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ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN: 0976-3376

Asian Journal of Science and Technology
Vol. 5, Issue 7, pp. 395-399, July, 2014

RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND INHIBITION STUDIES OF THE ETHANOLIC AND AQUEOUS EXTRACTS OF *VERNONIA AMYGDALINA* USING MICROBES ISOLATED FROM REFUSE DUNG HILL AS TEST ORGANISMS

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ARTICLE INFO

Article History:
Received 26th April, 2014
Received in revised form
01st May, 2014
Accepted 05th June, 2014
Published online 31st July, 2014

Key words:
Aqueous extract,
Ethanolic extract,
Inhibition,
Phytochemical,
V. Amygdalina,

ABSTRACT

Phytochemical analysis of the ethanolic and aqueous leaf, stem and root extracts of *V. amygdalina* showed that it contains saponins, cardiac glycosides, alkaloids, phenolics, flavonoids, terpenes and deoxy-sugar in different proportions. Out of the seven (7) phytochemicals present, only three (phenolics, flavonoids and de-oxysugar) were present in all the sample extracts, with variations in the other four (4). Five (5) bacteria (*Staphylococcus epidermidis, Bacillus subtilis, B. megaterium, Klebsiella aerogens and S. aureus*) and three (3) fungus *(Aspergillus flavus, A. fumigatus, Candida albicans)* were isolated from the refuse dunghill and used as test isolates against the sample extracts. The ethanolic extracts of *V. amygdalina* showed more inhibition activity against all the bacterial and fungal isolates than the aqueous extracts. In conclusion, the leaf, stem and root extracts of *V. amygdalina* is a good antimicrobial agent which has both cidal and static effects against bacterial and fungal isolates found in the environment, especially contaminated soils with highest antimicrobial potential observed in the ethanolic extracts

INTRODUCTION

The antimicrobial properties of many plants have been investigated by a number of researcher's worldwide (Lai, 2004; 2005; Latha and Kannabiran, 2006). Medical uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoction from the plants (Nair et al., 2005; Ogbulie et al., 2007). Medicinal plants were used as excellent antimicrobial agents because they possess a variety of chemical constituents. Recently, much attention is being directed towards extracts and biologically active compounds isolated from popular plant species (Prince and Prabakaran, 2011). *Vernoniaamygdalina,* a member of the Asteraceae family, is a woody shrub or small tree of 2-5m with petiolate leaf of about 6cm in diameter and elliptic in shape. The leaves are green with characteristic odour and bitter taste.*V. amygdalina*, is also known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo) (Odugbemi, 2006). The leaves are dark green coloured with a characteristic odour and a bitter taste. The species is indigenous to tropical Africa and is found wild or cultivated all over sub- Saharan Africa (Odugbemi, 2006). The stem, root and bark are used as chew sticks in many West African countries like Cameroon, Ghana and Nigeria (Burkill, 1985; Hamowia, 1994). The leaves are eaten, after crushing and washing thoroughly to remove the bitterness (Hamowia et al., 1994). *V. amygdalina* have been in many homes in the Eastern and Western parts of Nigeria as food especially in the preparation of soups.

The characteristic bitter taste is believed to have after taste of sweetness. The peeled stem is often used as chewing stick for cleaning the teeth and is very effective as anticaries (Yedjou, et al., 2008). The bitterness of the leaves is also exploited by nursing mothers to assist in weaning their babies by rubbing the juice on their breast (Iwu and kokwaro, 1996). The plants leaves and other parts have been used solely or mixed with other plants for the treatment of various suspected illnesses. It is also documented that *V. amygdalina* has been used traditionally in blood clotting and has elicited a significant reduction in blood glucose levels at post-prandial time point (Uchenna et al 2008). Fasola et al (2010) has also reported that *V. amydalina* has hypoglycemic activities. This work investigated the inhibitory studies of *V. amygdalina* using microbial isolates from dunghill as test organisms.

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MATERIALS AND METHODS

Collection and processing of plant parts

Fresh part of the plant (leaves, stems and roots) were collected from the wild of green forest vegetation at Ogbomoso after it has been identified by experts. Each plant parts was harvested and cleaned by washing under tap water. The stems and roots were cut into small pieces, dried at room temperature until when dry. The dried plant materials were further grinded into smaller pieces and then kept away from moisture by storing in a polyethylene bag until needed for extraction.

Extraction of plants parts

About 20g of each of the sample was weighed into a 500ml conical flask containing 200ml of the solvent. Extraction was done in a water-bath for about 60mins and left to stand over-night. The solution extract was filtered using No. 1 Whatman filter paper. The residue was withdrawn, while the filtrate was allowed to dry at 80^{o}C until the moisture and solvent content evaporated leaving the solid extract which was stored in a sterile universal bottle and preserved at 4^{o}C.

Collection of soil samples and isolation of micro-organisms

Soil samples were collected randomly from different parts of a refuse dunghill containing waste from various sources such as; hospital, agriculture, household, e.tc. Serial dilution of the sample was carried out followed by isolation of the organism using Nutrient agar (NA) for bacteria and Potatoes dextrose agar (PDA) for fungi. Pure colonies of the organisms were obtained, followed by identification of the organism obtained using standard technique as described by Cowan, 1999. The bacteria and fungi isolated were preserved inside slant bottles containing nutrient agar (NA) and potato dextrose agar (PDA), and then kept inside the refrigerator at 4^{o}C until when needed.

Phytochemical analysis of the extracts

Phytochemical analysis was carried out on the aqueous and ethanolic extracts using standard procedures to identify the constituents as described by Sofowara (1999), Harborne (1973).Different concentration of the crude extracts of each of the leaves, stem and root (50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml, 250mg/ml and 300mg/ml) were prepared into Empenderoff tubes for inhibition studies.

Inhibition study of the sample extracts

The antimicrobial activities of the sample extracts were done on bacteria and fungi isolated from the refuse dung-hill that receives wastes from different environmental sources. This was carried out using the standard agar well diffusion technique as described by Prashanth (2011). Different concentrations of the sample extracts were loaded on cut wells of pre-sterilized nutrient agar medium of each Petri-plate containing swabbed isolates and plates were incubated at 37^{o}C for 24-48 hours and 27^{0}C for 48-72 hours for bacteria and fungi respectively for observing zones of inhibition according to Prashanth et al (2011). A zone of clearance round each well signifies inhibition and the diameter of such zones were measured in millimeter (mm).

RESULTS AND DISCUSSION

Secondary compounds, which include tannins, saponins, cardiac glycosides and alkaloids, have been reported by Ahmad and Beg (2001) and other researchers, to be present in higher plants. Results of our preliminary phytochemical screening of the extracts of *V. amygdalina* revealed the presence of some of these compounds such as; saponins, cardiac glycosides, alkaloids, phenolics, flavonoids, terpenes and deoxy-sugar and the absence of tannins, steroids and phylobatannins as shown in table 1.

Table 1. Phytochemical Analysis of Aqueous and Ethanolic Extracts of *V.mygdalina*

Bioactive substance	Plant Extracts					
	LAE	LEE	SAE	SEE	RAE	REE
Saponins	++	-	++	-	++	+
Cardiac glycosides	+	-	+	-	++	-
Tannins	-	-	-	-	-	-
Steroids	-	-	-	-	-	-
Alkaloids	-	-	-	+	++	++
Phylobatannins	-	-	-	-	-	-
Phenolics	++	+	++	++	+	++
Flavonoids	+++	+	+	++	+	++
Terpenes	-	-	-	-	+	-
Deoxy-sugar	+++	++	+++	++	+++	+

Out of the seven (7) phytochemicals present, only three (3) (phenolics, flavonoids and de-oxysugar) were present in all the sample extracts, with variations observed in the other four (4).

LAE= leaf aqueous extract; LEE= leave ethanolic extract; SAE= stem aqueous extract;
SEE= stem ethanolic extract; RAE= root aqueous extract; REE= root ethanolic extracts;
(-) not present; (+) present in low concentration; (++) present at moderate concentration;
(+++) present at high concentration.

Five (5) bacteria (*Staphylococcus epidermidis, Bacillus subtilis, B. megaterium and Klebsiellaaerogens)* and three (3) fungus *(Aspergillusflavus, A. fumigatus, Candida albicans)* were isolated from the refuse dunghill and used as test isolates against the sample extracts. The sample extracts had profound activities against both Gram-positive and Gram negative bacteria. Our results showed different variations in the degree of antibacterial activities of the extracts against the all the test isolates; the outcome which supports the report of Ijeh and Adedokun (2006) that plant possesses antibacterial or antimicrobial substances. Tables 2 and 3 showed similar antimicrobial activities of the sample extracts against *S. aureus* and *S. epidermidis* respectively in which all the ethanolic extracts showed varying zones of inhibition (ZI), whereas only the root aqueous extract (RAE) of *V. amygdalina* showed the same with the exceptions of the leaf and shoot aqueous extracts. In addition, tables 4 and 5 also showed the same pattern of inhibiton of *B. subtilis* and *K. aerogenes* respectively in which there was inhibition in the root aqueous extract (RAE), although contrary to the aqueous extracts in which no inhibition was observed, except for leaf aqueous extract (LAE). Table 6 explains the antimicrobial activities of the sample extracts against *B. megaterium* in which ZI was recorded in both the ethanolic and aqueous sample extracts.

Table 2. Antimicrobial activities of extracts against *Staphylococcus aureus*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	2.45	1.09	1.60	ni	ni	ni
100	3.12	2.45	2.34	ni	ni	1.18
150	3.09	2.32	3.25	ni	ni	1.59
200	4.67	3.26	3.72	ni	ni	3.44
250	4.78	3.52	4.55	ni	ni	4.36
300	5.84	4.57	5.82	ni	ni	5.86

ni = no inhibition

Table 3. Antimicrobial activities of extracts against *Staphylococcus epidermidis*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	1.03	0.53	1.14	ni	ni	ni
100	1.12	1.05	2.45	ni	ni	1.34
150	2.56	3.21	3.38	ni	ni	1.68
200	3.87	4.24	4.41	ni	ni	3.32
250	3.90	4.76	4.78	ni	ni	3.71
300	4.88	5.42	5.93	ni	ni	4.82

ni = no inhibition

The ethanolic extracts of all the sample extracts gave the largest ZI in all the test isolates- the result of which is in alignment with the work of Ijeh and Adedokun (2006) and Akinyemi, (2005). Generally, the ethanolic extracts of *V. amygdalina* showed more activity against all the bacterial isolates than the aqueous extracts. It appears that the active compound responsible for antimicrobial properties do not decide easily in aqueous solution. Eloff (1998) had earlier reported that most active components are not water soluble. In addition, this may be due to the higher volatility of the ethanol which possesses greater extraction potential of the bioactive compounds from the samples Ibekwe et al (2000).

Table 4. Antimicrobial activities of extracts against *Bacillus subtilis*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	ni	ni	1.16	ni	ni	ni
100	ni	1.24	1.22	ni	ni	ni
150	1.42	2.26	2.34	ni	ni	ni
200	2.51	4.56	4.61	ni	ni	ni
250	3.24	8.62	8.51	ni	ni	ni
300	4.87	8.67	8.58	ni	ni	ni

ni = no inhibition

Table 5. Antimicrobial activities of extracts against *Klebsiella aerogens*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	1.52	ni	ni	ni	ni	ni
100	3.35	1.53	ni	ni	ni	ni
150	5.41	2.48	ni	ni	ni	ni
200	6.78	4.52	1.27	ni	ni	ni
250	8.25	5.32	1.83	ni	ni	ni
300	8.58	5.74	2.47	1.56	ni	ni

ni = no inhibition

Table 6. Antimicrobial activities of extracts against *Bacillus megaterium*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	1.45	2.03	3.16	1.26	2.04	1.53
100	3.21	5.12	4.28	3.27	5.27	3.28
150	3.54	7.22	5.43	3.58	7.22	4.61
200	4.55	8.18	5.50	5.18	8.31	8.53
250	5.68	8.54	5.50	5.23	8.45	8.57
300	5.73	8.73	6.34	5.51	8.67	8.62

ni = no inhibition

The antibacterial susceptibility test of *V. amygdalina* showed that the ethanolic extracts of the plants has higher inhibition onmicrobial pathogens such *E. coli*, *Bacillus cereus, Shigelladysentriae and Salmonella typhimurium* as compared to the aqueous extract with low inhibition. Moreover, the higher activity of the ethanolic extracts verifies the use of the ethanolic extraction method by local herbalists (Allero and Afolayan, 2006). One of the factors that affect microbial susceptibility is the concentration of the activity component; the more the concentration, the higher the activity of the chemical substance, thus higher concentrations of the extracts showed higher ZI (Table 2-9). The ethanolic sample extracts showed antifungal activity against *A. flavus*, *A. fumigatus* and *C. albicans* as shown in tables 7, 8 and 9 respectively, with the ethanolic extract showing greater inhibition as compared to the aqueous extract. Since the sample extract brought about a remarkable reduction in the growth of *A. flavus and A. fumigatus,* this suggests that the leaf, stem and root of *V. amygdalina* can might be used to make concoction for the treatment of diseases such as Aspergillosis and some fungal infections caused by the afore-mentioned organisms. It is possible that the presence of some impurities in the crude sample extracts might have lowered the inhibition potency of the crude extracts, which when removed will exhibit higher potency with greater inhibition even if compared with the conventional commercial antibiotics (Slayer and Whiff, 1994). Therefore, if the crude sample extracts is further purified, the active components might give higher and clearer zones of inhibition.

Table 7. Antimicrobial activities of extracts against *Aspergillus flavus*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	2.41	1.36	1.39	ni	ni	1.17
100	2.85	1.47	2.41	ni	ni	2.25
150	3.56	1.62	3.38	ni	ni	3.41
200	5.38	1.68	3.74	ni	ni	3.67
250	5.42	3.52	4.27	ni	ni	3.87
300	5.90	6.42	5.83	1.51	ni	4.53

ni = no inhibition

Table 8. Antimicrobial activities of extracts against *Aspergillus fumigatus*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	ni	2.05	ni	ni	ni	1.17
100	ni	2.17	1.48	ni	ni	2.36
150	ni	2.36	1.66	ni	ni	2.71
200	ni	2.52	2.60	ni	ni	3.29
250	ni	3.57	3.38	ni	ni	3.47
300	ni	3.83	4.74	ni	ni	4.18

ni = no inhibition

Table 9. Antimicrobial activities of extracts against *Candida albicans*

Extract concn. mg/ml	Zone of inhibition (mm)					
	LEE	SEE	REE	LAE	SAE	RAE
50	2.28	2.34	1.38	ni	ni	1.58
100	2.64	3.33	1.67	ni	ni	2.41
150	5.38	3.75	2.52	ni	ni	3.84
200	5.75	3.78	3.36	ni	ni	4.59
250	5.83	4.52	3.85	ni	ni	5.67
300	6.41	5.72	4.64	ni	ni	5.85

ni = no inhibition

Conclusions

In summary, the leaf, stem and root extracts of *V. amygdalina* is a good source of antimicrobial agent which has applications in chemotherapy with both cidal and static effects against bacterial and fungal isolates found in the environment, especially in contaminated soils. This work also revealed that ethanol is a better solvent in the extraction of bio-active ingredients embedded in *V. amygdalina*. The antimicrobial study of the plant extracts demonstrated that traditional medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of this plant in traditional medicine suggests that they represent an economic and safe alternative to treat infectious diseases (Yedjou, et al., 2008). The plant can thus be recommended for the treatment of diseases caused by microorganisms. Although, the scope of this work did not extend to purification of the crude extracts. If the extracts are further purified, an increase in their antimicrobial activity is likely to be enhanced. Finally, the structure of the bioactive components can further be investigated with the view to using it, in the production of synthetic drugs.

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