 g/l) was added and incubated for 2 hrs at room temperature. The absorbance was measured at 765 nm using a microtitre plate reader (Multimode detector DTX 880, Beckman-Coulter). Tannic acid (0 – 10 mg/ml) was used for plotting a standard curve. Results were expressed as tannic acid equivalents (TAE)/g dry weight of plant material.

Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F254). The TLC plates were developed with one of the three eluent systems, i.e., ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/-ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour by lining the tank with filter paper wetted with the eluent.

TLC analysis of the extracts

Visible bands were marked under daylight and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600) before spraying with freshly prepared *p*-anisaldehyde (1 ml *p*-anisaldehyde, 18 ml ethanol, 1 ml sulphuric acid) or vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagents (Stahl, 1969). The plates were carefully heated at 105°C for optimal colour development.

Bioassays

Antiproliferative activity

The antiproliferative activity of the extract was evaluated on Jurkat (clone E6-1) T cells. Jurkat T cells (ATCC, TIB-152) were grown in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) and 1% penicillin, streptomycin, neomycin (PSN) cocktail at 37°C in a 5% CO₂ 95% humidified atmosphere. For cytotoxicity effects, Jurkat T cells were seeded at 2×10^4 cells/ml in a 12-well culture flask and then treated with different concentrations (0; 100; 200, 400, 800 µg/ml) of the extract. The extract was filtered (Millex 0, 22 µm filter) to sterility before treating the experimental cultures. Cell viability and number were assayed at 24hrs interval for 72 hrs using trypan blue dye exclusion method (Altman *et al.*, 2008). The assay is based on the principle that live cells possess intact cell membranes that excludes trypan blue dye, whereas dead cells allow the dye to penetrate through, thus staining the dead cells blue. The cell suspension is simply mixed with the dye and then visually examined under a light microscope to determine whether cells take up or exclude the dye; a viable cell will have a clear cytoplasm whereas nonviable, dead cells will have a blue cytoplasm. Dimethyl sulphoxide (DMSO) (0.01%) was used as a control in addition to untreated cell control, since the extract was suspended in DMSO.

Free radical scavenging activity

Qualitative 2, 2 -diphenyl-2-picrylhydrazyl (DPPH) assay on TLC

The compounds present in extracts were separated by TLC as described earlier. The chromatograms were dried in a fume hood. To detect antioxidant activity, chromatograms were sprayed with 0.2% 2, 2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma®) in methanol, as an indicator (Deby and Margotteaux, 1970). The presence of antioxidant compounds were detected by yellow spots against a purple background.

Quantitative DPPH radical scavenging activity assay

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of *Senna italica* fractions were determined according to the method described by Katsube *et al.* (2004). The assay involves the measurement of the disappearance of the coloured free radical, DPPH, by spectrophotometric determination. Serial dilution from 2 to 250 µg/ml was done using distilled H₂O; 50 µl of the prepared concentrations was pipetted into a 96-well plate. An equivalent volume of ascorbic acid (vit C) at a concentration of 250 µg/ml was used as a positive control. One hundred and eighty five microliters of DPPH solution dissolved in 50% methanol in water was added to each well and the plate gently shaken for 20 mins at room temperature. The change in absorbance at 550 nm was measured using a microtitre plate reader (BIO-RAD model 550, Japan). The decrease in absorbance indicated the increase in radical scavenging activity.

Antibacterial activity

Quantitative antibacterial activity assay by minimum inhibitory concentration (MIC)

The microplate serial dilution method (Eloff, 1998a) was used to determine the minimum inhibitory concentration (MIC) of extracts against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. Extracts (10 mg/ml) were dissolved in acetone and serially diluted with sterile water in microplates in a laminar flow cabinet. The same volume of an actively growing culture of the test bacteria was added to the different wells and cultures were grown overnight in 100% relative humidity at 37°C. As an indicator of growth, 40 µl of 0.2 mg/ml of p-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells. Growth was indicated by a violet colour of the culture. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control acetone had no influence on the growth at the highest concentration used (25%). Ampicillin was used as positive control. Densities of bacterial cultures for use in the screening procedures were as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml.

Total activity of the extracts

The total activity in ml/g was calculated by dividing the MIC value with the quantity extracted from 1 g of plant material. The resultant value indicates the volume to which the extract can be diluted and still inhibits the growth of the bacterial isolate (Eloff, 2004).

Qualitative antibacterial activity assay by bioautography

The bioautography procedure described by Begue and Kline (1972) was used. TLC plates were prepared, developed in the different solvent systems and dried for 2 to 3 days under a stream of air to remove residual solvent, which might inhibit bacterial growth. The plates were sprayed with one of four of test bacterial cultures. Ten milliliters of highly dense fresh bacteria culture was centrifuged at 5300 x g for 20 mins to concentrate the bacteria. The supernatant was discarded and the combined pellet resuspended in 2 – 4 ml of fresh Müller-Hilton broth. The plates were sprayed with the concentrated suspension until they were just wet, air-dried to remove excess liquid, and incubated overnight at 37°C in 100% relative humidity. After incubation, plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma Chemicals). Clear zones on chromatograms indicated inhibition of growth after incubating for about 1 h at 37°C (Begue and Kline, 1972).

Results and discussion

The continuous development of antibiotic resistance of pathogenic microorganisms is a major health concern worldwide. The screening of plant materials and their isolated substances for new antimicrobial compounds represent an important source for new effective medicines. In this study, the *Senna italica* acetone extract was used to evaluate its inhibitory properties on selected pathogenic bacteria. Two standardized techniques were used to determine the quantitative (microdilution method) and qualitative (bioautography) antibacterial activity. .

Table 1: The yield from 150 g and the total phenolic content of the acetone extract of the roots of *Senna italica*

Yield (g)	Yield (%)	Phenolic content TAE/g dry weight (mg/ml)	% Phenolic content (w/w)
2.8	1.87	24.08	0.86

Table 2. Minimum inhibitory concentrations (MICs) values of the acetone extract of *Senna italica* tested on various microorganisms

Microorganism	MIC value (mg/ml)	
	Acetone extract	Ampicillin
<i>P. aeruginosa</i>	0.16	0.13
<i>E. faecalis</i>	0.078	0.16
<i>E. coli</i>	0.16	0.08
<i>S. aureus</i>	0.078	0.16
Average	0.12	0.13

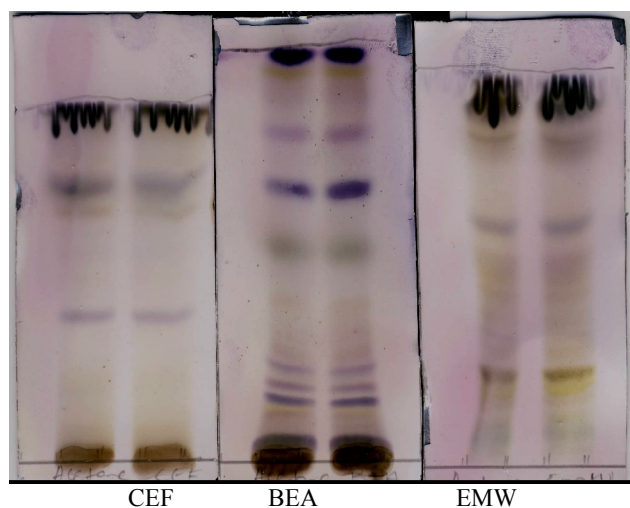


Figure 1a: The chromatograms of crude acetone root extract of *Senna italica* separated by the three different solvent systems. with BEA (non-polar), CEF (intermediate polarity) and EMW (polar). The individual compounds were visualized with *p*-anisaldehyde.

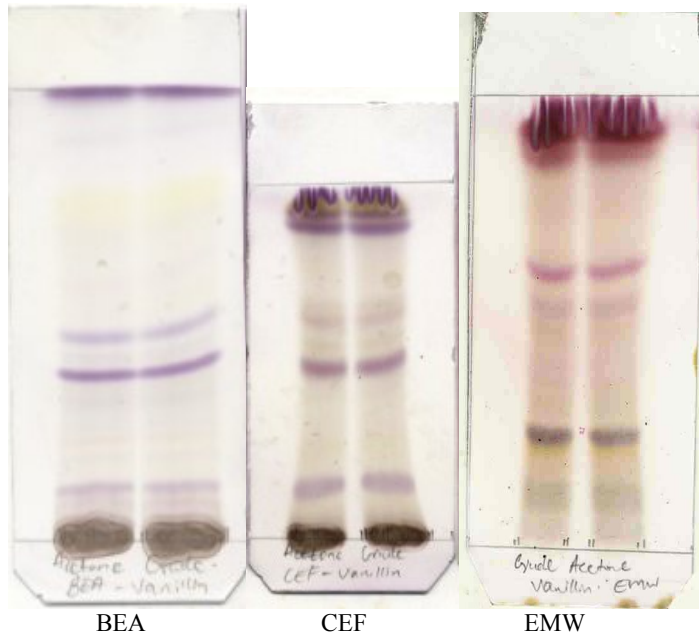


Figure 1b: The chromatograms of crude acetone root extract of *Senna italica* separated with BEA, CEF and EMW and visualized with vanillin-sulphuric acid.

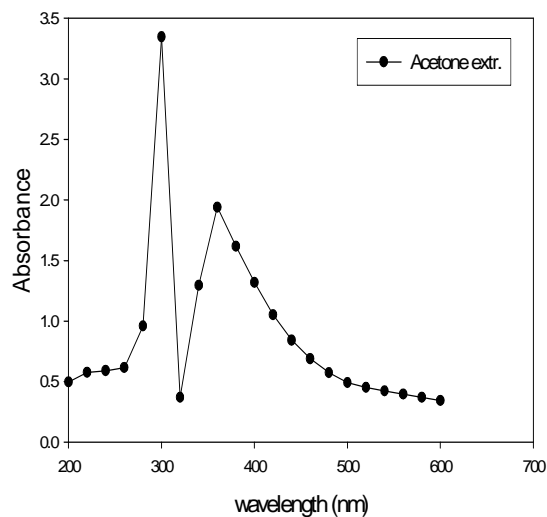


Figure 2: The UV-Visible spectral profile of the acetone extract of the roots of *Senna italica*. Major absorption of light was shown at a range of 240 nm to 450 nm which suggests the presence of flavonoids, prosalins or caretenoids.

Roots of *Senna italica* (150 g) was extracted with acetone and yielded 2.4 g (1.87%) material (Table 1). The choice of the extraction solvent for biological activity screening is a very important step. This choice is influenced by the type of bioassays envisaged and the ease of working with a particular extractant. Practitioners of indigenous medicine mainly use water (boiled water) to extract bioactive compounds. The use of water as the extractant is mainly based on its non-toxic nature towards human beings and, in particular, it is the only available extractant at the disposal of rural people. In the present study, we chose acetone as our extractant for a number of reasons, *viz*: acetone was found to be a good extractant because of its nontoxicity towards test organisms during bioassays; its ability to extract compounds across different polarities and its easiness to remove from extracts (Eloff, 1998b).

The total phenolic content of the extract as determined by the Folin-Ciocalteu method was 0.86% (Table 1). The determination of the total phenolic content of the plant's extract through the Folin-Ciocalteu and

other methods is not absolute. However, it is very significant to determine the phenolic compounds contributing to the biological activities of the extract. The acetone extract of *Senna italica* showed reducing capacity relative to tannic acid, thus signifying the presence of phenolic compounds. We can therefore deduce that some of the biological activities of *Senna italica*, as demonstrated in the current study, could be due to the presence of these phenolic compounds. The linkage between the biological activity of plant extracts and their phenolic content has been reported before (Frankel et al., 1995).

The three mobile phase systems (BEA, CEF and EMW) used in this study separated compounds according to their polarities and were visualized with the anisaldehyde (Figure 1a) and vanillin spray reagents (Figure 1b). The acetone extract of the roots of *Senna italica* contains compounds with a wide range of polarities as seen by the separation of compounds by the three mobile phases with wide differences in polarity. This observation supports the conclusion by Eloff (1998b) that acetone is a good extractant in phytochemistry studies as it extracts compounds of widely varying polarity.

The UV-Visible spectral profile of the acetone extract of *Senna italica* (Figure 2) shows the presence of a number of compounds. These compounds absorbed light mostly within the range of 280 nm to 450 nm which could be due to the presence of flavonoids. Our observations, as shown in Figure 2, suggest the presence of phenolic type of compounds which absorb light between 280 nm and 450 nm. This observation provides a qualitative confirmation of the phenolic content of the extract as determined by the Folin-Ciocalteu method (Table 1).

The antiproliferative properties of the acetone extract of the roots of *Senna italica* were evaluated on cancerous Jurkat T cells (Figures 3a and 3b). It was demonstrated that the acetone extract inhibited the proliferation and viability of Jurkat T cells in a dose- and time-dependent manner. The observation of cancerous cell-death is a bioassay model that indicates the potential of the extract to inhibit the progression of cancer. The progression of cell proliferation is halted by the arrest of the cell division cycle at one of the checkpoints (either G₁/S or G₂/M interphases) in the cell division cycle. The arrest is mainly triggered by the irreparable or repairable damage in the cell's DNA. In case of an irreparable DNA damage, the cell death pathways are triggered. The cell death could either be apoptotic or necrotic. The apoptotic cell death is the preferred mode of cellular demise than the necrotic pathway. The determination of the mode of cell death triggered by the extract of *Senna italica* is not dealt with in this study and follow-up studies are in progress to explore this aspect.

Dietary antioxidants capable of scavenging free radicals are believed to reduce the risk of disease and hence it is important to determine the radical-scavenging effects of antioxidants in *Senna italica*. This study demonstrated that the roots of *Senna italica* possess compounds with antioxidative activity against the free radical, DPPH. This is demonstrated by several yellow bands appearing on the chromatograms against the purple DPPH background (Figure 4a). The radical scavenging activity was further quantified using vitamin C as the standard (Figure 4b). The free radical scavenging activity of the plant extract contributes to the plant's antioxidant properties. Previous study indicated that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc) and aromatic amines reduce and decolourise DPPH through their hydrogen donating ability (Abdille et al., 2005). Thus, the acetone extract of the roots of *Senna italica* may possess a similar group of compounds with hydrogen donating ability which reduced DPPH (Figure 4a).

The number of antibacterial compounds in the acetone extract of the roots of *Senna italica* was determined using the bioautography method (Begue and Kline, 1972) and the activity was quantified through the microtitre plate method (Eloff, 1998a). The current study demonstrated that the acetone extract of *Senna italica* had a similar antibacterial activity against Gram-negative and Gram-positive bacteria (Table 2); MIC values of the extract against *P. aeruginosa*, *E. faecalis*, *E. coli* and *S. aureus* were 0.16, 0.078, 0.16 and 0.078 mg/ml, respectively. The results indicate that the activity is probably not related to the cell membrane metabolism because differences in sensitivity between Gram-positive and Gram-negative bacteria are frequently attributed to the differences in membrane morphology between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane containing lipopolysaccharide components and the Gram-positive bacteria only have an outer peptidoglycan layer which is not as an effective permeability barrier as the former.

Bioautography was used to determine the number of antibacterial compounds in order to obtain more information on the diversity of antibacterial compounds which maybe present in the acetone extract of *Senna italica*. Inhibition zones indicative of antibacterial activity are observed as white spots on a purple-red background. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds which inhibited the growth of the tested bacteria.

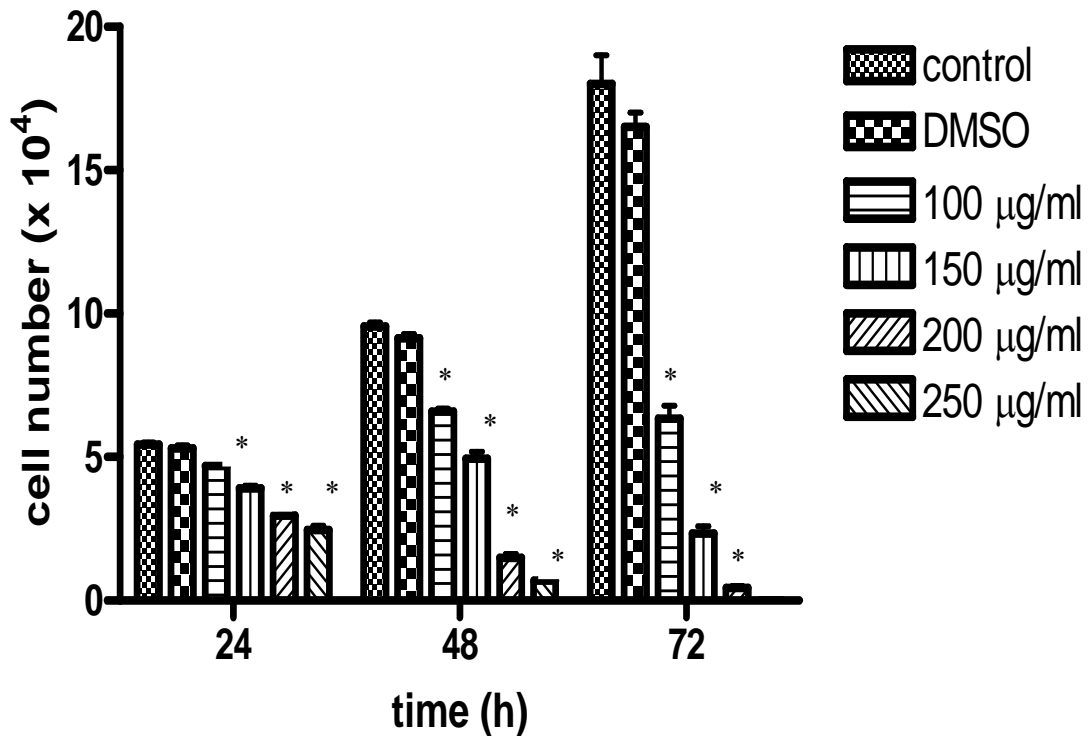


Figure 3a: The effect of the acetone extract of the roots of *Senna italica* on the proliferation of Jurkat T cells treated with increasing concentrations of crude acetone extract at 24 h intervals (* $p < 0.01$). Cell growth was inhibited in a dose- and time-dependent manner.

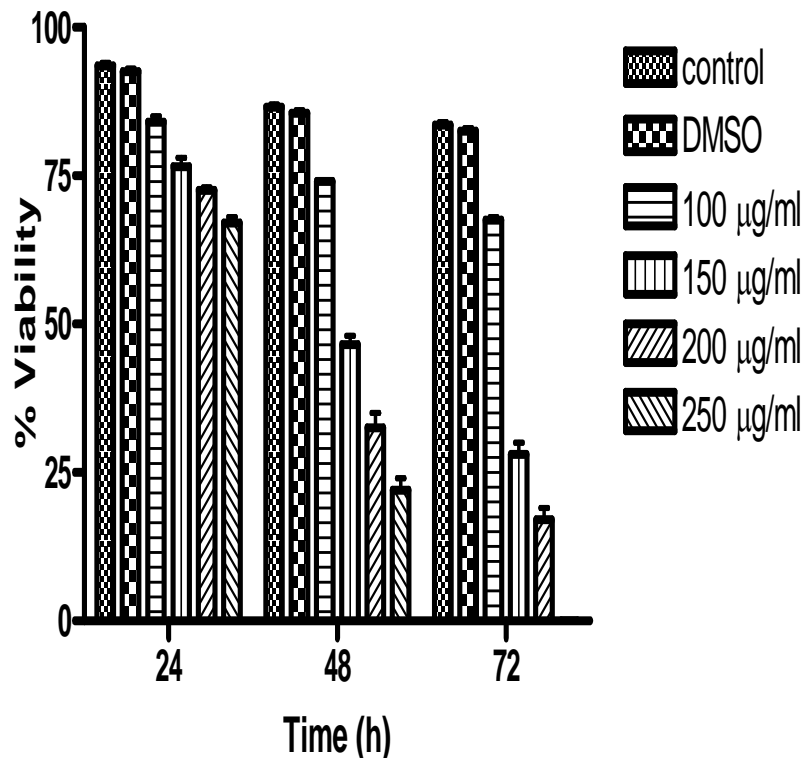


Figure 3b: The effect of the acetone extract of the roots of *Senna italica* on viability of Jurkat T cells treated with increasing concentrations of crude acetone extract at 24 h intervals (* $p < 0.01$). Cell viability was reduced in a dose- and time-dependent manner.

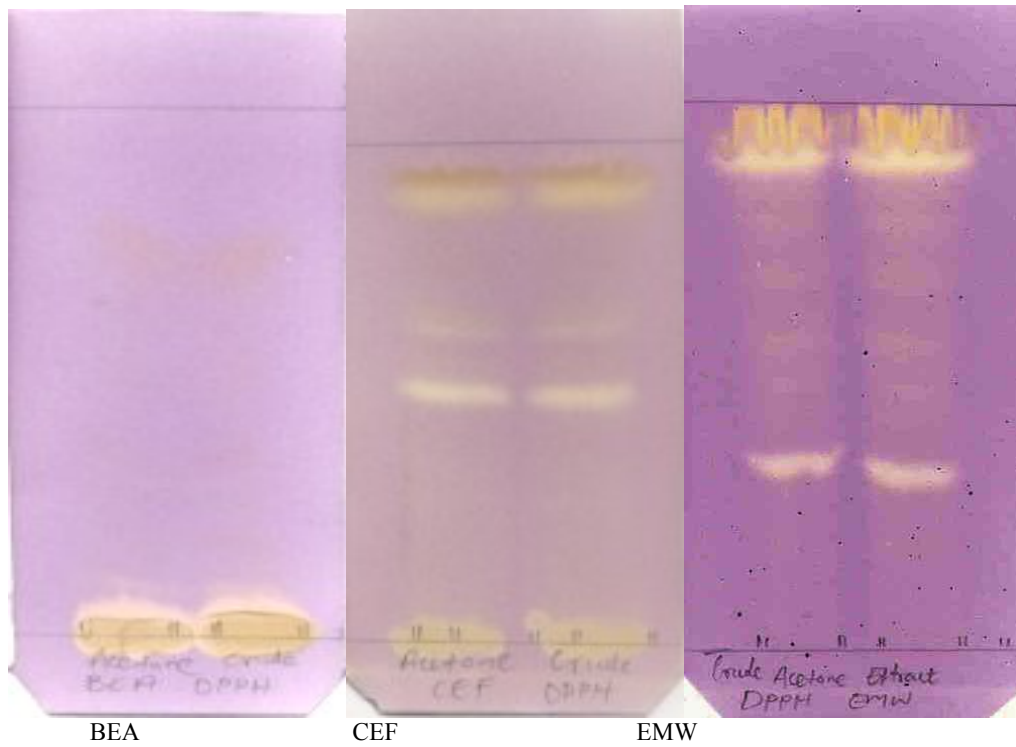


Figure 4a: The free radical scavenging power of the acetone extract of the roots of *Senna italica* evaluated by the DPPH assay. The yellow zones on the chromatograms developed with the different solvent systems indicate compounds with free radical scavenging activity.

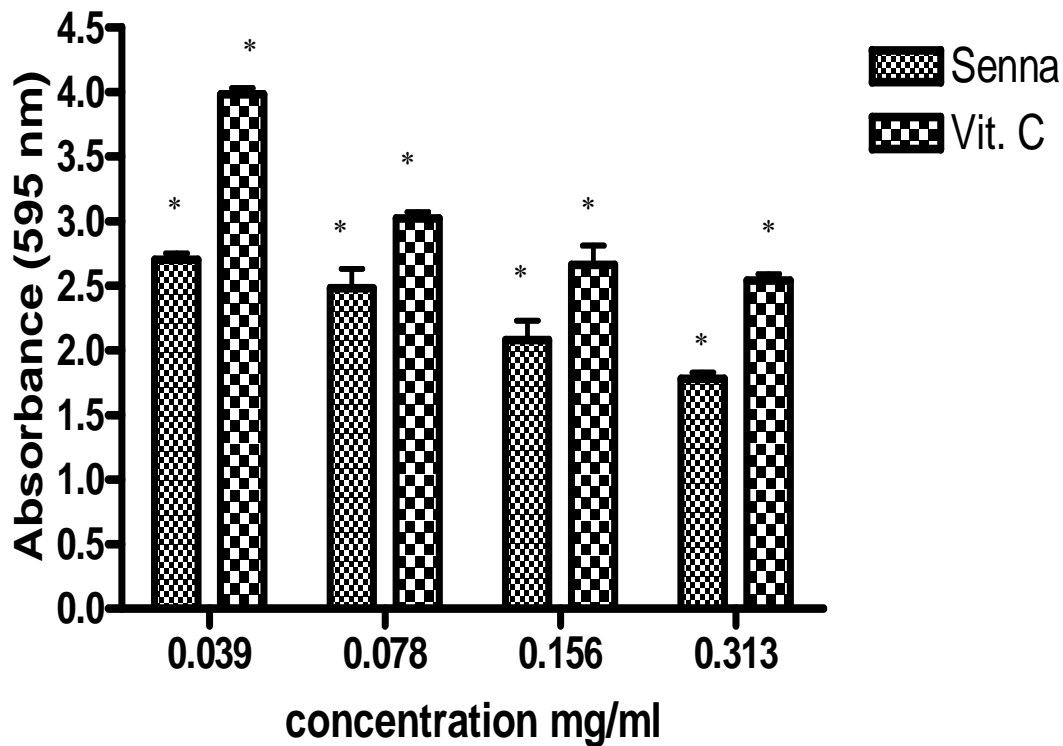


Figure 4b: The comparison of the free radical scavenging power of the acetone extract of the roots of *Senna italica* with vitamin C (* $p < 0.01$). Low absorbance values indicate high free radical scavenging power.

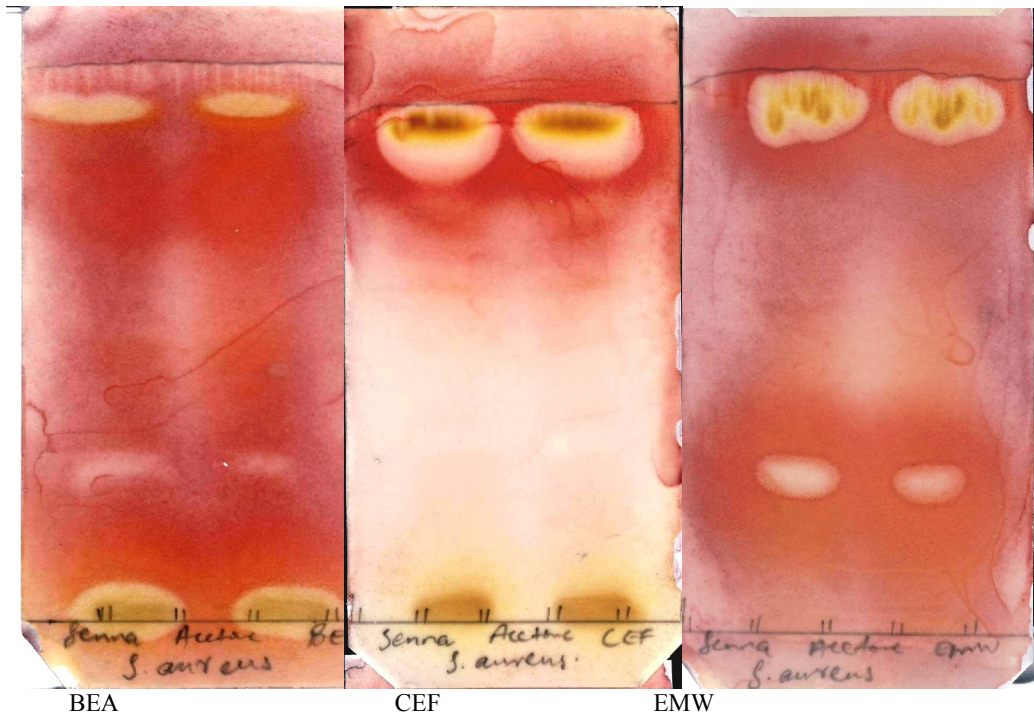


Figure 5a: The antibacterial activity of the acetone extract of the roots of *Senna italica* evaluated on *E. faecalis*. The clear zones on the bioautograms indicate areas where inhibition of bacterial growth occurred.

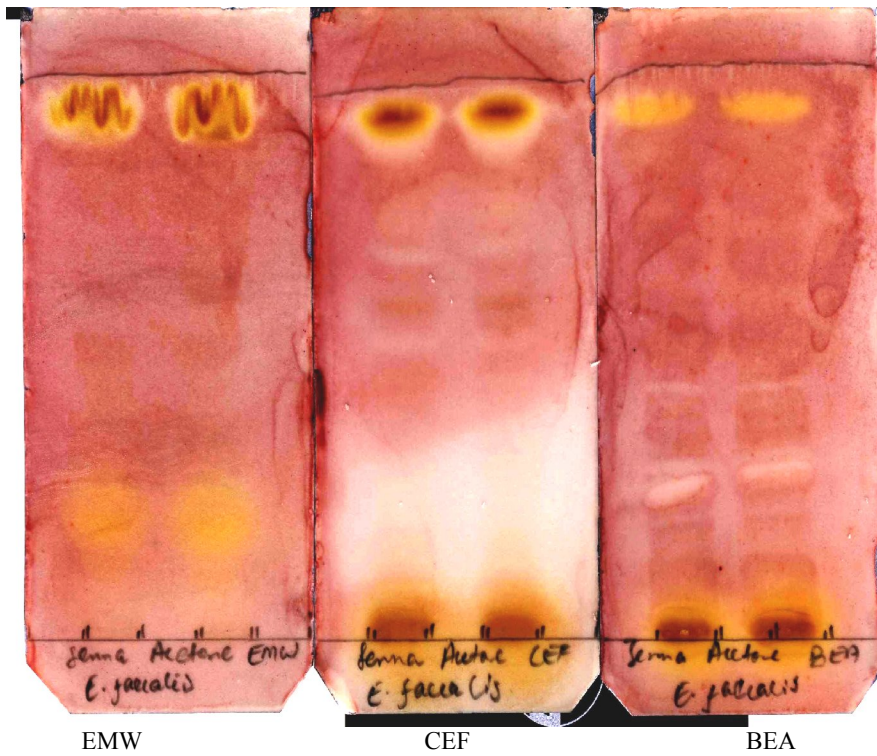


Figure 5b: The antibacterial activity of the acetone extract of the roots of *Senna italica* evaluated on *S. aureus*. The clear zones on the chromatograms indicate areas where inhibition of bacterial growth had occurred.

Bioautograms demonstrated strong inhibition zones for *Senna italica* against the growth of *E. faecalis* (Figure 5a) and *S. aureus* (Figure 5b). The clear zones were located at different R_f values on bioautograms suggesting that more than one compound is involved in the antibacterial activity. There was no inhibition zone present on the bioautochromatograms of *Senna italica* acetone extracts covered with *E. coli* (results not shown). A number of antimicrobial compounds were observed with distribution across a range of polarities. All solvent systems which were used in bioautography assays (i.e., BEA, CEF and EMW) separated compounds with antibacterial activity. In some cases organisms did not grow too well and it was difficult to detect inhibition zones i.e., CEF (Figure 5a). The most likely explanation is that there were still traces of formic acid left on the chromatogram that inhibited the bacterial growth. Observations made on figures 5a and 5b suggest that the extract contains several antibacterial compounds that may be worth isolating and identifying.

The crude extract had an activity close to that of ampicillin. The total activity was calculated by dividing the quantity obtained from 1 g of plant material (1.87% = 18.7 mg/g) with the MIC in mg/ml (Eloff, 2004). The values of 234 and 117 ml/g indicates that the antibacterial compounds present in 1 g could be diluted to more than 100 ml and would still kill the test organisms. This proves the substantial value of such an extract if there are no safety problems.

Conclusion

The results of the present work indicate that the *Senna italica* extract had substantial antibacterial activity in the same order as ampicillin for the four test organisms. The *Senna italica* extract also had antiproliferative and antioxidant properties. These findings support the use of this plant in folk medicine for the treatment of some diseases that are related to bacterial infections. Further studies aimed at isolating and characterizing active compound(s) responsible for the antibacterial effects from *Senna italica* are currently underway (Gololo, 2008).

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