PHYTOCHEMICAL, ANTIMICROBIAL AND ANTIMALARIAL ANALYSES OF *Citrus medico. LEAVES* AND *Parinari polyandra* STEM BARK

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Abstract

Ground leaves of Citrus medica and stem bark of Parinari polyandra were separately extracted with methanol using the Soxhlet extractor. Tlie phytochemical screening of the crv,dc extracts of the plants showed the presence of flaoonoids, alkaloids, tannins, cardiac glycosides, phenols, resins and carbohydrate. When compared with tetracydine and ampicillin, P. polyandra was less active but showed a better antimicrobial activity than C. medica and inhibited the growth of Staphylococcus aurcus, Escherichia coli and Salmonella aerugunosa with minimum inhibitory concentration of 2.5 mg/ml, 5.0 mg/ml and 2.5 nig/ml respectively. Antimalarial screening of P. *polyandra showed activity against plasmodium berghei in mice. The mice with the parasite lived longer when* P. *polyandra extract was intraperitoneally administered compared to the untreated injected mice. On the other hand, the extract of C. medica showed little activity against plasmodium.*

Keywords: Antimalarial, antimicrobial, medicinal plants, photochemistry

INTRODUCTION

Malaria is caused by *plasmodium*, transmitted to man through the anopheles mosquito. It is one of the fatal diseases in the world, especially in the tropics and is endemic in some 102 countries with more than half of the world population at risk (Smyth, 1994). In Nigeria, malaria is endemic throughout the country, and costly to treat. World Health Organization (WHO) estimated malaria mortality rate for children under five years in Nigeria at 729 per 100, 000. In the year 2004, the Ministry of Health reported that malaria is responsible for 10% of deaths in pregnant women (Government in Action, 2005).

The use of plant-derived drugs for the treatment of malaria has a long and successful tradition, as medicinal plants have been used in the treatment and prevention of malaria in various parts of the world. Quinine extracted from the bark of the cinchona tree was used as an antimalarial agent as early as 1632 and by the 19th Century it was still the only known antimalarial agent (Baird *et al*, 1996). Primaquine and quinacrine were produced after the First World War; chloroquine followed thereafter in 1934 (Thomson & Werbel, 1972), and by 1946 it was designated the drug of choice for treatment of malaria (Coatney, 1993). The ineffectiveness of antimalarials, including chloroquine, in combating malaria has led to frontier researches, which have produced new and effective antimalarial drug, such as Artemisin (WHO, 2000).

The research and usefulness of medicinal plants may hold the key to new and effective antimalarial drugs (UNESCO, 1998). In Nigeria, researches are ongoing on indigenous medicinal plants to combat malaria including *Citrus medica, Gossypium barbadense, Ocimumgratissimum* (Toluef *al., 2007).*

The essential oils of *citrus* plants have been reported to effectively inhibit the growth of *phaeoramularia anlogensis* (Dangamo *et al.,* 2002), and *Propionibacterium acnes* (Luangnarumitchai *et al,*
2007) responsible for inflammatory skin diseases. The essential oil of *Citrus medica* L. showed antifungal effect on storage fungi of *Arachis hypogea* L. stored for 6 months; the oil inhibited all the 14 fungi species tested (Essien & Essien, 2002), retarded the availability of moisture to spoilage organisms (Nwachukwu and Umechurupa, 2001), but did not exhibit any adverse effect on seed germination or early seedling growth of groundnut (Essien & Essien, 2002). The peel extract of citrus fruits contain the highest concentration of flavonoids as compared to its juice or pulp (Anagnostospoulou *et al, 2006).* Antioxidant and pharmacological effects of citrus peels of different species such as *Citrus sinensis, Citrus medica, Citrus paradisi, Citrus reliculata* and *Citrus junos* are well documented (Alicia *et al,* 2005; Parmar & Kar, 2008; Yi *et al,*

2008). Sood *et al* (2009) reported that *Citrus decumana* peel extract possessed ameliorative potential in stress induced peptic ulcer in rat. The methanolic extract of the stem bark of *Parnari polyandra* revealed significant ($P < 0.05$) anti-nodceptive and anti-inflammatory effects in the rats tested. The presence of flavonoids, tannins, and saponin glycoside in the extract suggested their pharmacological activity (Vongtau, 2004). The antimalarial activity of *Parinari polyandra and Citrus medica* leaves against *plasmodium faldparum* have been reported (Adebisi, 1999; Alex, 2005). Apart from allergic reactions, chloroquine remains the cheapest drug of choice for malaria treatment in Nigeria. The resistance of malaria parasites to different antimalarials have been widely reported (Cheesbrough, 1999; Ejov, 1999; Mary *et at*, 2001; Etonihu *et al*, 2006). At the same time, the anopheles mosquitoes have developed resistance to many insecticides (Srisilam & Veershani, 2003). Thus it is important to search for new anti-malarial compounds, either synthetic or natural compounds that kill either the vector or parasite. In this work, the crude extracts of leaves of Citrus *medica* L. and stem of *Parinari polyandra* B. were screened for phytochemicals, antimicrobial and antimalarial activities.

MATERIALS AND METHODS

Collection, Identification and Preparation of

Plant Samples

Leaves of Citrus *medica L.* and stem bark of *Parinari polyandra* were collected at the Herbarium of the National. Institute for Pharmaceutical Research Development (NIPRD) at Idu in Abuja, Nigeria. The plant samples were dried at room temperature, ground to powder with a clean wooden mortar and pestle to have a good surface area, and properly stored in an air-tight container for further analyses.

Solvent Extraction of the Plant Samples

Each of the powdered samples (200 g) of *Citrus medica* and *Parinari polyandra was* extracted in 1.8 litres of methanol in a Soxhlet extractor at 50 - 70°C for 8 h. The extract was filtered using muslin cloth, followed by suction filtration, concentrated using a rotary evaporator and dried in an evaporating dish over a water bath. The weighed methanol extracts of C. *medica* and P. *polyandra was 4.6 g* and 5.2 g, respectively.

Phytochemical Screening of the Extracts

The phytochemical tests included tests for carbohydrates (Trease & Evans, 1989), flavonoids (Gessiman, 1962), cardiac glycosides (Trease &Evans, 1989), anthraquinone dervatives (Trease & Evans, 1989), resins and tannins (Trease & Evans, 1989), saponins (Sofowora, 1993), alkaloids (Trease & Evans, 1989), phenols (Trease & Evans, 1989), and sterols (Sofowora, 1993).In *Vivo* Antimalarial Tests on the Specimens Eight Swiss albino mice of 5 kg body weight i chosen and divided into four groups of 21

A donor mouse infected with rodent parasite, *Plasmodium berghei* (Parasitemia of. 20 - 30%) was anaesthetized with trichorouieUuae (CHClj) and blood was collected through conU puncture with a sterile and apyrogenic disposable needle and syringe. An innoculum of 0.2 cm* was given to each of the 8 clean mice intraperitoneally. The animals were initially left for 72 h for the infection to be established and microscopy of tail blood smear was done to determine the level of parasitemia. The route of administration was subcutaneous. Treatment with the plant extracts (5 mg/kg body weight), chloroquine (5 mg/kg) and normal saline (20 ml/g) respectively commenced on day 3. The parallel test with chloroquine (CQ PO4 purchased from Sigma, USA) was to serve as reference. The fourth group administered with normal saline served as control. While being fed on their normal food and clean water, the mice were monitored to determine how long they could survive (Peters, 1985).

Antimicrobial Screening of Extracts of Citrus *medica* L and *Parinari polyandra* B The disc diffusion method was used for the antimicrobial screening of *Pseudomonas aerugunosa, Staphylococcus aureus, Escherichia coli, and Salmonella aerugunosa*. 3 h culture of these organisms were prepared and left overnight so as to reduce their concentration due to their rate of multiplicity. Nutrient agar was also prepared and poured into 8 similar, clean and dry petri dishes and then allowed to solidify. Serial dilution of the overnight culture was made and poured over the solidified nutrient agar. This was the inoculation of the organism. The plates were allowed to set for about 30 minutes on the work bench. Different concentrations 20 mg/ml, 10 mg/ml, 2.5 mg/ml, 1.25 mg/ml, and 0.625 mg/ml of methanolic extracts of the stem bark of P. *polyandra* and leaves of C *medica* were prepared (Bauer *et al*, 1966; Emmanuel, 2003). Four cup wells were then sealed with a cork borer in each of the 8 petri dishes. The cup .wells were then sealeid with a drop of nutrient agar. This was to prevent either the extract or the standard from moving underneath the wells. Few drops of each extract and standard were injected into the wells and allowed for another 30 minutes, after which the plates were incubated at 37°C for 24 h (Perez *et al*, 1990). Three controls were also incubated alongside the plates. This was to aid in the identification of the incubated organism and for easy comparision. These controls were Organism Viability Control, Extract Sterility Control, and Agar Sterility Control.

MtKurement of Zone of Inhibition

After overnight incubation, the diameter of each *toot* of inhibition was measured with a sterilized otiper. The zones of inhibition on the media containing blood are measured from the top surface *d* the plate with the top removed. It is convenient to use a ruler with a handle attached for these measurements, holding the ruler over the surface of tie disk when measuring the inhibition zone. Care was taken not to touch the disk or surface of the agar to avoid cross-contamination. The zones of inhibition (in mm) were measured from the edges of the last visible colony-forming growth and interpretation of susceptibility was obtained by comparing the results with standard zone sizes (Bauer *et al,* 1966; Emmanuel, 2003).

RESULTS AND DISCUSSION

The results of phytochemistry, antimicrobial, and antimalarial tests of Citrus *medica* and stem bark of *fgmaripolyandra arepresented in Tables 1* to 5. Table 1 shows the presence of alkaloids, phenols, tannins, cardiac glycosides, carbohydrate in the crude methanolic extract of both plants. Numerous work on citrus peels of different species, like *C. anensis, C. medica, C. parodist, C. reticulata* and C jimos have shown the presence of phytochemicals such as alkaloids, tannins, saponins, flavonoids, terpenoids and phenolic acids (Alicia *et al.*, 2005; Parmar & Kar, 2008; Yi *et al*, 2008; Audu & Adewale, 2007). Alkaloids are one of the characteristic secondary metabolites in leaves of citrus plants and bark of *Parinari polyandra*. Alkaloids are known to possess antimalarial properties and may be the reason why *C.medica and* P. *polyandra* are commonly used indigenous plants for malarial treatment (Alex, 2005; Adebisi, 1999). Flavonoids are known to be synthesized by plants in response to microbial infection. This could be the reason why P. *polyandra* and *C. medica* extract exhibit anti-inflammatory effects and in treating arthritis and rheumatism. Tannins (commonly referred to as tannic acid) are antimicrobial agents that have been reported to prevent the development of microorganisms and hasten the healing of wounds and inflamed mucous membrane by precipitating microbial protein. The growth of many fungi, yeasts, bacteria, and viruses were inhibited by this compound (Parmar & Kar, 2008). Furthermore, tannins have been reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phytotherapeutically, tannin-containing plants are used to treat nonspecific diarrhoea, inflammations of mouth, throat and slightly injured skins. The antimicrobial action of C. *medica* may be attributed to the presence of flavonoids (Essien & Essien, 2002). Cardiac glycosides are known to act directly on the heart muscles and are therefore useful for the treatment of heart disorder, and beneficial on cardiac axhythmias. The presence of cardiac glycosides in methanolic extracts of both plants indicated that the plants may be useful in the treatment of congestive heart failure (Trease & Evans, 1989).

The. results of preliminary antiplasmodial/ antimalarial tests (Tables 2 and 3) showed that *C. medica* had no appreciable activity against *Plasmodium berghei* infected mice compared to P. *polyandra.* The animals treated with normal saline which served as control died while those treated with chloroquine diphosphate were cured at day 16. Among the mice treated with the plant extracts, chloroquine and normal saline, the curative effect were observed as a function of survival period in days. Those treated with normal saline were found to have survived in an average of 7 days. The highest survivors were those treated with chloroquine; while those treated with the plant extracts showed a survival tendency above those of the normal saline, but below the chloroquine-treated mice. The difference in the survival period of the mice treated with plant extracts at single and double doses was found to be almost insignificant. Tables 2 and 3 also show a slightly higher survival period among the female than male mice.

The only activity against S. aureus was at 20 mg/ml of crude extract o/C. *medica. P. polyandra had no activity against* P. *aeruginosa, but was active against S. aureus, E. coli and S. aeruginosa with MIC of 2.5 mgftnl, 5 mg/ml and 2.5 mg/ml, respectively (Table 4). The* differences in the inhibitory effect of the plant extracts may be attributed more to variation in the intrinsic properties of the plants than the individual adaptability of the microbial species to the toxic nature of the extracts. This is in agreement with the findings of Kubmarawa *et al*. (2007) and Nwachukwu & Umechuitfpa, 2001. Tannins and flavonoids inhibit the growth of many fungi, yeasts, bacteria, and viruses, and are biologically active against S. *aureus* and E. *coli* (Harborne, 1993). .Consequently, the high microbial activity of P. *polyandra against the test organisms may be indicative of their tannins level when compared to C. medica. However,* when compared with standard antibiotics like tetracycline and ampicillin with MIC of 0.8 mg/ml (Table 5), *Parinari polyandra was less active against the test organisms (Etonihti et al., 2008).*

Table 1: Phytochemicals in the methanolic extract of leaves of *Citrus medica* and stem bark of *Parinari polyandra*

Tests	*Citrus medica* L.	*Parinari polyandra* B.
Saponins	–	–
Flavonoid	+	+
Alkaloids	+	+
Tannins	+	+
Cardiac glycosides	+	+
Phenols	+	+
Terpenes	–	–
Sterols	+	+
Resins	+	–
Anthraquinones	–	–
Carbohydrate	+	+

+ means present; – means absent

Table 2: Preliminary antiplasmodial (*P. Berghei*) screening of methanolic extract of *Citrus medica and Parinari polyandra*

	Treatment Dose (mg/kg b.w) i.p	Parasitamia (a)	(b)
C. medica	300	+++	++
P. polyandra	300	+	++
CQ PO4	5	+	+
*Normal Saline	20ml	+++	+++

b.w = body weight; i.p = intraperitoneal; a = male mice; b = female mice;
* = died; + = slightly present; ++ = moderately present; +++ = highly present

Table 3: Survival period (in days) of parasitized mice treated with *C. medica* and *P. polyandra* methanolic extracts

	Treatment Dose (mg/kg b.w) i.p	Parasitamia (a)	(b)
C. medica	150	11	13
	300	15	17
P. polyandra	150	14	14
	300	18	19
CQ PO4	5	23	23
*Normal Saline	20ml	6	7

b.w = body weight; i.p = intraperitoneal; a = male mice; b = female mice; * = died

Table 4: Antimicrobial activity of crude methanolic extract of *Citrus medica* and *Parinari polyandra*

Test Organism	Concentration (mg/ml) 20	10	5	2.5	1.25	0.625
			Citrus medica			
S. aureus	+	–	–	–	–	–
E. coli	–	–	–	–	–	–
P. aeruginosa	–	–	–	–	–	–
S. aeruginosa	–	–	–	–	–	–
			Parinari polyandra			
S. aureus	+	+	+	*	–	–
E. coli	+	+	*	–	–	–
P. aeruginosa	–	–	–	–	–	–
S. aeruginosa	+	+	+	*	–	–

+ = activity; – = no activity; * = minimum inhibitory concentration (MIC)

Table 5: Antimicrobial activity of Tetracycline and Ampicillin on test organisms

Test Organism	Concentration (mg/ml) 2	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.5
5. *aureus*	+	+	+	+	+	+	4-	*	—	-
S. subtillis	+	+	+	+	4-	+	4-	*	—	-
E. coli	+	+	+	4-	+	+	4-	*	—	-
P. aeruginosa	-	-	—	-	-	—	4-	*	-	—
C. *albicans*	+	+	+	4-	+	4-				

+ s activity; - s no activity, * = minimum inhibitory concentration (MIC)

Source: Etonihu *et al.* (2008)

CONCLUSIONS

The phytochemical screening of the crude methanolic extracts of leaves of *Citrus medica* and stem bark of *Parinari polyandra* showed the presence of flavonoids, alkaloids, tannins, cardiac glycosides, phenols, resins and carbohydrate. Although P. *polyandra* was less active than tetracycline and ampicillin, it showed a better antimicrobial activity than *Citrus medica* and inhibited the growth of *Staphylococcus '^aureus, Escherichui coli and Salmonella aeruqunosa* with minimum inhibitory concentration of 2.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml, respectively. Antimalarial screening of P. *polyandra* showed activity against plasmodium berghei in mice. The mice with the parasite lived longer when P. *polyandra* extractwas intraperitoneally administered compared to the untreated infected mice. On the other hand, the extract of C. *medica* showed little activity against plasmodium.

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