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SYNERGISTIC IN-VITRO ANTHELMINTIC POTENTIALS OF VERNONIA AMYGDALINA DELILE STEM AND CARICA PAPAYA LIN. SEEDS

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ABSTRACT

Vernonia amygdalina and *Carica papaya* are medicinal plants used ethnobotanically for treatment of various conditions including helminthes infestations. Ethanol extracts of *V. amygdalina* stem (EVAS) and *C. papaya* seeds (ECPS) and chloroform extracts of *V. amygdalina* stem (CVAS) and *C. papaya* seeds (CCPS) were evaluated for synergistic in-vitro anthelmintic activity. After cold maceration of both plant materials, percentage yields of 2.398%, 4.494%, 2.480% and 3.892% were obtained for CVAS, EVAS, CCPS and ECPS respectively, which could be due to polarity difference of solvents used. Preliminary phytochemical screening on the extracts from both plants revealed the presence of phyto-constituents such as tannins, saponin glycosides, reducing sugars, alkaloids, steroids and flavonoids in all the plant extracts (EVAS, ECPS, CVAS and CCPS). There was the presence of terpenoids in EVAS and ECPS but absent in CVAS and CCPS. All the extracts (EVAS, ECPS, CVAS and CCPS) however showed the absence of anthraquinones, cardiac glycosides and cyanogenetic glycosides. Five concentrations (18.75, 37.5, 75, 150, 300 mg/ml) of each extract were evaluated by determining the effects of the extracts on the paralytic and death times of *Pheretima posthuma* using piperazine citrate (15mg/ml) and albendazole (20mg/ml) respectively as reference drugs. Ethanol extracts of both plants were added in a ratio of 1:1,

and their chloroform extracts also in the same ratio to achieve the final concentrations (18.75, 37.5, 75, 150, 300 mg/ml) and was assessed for any synergistic effect. The extracts demonstrated a concentration dependent anthelmintic activity as (EVAS + ECPS) > (CVAS + CCPS) > ECPS > CCPS > EVASB > CVASB.

KEYWORDS: Anthelmintic, synergistic, *Vernonia amygdalina*, *Carica papaya*, *Pheretima posthuma*.

INTRODUCTION

Helminthiasis is among the most common infections and a major public health concern to humans and livestock.[1] Helminthiasis is a medical condition in which a part of the human or animal body is infected with parasitic worms (helminths). The worms which infest the intestines of humans include; tapeworms (*Taenia solium*), hookworm (*Ancylostoma duodenale*), flukes (*Schistosoma hematobolium*), and roundworm (*Ascaris lumbricoids*).[2] These infections are as a result of poor sanitation and poor hygiene.

The worms mostly live in the gastrointestinal tract and may bore into other organs such as the liver.[3] These helminths in the human and animal host system produce harmful toxins and deprive the host of food which lead to anemia. Other effects such as impaired memory, reduced physical fitness, gastroenteritis, stunted growth and blindness, pneumonia are also encountered by the host.[1,4,5] Helminthes infections are the major cause of limb and genital deformities found in impoverished area of developing countries.[6] In the list of infectious diseases that cause death worldwide, acute lower respiratory tract infections, HIV/AIVs, diarrheal diseases, tuberculosis, helminthes and malaria predominate.[7] The world's population affected by helminths is estimated to be 60% to 80% of which a large percentage is in developing countries.[8]

Worm infestation of human and livestock are mostly controlled by treatment with three major classes of drugs. These are the macrocyclic lactone, benzimidazoles and nicotinic acetylcholine agonist. However, some worms have developed resistance to all the three classes of drugs.[9,10] The rapid proliferation of parasitic and micro-organisms resistance has raised the need for the use of drug combinations to maintain clinical efficacy and combat the evolution of resistance.[11]

Despite the prevalence of worm infestations, the discovery rate by the pharmaceutical industry for effective anthelmintic agents is low. One reason for this development is that nations that are mostly affected by worms have little money to invest in effective drug discovery.[12]

Over the years, a number of medicinal plants have been useful in the treatment of parasitic infections and the demand for the use of herbal medicine has increase, due to their effectiveness, safety, availability, and accessibility.[1] However, there is little or no scientific evidence to support the traditional use of these plants. It is based on this background that several researches have been mounted to evaluate medicinal plants for their claimed traditional uses.[13]

Vernonia amygdalina of the family Asteraceae is commonly known as bitter leaf because of its bitter taste. It is a small ever-green shrub that grows in most parts of Africa.[14] The bitter leaf plant grows to a height of 2-5m with a rough bark bearing green leaves which are elliptical in shape and of about 20cm long.[15,16] No seeds are produced and thus distributed by cutting.[17] The various parts of *V. amygdalina* have been recommended for the treatment of helminths, malaria, skin infections, anemia, diabetes, and cancer among many diseases.[18,14]

Carica papaya of the family Caricaceae is known commonly as pawpaw. It is a soft wooded perennial plant without branches that lives for about 5-10 years.[19] The plant has a height of 8-10m with a soft, hallow cylindrical trunk of 30cm in diameter at base and 5cm diameter at the crown.[20] The leaves are large and palmately lobed with entire margins having 0.3-0.9m petioles. The fruits are oval to oblong in shape with smooth-skin and may vary in size dependent of the cultivar. The fruits contain black seed with a spicy taste when chewed.[21] The various parts of pawpaw (leaves, seeds, fruits, latex, flowers and roots) have been recommended for the treatment of helminths, skin diseases, liver cirrhosis, some cancers, high blood pressure, chronic indigestion among other indications.[22]

Most research work undertaken on these two plants have focused on their leaves.[23] The aim of this research work is to assess the individual and combined extracts effect of *Vernonia amygdalina* stem and *Carica papaya* seeds on helminths.

MATERIALS AND METHODS

Materials

All chemicals and reagents employed in the study were of analytical grade; solvents (chloroform, ethanol and methanol) were obtained from Sigma-Aldrich, USA, Albendazole Medrich Pharmaceutical, Piperazine citrate GlaxoSmithKline Pharmaceutical Limited, acetic anhydride, iodine crystals, Diphenyl-picryl-hydrazyl (DPPH) and concentrated sulphuric acid from Sigma-Aldrich, USA, Mayer's reagent and dragendroff's reagent.

Plant material collection and authentication

The fresh stem of *V. amygdalina* and seeds of *C. papaya* (obtained from the ripped fruits) were harvested from Asakaraka-Kwahu (Eastern Region of Ghana) in January 2016 with the help of Mr. Asare a local herbalist. The collected stem was authenticated at the Department of Herbal Medicine, in the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST). Voucher specimen numbers KNUST/HM/2016/SM005 and KNUST/HM/2016/S001 were respectively assigned.

Processing of plant material

The stem of *V. amygdalina* and seeds of *C. papaya* were thoroughly washed to remove debris. The washed stem of *V. amygdalina* was chopped into smaller pieces. Both plant samples were air dried for two weeks. Each dried sample was milled into fine powder using a mechanical grinder. The powders were weighed, labelled and stored in a clean air-tight container.

Extraction of plant material

Cold maceration was employed in the extraction of the two powdered plant materials. 500g each of stem of *V. amygdalina* powder and seeds of *C. papaya* powder was weighed into two separate maceration flasks and extracted serially with 2L each of chloroform and ethanol for 72 hours while shaking on an orbital shaker (Gallenkamp, England) at room temperature. Each sample was filtered after 72 hours and the filtrate was concentrated by the use of rotary evaporator (R-114, Buch-Switzerland) at reduced temperature and pressure. The extracts were labelled as: Chloroform extract of *V. amygdalina* stem (CVAS) and Chloroform extract of *C. papaya* seed (CCPS), Ethanol extract of *V. amygdalina* stem (EVAS) and Ethanol extract of *C. papaya* seed (ECPS) and each weighed and stored in a desiccator till further use.

Collection of Worms

Adult earthworms *Pheretima posthuma* of the class Annelida were selected for use in this experiment. Worms with lengths 6-12cm and width 0.2-0.3cm were obtained from the damp, cool and covered area of a flower garden at Moitso in Ghana, washed with normal saline and transferred into a transparent glass container with a perforated cover to prevent suffocation. The earthworms were authenticated at the Department of Anatomy of Central University.

Preliminary Qualitative Phytochemical Analysis

The chloroform and ethanol extracts of *V. amygdalina* stem and *C. papaya* seeds were screened for the presence of phytochemicals such as tannins, glycosides, saponins, terpenoids and steroids using standard procedures.[24,25]

***In-vitro* Anthelminthic Activity**

The anthelminthic assay was carried out by the method described by Qureshi [26] with little modifications. The adult earthworm (*Pheretima posthuma*) was selected for the in-vitro assay due to its physiological and anatomical resemblance with intestinal roundworms parasites of human and animal, their easy availability and their widely used for evaluation of anthelmintic assay.[27,28] The washed earthworms were grouped into five worms per petri dish and treated with 10ml each of various concentrations of extracts and reference drugs as follows; Normal saline (0.9%) as negative control, Albendazole (ALB) (20mg/ml) as reference drug for death (positive control), Piperazine citrate (PZN) (15mg/ml) as a reference drug for paralysis (positive control), five concentrations (18.75, 37.5, 75, 150 and 300 mg/ml) each of the chloroform and ethanol extract, combining the ethanol extracts of both plants (*V. amygdalina* stem and *C. papaya* seeds) in the ratio 1:1 and combining the chloroform extracts of both plants (*V. amygdalina* stem and *C. papaya* seeds) also in the ratio 1:1. After treatment, the individual worms in each group were observed for the time taken for the various concentrations of extracts and reference drugs to cause paralysis and death. Paralysis was said to have occurred when the worm does not revive even in normal saline or when slow or no movement of any sort could be observed except when the worm was shaken vigorously. Death is said to have occurred when the worms lose total motility even upon vigorous shaking followed by fading of body colour to pale pink.

Statistical Analysis of Results

The results obtained were analyzed using Graphpad Prism windows version 6 (Graphpad software, San Diego, CA, USA) by two way ANOVA, followed by bonferroni post-test analysis.

RESULTS

Percentage yield

The results of the calculated percentage yields of the ethanol and chloroform extracts of *V. amygdalina* stem and *C. papaya* seeds are showed in Table 1 below.

Table 1: Percentage yield of different solvent extracts of the two plant materials

Extracts	Percentage Yields (%)
Chloroform extract of *V. amygdalina* stem (CVAS)	2.40
Ethanol extract of *V. amygdalina* stem (EVAS)	4.50
Chloroform extract of *C. papaya* seeds (CCPS)	2.46
Ethanol extract of *C. papaya* seeds (ECPS)	3.89

Preliminary phytochemical screening

Table 2 below shows the results obtained for the preliminary qualitative phytochemical screening carried out of the ethanol and chloroform extracts of *V. amygdalina* stem and *C. papaya* seeds. The results revealed the presence of some secondary plant metabolites such as; saponin glycosides, tannins, flavonoids, steroids, terpenoids, alkaloids and the absence of cyanogenetic glycosides, cardiac glycosides and anthraquione glycosides in both plant materials.

Table 2: Preliminary qualitative phytochemical analysis of ethanol and chloroform extracts of *V. amygdalina* stem (VAS) and *C. papaya* seeds (CPS).

Test	Results			
	Ethanol Extract		Chloroform Extract	
	EVAS	ECPS	CVAS	CCPS
Tannins	+	+	+	+
Reducing sugar test (glycosides)	+	+	+	+
Saponin glycosides	+	+	+	+
Cyanogenetic glycosides	-	-	-	-
Cardiac glycosides	-	-	-	-
Anthraquinone glycosides	-	-	-	-
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Terpenoids	+	+	-	-
Steroids	+	+	+	+

Key: (+) = **Presence** (-) =**Absence**

***In-vitro* Anthelmintic assay**

Table 3 shows that the ethanol and chloroform extracts of *V. amygdalina* stem (VAS) and *C. papaya* seeds (CPS) demonstrated a concentration dependent anthelmintic activity with a reduction in time of paralysis and time of death upon increase in the concentration of the extracts. Normal saline was used as a negative control, Piperazine citrate (PNZ) and Albendazole (ABZ) were used as positive controls for paralysis and death respectively.

Table 3: Paralysis and death time of the various concentrations of reference drugs and plant extracts against *P. posthuma* All the values are expressed in Mean ± SEM (n=5).

Groups	Treatment	Concentration (mg/ml)	Paralysis Time (mins)	Death Time (mins)
1	Normal saline	0.9	-	-
2	Albendazole	20	-	1.06 ± 0.14
3	Piperazine citrate	15	12.40 ± 0.15	53.06 ± 0.32
4	Ethanol extract of *V. amygdalina* stem (EVAS)	300 150 75 37.5 18.75	6.12 ± 0.35 12.83 ± 0.86 22.20 ± 1.48 24.39 ± 1.10 45.22 ± 4.06	11.16 ± 0.57 24.29 ± 1.34 43.24 ± 1.63 53.06 ± 2.88 78.75 ± 4.04
5	Ethanol extract of *C. papaya* seeds (ECPS)	300 150 75 37.5 18.75	3.49 ± 0.27 4.28 ± 0.19 6.69 ± 0.68 11.47 ± 0.66 18.85 ± 1.57	10.61 ± 0.66 14.58 ± 1.10 19.75 ± 0.73 23.80 ± 0.78 28.80 ± 1.40
6	Chloroform extract of *V. amygdalina* stem (CVAS)	300 150 75 37.5 18.75	4.34 ± 0.71 10.16 ± 0.37 11.95 ± 0.28 14.85 ± 1.02 20.01 ± 1.70	13.22 ± 0.98 18.70 ± 2.51 41.74 ± 2.21 52.96 ± 2.21 89.28 ± 2.87
7	Chloroform extract of *C. papaya* seeds (CCPS)	300 150 75 37.5 18.75	5.83 ± 0.96 12.07 ± 1.75 19.63 ± 0.95 28.01 ± 2.56 38.86 ± 0.95	8.67 ± 0.92 17.81 ± 1.45 27.71 ± 1.10 34.30 ± 1.95 42.95 ± 1.34
8	Combination of (EVAS) + (ECPS) in ratio 1:1	300 150 75 37.5 18.75	2.76 ± 0.19 4.73 ± 0.29 6.08 ± 0.30 7.56 ± 0.31 16.15 ± 1.45	7.09 ± 0.43 9.93 ± 0.93 14.44 ± 0.64 19.62 ± 0.81 24.67 ± 1.12
9	Combination of (CVAS) + (CCPS) 1:1	300 150 75 37.5 18.75	5.34 ± 0.52 7.77 ± 1.09 17.35 ± 1.14 20.85 ± 0.52 24.84 ± 0.81	7.00 ± 0.98 13.47 ± 2.16 21.18 ± 0.60 24.92 ± 1.13 28.85 ± 0.48

DISCUSSIONS

Medicinal plants have for decades shown to be effective against many infections of humans and animals due to the presence of important secondary plant metabolites. These secondary metabolites such as alkaloids, tannins, flavonoids, glycosides, steroids among others possess intrinsic abilities which help in managing and curing these infections.[29] Research has shown that many of these phytochemical constituents isolated from different parts of plants possess significant biological activities.[30]

After extraction, concentration and drying of the extracts, the percentage yields were determined. From Table 1, it was observed that the percentage yields for the ethanol extracts for both plant samples were higher than their chloroformic extracts. This may be due to the fact that the plant samples contained higher quantities of polar constituents which were soluble in the much polar solvent (ethanol).

Results from the preliminary phytochemical investigation (Table 2) revealed the presence of phyto-constituents such as tannins, saponin glycosides, reducing sugars, alkaloids, steroids and flavonoids in all the plant extracts (EVAS, ECPS, CVAS and CCPS). Terpenoids were detected in EVAS and ECPS but absent in CVAS and CCPS. All the extracts (EVAS, ECPS, CVAS and CCPS) however showed the absence of anthraquinones, cardiac glycosides and cyanogenetic glycosides.

The various parts of *Carica papaya* have been reported to possess useful phytochemicals which have shown different pharmacological activities. The seeds are rich in alkaloids (carpaine), glucosides (benzylglucosinate and glucotropacolin), cyanogenetic glycoside (benzylisothiocynate), sterol (β-sistosterol) and glycoside (sinigrin and caricin). These phytochemicals has been reported to be responsible for the anthelmintic, antibacterial, anticancer and other acclaimed pharmacological activities observed with its use.[31] Though previous work has suggested the presence of anthraquinons and cyanogenetic glycosides in the seeds of *C. papaya*,[31] results from Table 2 shows otherwise. Phytochemicals isolated from *V. amygdalina* which have shown important pharmacological activities include; sesquiterpene lactones (vernolide and vernodalol) demonstrating antimicrobial activity against selected strains of microorganisms,[32] steroidal constituents against *plasmodium falciparum*, flavonoids (Luteolin 7-O-β-glucuronoside and Luteolin 7-O-olucoside) with antioxidant properties, stigmastone type of saponins (vernioside A, B and C) having effect on weight, liver and cholesterol level.[33,17] Work done on the stem of *V. amygalina* by Okeke[34]

showed the presence of alkaloids, tannins, saponins, anthocyanis and flavonoids but the absence of sterols, triterpenes and reducing sugars. The differences in the previous studies and the present study as in Table 2, may also be due to differences in geographical location, the time of collection, variation in mineral composition of the soil, climate, season, methods of drying and extraction of the plant samples.[35]

The anthelmintic results from the study as shown in Table 3 above revealed a concentration dependent anthelmintic activity with a significant reduction in the time of paralysis and death upon increase in the concentration of all the extracts. Comparing the individual extracts (EVAS, ECPS, CVAS and CCPS), it was observed that the ethanol extracts showed a better anthelmintic activity as compared to the chloroform extracts. The ethanol extract of *C. papaya* however, showed a better activity than that of *V. amygalina*. The chloroform extract of *V. amygalina* demonstrated a better activity than that of *C. papaya*. The combined extracts showed a better anthelmintic activity compared to the individual extract. The combined extracts of EVAS + ECPS (the ethanol extracts of *V. amygalina* and *C. papaya*) showed a much reduction in time of paralysis and death as compared to CVAS and CCPS (the chloroform extracts of *V. amygalina* and *C. papaya*). It have been reported that the inhibitory effect of two drugs in combination can be larger than expected from their individual effects, corresponding to the synergistic interactions between the drugs respectively.[36] Synergistic interactions are usually thought of as advantageous because for a certain amount of drug, they effectively cause inhibition of drug-sensitive growth pathogens more.[37] Researchers have reported that some phytochemicals such as tannins, glycosides and alkaloids have exhibited activity against helminthes. It has been reported that tannins produce helminthic activity by binding to free proteins in the gastrointestinal tract of the helminthes or glycoprotein on the cuticle of the parasite leading to death.[38] Another research reported that parasitized ruminants that grazed on forage with high tannin content had very low faecal egg count as compared to those who feed on low tannin content forage.[39,30] Some phenolic compounds have also shown anthelmintic activity by uncoupling oxidative phosphorylation. This hinders the energy production in the helminths causing death.[40] Some alkaloids have also been reported to possess anthelmintic activity by acting on the central nervous system.[30,41]

The anthelmintic activity demonstrated by the plant samples may be attributed to the presence of the phytochemical constituents shown in Table 2. The plants under investigation

could therefore be potential sources of anthelmintic agents while a combination of both plants could provide a more potent activity than the use of the individual plants.

CONCLUSION

The study has revealed the presence of important phytochemicals in the ethanolic and chloroformic extracts of the fresh stem of *V. amygdalina* and seeds of *C. papaya* plant samples. All the extracts exhibited potent anthelmintic activity with the combined extracts demonstrating a synergy with better activity than the individual extracts.

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Competing Interest

The authors declare that they have no competing interests.

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