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Full Length Research Paper

PRELIMINARY STUDIES ON THE ANTIMALARIAL PROPERTIES OF THE PEELS OF *CITRUS MAXIMA* LINN

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ABSTRACT

Even though *Citrus* plants have antimalarial properties, there is no report of such about peels of *C. maxima*. This study explored fractions of peels of *C. maxima* for the presence of antimalarial activity using murine models. The crude extract was tested at 3 dose levels (125, 250, 500 mg/kg/b.w./day) and fractions at 2 dose levels (250 and 500 mg/kg/b.w./day). All treatments lasted 7 consecutive days. In the experimental design and layout, blood samples were collected from tails of animals on day 4 and day 7. Over 89% of the parasite was cleared by 125 mg/kg dose on day 7. The highest clearance at day 4 and day 7 were at 500 mg/kg aqueous fraction (84.85%) and 500 mg/kg ethyl acetate fraction (97.96%), respectively. Only the aqueous fractions (250 and 500 mg/kg) had clearances > 80% on day 4. Other fractions having clearances > 50% were 500 mg/kg n-butanol (61.90%) and 500 mg/kg n-hexane (59.57%). Results of day 7 show the presence of antimalarial principles in all the fractions. This study demonstrated that the fruit peels of *C. maxima* possess antimalarial activity. More studies are needed to identify the mechanisms of action and active compounds involved.

Keywords: *Citrus maxima*, antimalarial activity, parasite clearance, *Plasmodium berghei*

INTRODUCTION

There are reasons to suspect that the *Citrus* genus possess antimalarial property. The *Citrus* genus of plants has been reported to possess several pharmacological activities. The genus comprises a number of species which includes *C. limon* (lemon), *C. medica* (citron), *C. limetta*, *C. aurantium* (sour

orange), *C. paradisi* (grapefruit), *C. reticulata* (mandarin, tangerine), *C. clementina* (clementine) and *C. sinensis* (sweet orange) (Favela-Hernández *et al*., 2016). Activities found in the fruit include antioxidant (Mehmood *et al*., 2015; Singh *et al*., 2010), anticancer (Benavente-García and Castillo, 2008; Wang *et al.*, 2014), anti-aflatoxigenic (Singh *et al*., 2010), cardiovascular (Benavente-García and Castillo, 2008), anti-inflammatory (Benavente-García and Castillo , 2008; Mohanty *et al*., 2015), larvicidal (Murugan *et al*., 2012), pupicidal (Murugan *et al*., 2012), and insect repellent (Murugan *et al*., 2012) activities. Antimicrobial properties in literature include antibacterial (Mehmood *et al*., 2015) and antifungal (Singh *et al*., 2010). These activities have been demonstrated against *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Proteus vulgaris, Staphylococcus epidermidis, Aspergillus flavus* and *Trichophyton* (Khan *et al*., 2012). These properties have been found in the fruit peel (Dhiman *et al.*, 2012; Mehmood *et al*., 2015; Mohanty *et al*., 2015; Wang *et al*., 2014), essential oils (Singh *et al*., 2010), and pulp (De Moraes Barros *et al*., 2012).

C. maxima, though native to Southeast Asia, can be found in many tropical and semi-tropical countries. It is also referred to as *Citrus decumana* or *Citrus grandis*. It has large leaves, flowers, and fruits, and is commonly called pummelo, pommelo, shaddock, pumelo, and pomelo. *C. maxima* is a plant with pharmacological properties such as antioxidant (Chowdhury *et al*., 2015; He *et al*., 2012; Mäkynen *et al*., 2013), antihyperlipidemia (Mäkynen *et al*., 2013) and prevention of metabolic disorders (Ding *et al*., 2013). Some of the studies on the fruit peel include flocculating and suspending properties (Piriyaprasarth and Sriamornsak, 2011), preparation of pectin (Sotanaphun *et al*., 2012), and sorption of dye and oil (Argun *et al*., 2014; Chai *et al*., 2015; Hameed *et al*., 2008). The peels of pummelo (*C. maxima*), lemon (*C. limon* (L.) Burm. f.), grapefruit (*C. paradisi* Macfayden), bergamot (*C. bergamia* Rissoet Poit.), bitter orange (*C. aurantium* L.), sweet orange (*C. sinensis* (L.) Osbeck), and mandarin (*C. reticulata* Blanco) possess antimicrobial properties (Gülay Kirbaşlar *et al*., 2009; Mathur *et al*., 2011). *Citrus* plants suspected to possess antimalarial properties include *C. limetta* fruit peels (Mohanty *et al*., 2015), *C. limon* (Ruiz *et al*., 2011), and *C. paradise* (Ruiz *et al*., 2011). Incidentally, activity against the malaria parasite vectors were found in the peels of *Citrus* plants (Murugan *et al*., 2012).

Even though *Citrus* plants have been associated with antimalarial properties, published literature does not show any study on antimalarial property of the peels of *C. maxima.* This study is a heuristic attempt to screen the fractions of the peels of *C. maxima* for the presence of antimalarial activity as well as provide some pharmacognostic markers for the peels used.

MATERIALS AND METHODS

Preparation of plant materials

The fresh fruits of *C. maxima* were harvested, peeled and the peelings were chopped with knife, dried and ground to powder. Crude extraction was with 95% methanol. The powder was extracted by

maceration using 95% methanol and subsequently filtered using Whatman filter paper No.1. The crude extract was fractionated using 4 solvents namely n-hexane, ethyl acetate, n-butanol and water. The crude extract and fractions were dried *in vacuo* by evaporating in rotary evaporator under reduced pressure and subsequently stored at 4°C.

Phytochemical screening

Phytochemical screening of the crude extract was carried out using standard procedures (Trease and Evans, 1989).The moisture and ash contents were also determined using standard laboratory procedures (AOAC, 1990).

Parasite inoculation

Parasite inoculation was done using a previously described method (Ihekwereme *et al*., 2016). Briefly, animals were quarantined 7 days prior to infection. Standard inocula of 1 x 10^7 *P. berghei* infected erythrocytes in 0.2 ml were prepared by diluting infected blood (from donor mouse) with 0.9% normal saline. Each mouse was inoculated by intra-peritoneal injection with a blood suspension (0.2 ml) containing 1x10^7 parasitized erythrocytes (Moll *et al*., 2008).

Effect of crude extract and fractions on curative antimalarial test

The evaluation of the curative potential of the extract in Swiss albino mice was done using the method described by Ryley and Peters (Ryley and Peters, 1970). Infected animals were divided into 5 groups (n = 5) when the level of parasitemia was observed to be > 4%. The crude extract was tested at 3 dose levels (125, 250, and 500 mg/kg/b.w./day). The fractions were tested at 2 dose levels (250 and 500 mg/kg/b.w./day). Positive (0.95 mg/kg of a co-formulated tablet containing 20 mg artemether and 120 mg lumefantrine) and negative (distilled water) control groups (n = 5) were used. All treatments lasted 7 consecutive days (day 1-7). Blood samples were collected from the tip of the tails of the animals on day 4 and day 8.

Parasitemia Monitoring

Parasitemia was monitored using a previously described method (Arrey Tarkang *et al*., 2014). Briefly, blood samples were collected from the tip of the tails of the animals. Thin blood films were dried, and fixed (for 15 min) using methanol, and subsequently stained with 10% Giemsa for 25 min. Stained film was washed off using phosphate buffer, pH 7.2 and allowed to dry. The film was immersed in oil and viewed at x100 magnification. The parasitemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in random fields of the microscope (Toma *et al*., 2015).

Average percentage parasitemia was calculated using the formula:

$$\% \text{ Parasitemia} = \frac{\text{Total number of parasitized erythrocytes}}{\text{Total number of erythrocytes counted}} \text{ X } 100$$

Average percentage of chemo-suppression (or parasite suppression) was calculated using the formula:

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in test group}}{\text{Parasitemia in negative control}}$$

Data analysis

The results were presented as the mean ± SEM (standard error of mean) for each group of experiments. The test groups were compared with the negative control group using one-way analysis of variance (ANOVA). All data were analyzed at a 95% confidence interval. P-value less than 0.05 was considered statistically significant.

RESULTS

Table 1 shows the results of the crude extract. The least dose used (125 mg/kg) cleared over 89 % of the parasite on day 7. The highest recorded value was 95.87 % on day 7 at 500 mg/kg dose; compared to 98.71 % clearance recorded from the positive control.

Even though, there were differences in parasite clearance of the crude extract at 125 and 250 mg/kg dose on day 4 (46.55%, 61.51%), the same level of parasite level was observed on day 7 (89.96%, 89.96%). At 500 mg/kg dose, a high level of clearance (82.65% and 95.87%) was sustained. Comparing the results of the crude on day 4 and day 7, lower values were recorded on day 4 at 125 and 250 mg/kg doses.

Table 2 shows the results of the fractions. The highest clearance at day 4 was at 500 mg/kg aqueous fraction (84.85 %), while 500 mg/kg ethyl acetate fraction (97.96 %) gave the highest parasite clearance at day 7. All the fractions had between 87% and 98% clearance on day 7. At 500 mg/kg, the range for the 4 fractions at day 4 was between 41.48% and 84.85% clearance as against that of the crude extract (82.65%). At 250 mg/kg, the range for the 4 fractions at day 4 was between 29% and 80.95% clearance while the crude extract had 61.51%. Only the aqueous fractions (250 and 500 mg/kg) had clearances > 80 % at both doses on both days. Other fractions having clearances > 50% were 500 mg/kg n-butanol (61.90%) and 500 mg/kg n-hexane (59.57 %). On comparison, the 250 mg/kg aqueous fraction had 80.95 % on day 4 while the crude had 61.51% on same day.

Generally, the performance of the fractions on day 7 was similar to the crude. At 250 mg/kg, the range for the four fractions on day 7 was between 87.3% and 97.0% clearance as against that of the crude (89.96%). Similarly, at 500 mg/kg, the range for the 4 fractions at day 7 was between 94.29% and 97.96% clearance as against that of the crude (95.87%).

Table 1: Mean *Plasmodium berghei* blood levels before and after treatment with crude extracts of *Citrus maxima*

Treatment	Mean parasit*a*emia			% parasite clearance	
	Basal parasitaemia (72 h post infection)	day 4 (Mean)	day 7 (Mean)	day 4	day 7
NC (10 ml/kg DM)	11.55 ± 0.08	9.55 ± 2.06	9.05 ± 2.19	17.37	21.65
PC (0.95 mg/kg ACT)	11.65 ± 0.05	0.90 ± 0.39	0.15 ± 0.14	92.28	98.71
125 mg/kg peel extract	11.60 ± 0.06	6.20 ± 1.15	1.20 ± 0.79	46.55	89.66
250 mg/kg peel extract	11.95 ± 0.04	4.60 ± 0.85	1.20 ± 0.48	61.51	89.96
500 mg/kg peel extract	12.10 ± 0.06	2.10 ± 0.52	0.50 ± 0.40	82.65	95.87

Results are expressed as mean ± SEM, n = 5.

NC= negative control / 0.09 ml distilled water, PC= positive control

Table 2: Mean *Plasmodium berghei* blood levels before and after treatment with fractions from *Citrus maxima*

Treatment	Dose	Mean parasit*a*emia			% parasite clearance	
		Basal parasitaemia (72 h post infection)	day 4	day 7	day 4	day 7
NC		5.20 ± 0.46	8.12 ± 0.60	10.90 ± 0.73	0.00	0.00
PC		7.10 ± 0.34	0.20 ± 0.12	0.00 ± 0.00	97.18	100
n-hexane fraction	250 mg	7.00 ± 0.58	4.00 ± 0.58	0.30 ± 0.17	42.85	95.71
	500 mg	9.40 ± 0.40	3.80 ± 0.54	0.30 ± 0.03	59.57	96.81
Ethyl acetate fraction	250 mg	10.00 ± 1.00	5.70 ± 0.44	0.30 ± 0.17	43.00	97.00
	500 mg	9.80 ± 0.10	5.70 ± 0.70	0.20 ± 0.17	41.84	97.96
Butanol fraction	250 mg	7.90 ± 0.74	5.60 ± 0.84	0.70 ± 0.17	29.11	91.13
	500 mg	10.50 ± 0.76	4.00 ± 0.58	0.60 ± 0.44	61.90	94.29
Aqueous fraction	250 mg	6.30 ± 0.60	1.20 ± 0.41	0.80 ± 0.13	80.95	87.30
	500 mg	6.60 ± 0.83	1.00 ± 0.35	0.30 ± 0.16	84.85	95.45

Results are expressed as means ± SEM, n = 5.
NC= negative control / 0.09 ml distilled water; PC= positive control

Phytochemical test on the crude showed the presence of alkaloids, saponins, tannins, flavonoids, terpenoids, glycosides, proteins, carbohydrate, and reducing sugars. The test did not detect the presence of steroid. Quantitative tests (Table 3) show the crude contained large quantities of alkaloids and tannins. The results of the proximate analysis and yield of the fractions of the powdered peel of *C. maxima* are presented on Table 4 and Table 5 respectively.

Table 3: The result of the quantitative phytochemical screening of the powdered peel of *Citrus maxima*

Phytochemical	Values (%)
Flavonoid	0.12
Alkaloid	16.49
Tannin	14.30
Saponin	2.30

Table 4: The result of the proximate analysis of the powdered peel of *Citrus maxima*

Parameter	Values (w/w)
Total ash	8.5
Acid insoluble ash	4.5
Water insoluble ash	3.0
Moisture content	9.0

Table 5: The yield of the fractions

Fraction	Yield (%)
Ethyl acetate	19.1
Butanol	13.0
n- Hexane	10.0
Aqueous	12.0

DISCUSSION

As previously stated, there are reasons to believe the *Citrus* plants, especially the fruit peels, possess antimalarial properties. Members of its genus possess antimicrobial

and antimalarial properties. Plants such as tangelo, mandelos, oroblanco and melogold are hybrids from *C. maxima* or its genetic material and other plants. It makes sense to suspect that *C. maxima* may as well exhibit antimalarial properties since plants within the same genus share similar phytochemicals and phytocompounds.

The results of the crude at 125 mg/kg (89.66%) on day 7, suggest the crude may still maintain high activity at lower doses. The similar parasite level which was recorded by the crude extract at 125 and 250 mg/kg doses on day 7 suggested the extract may be slow-acting. It also shows that a 7-day pomelo peel herbal treatment may not need to go beyond 125mg/kg dose. However, a 3-day treatment may possibly be achieved at 500 mg/kg.

Comparing results of day 4, the 250 mg/kg aqueous fraction (80.95%) had better performance than the crude of the same dose (61.51%). This is a sign most of the rapidly active constituents may be present in this fraction. Nevertheless, aggregate effect of the samples between day 4 and day 7 seems to show multiple antimalarial constituents in the crude. This is inferred from the results of the respective fractions which show activity irrespective of the fact that each fraction contains constituents with diverging polar or solubility properties.

A look at the result on day 7 may not help in identifying where most of the antimalarial constituents lie since values for all the fractions lie within a narrow range. On day 4, the aqueous fraction appears to have most of the active principles as it maintained clearance rates > 80% at tested doses. Taken together, it appears there is a preponderance of the antimalarial principles in the aqueous fraction. This opinion is strengthened by the observation that only the aqueous fraction maintained a high parasite clearance rate at both doses on both sampling days. Furthermore, on day 4, the aqueous fraction (80.95% and 84.85%) maintained a higher performance than the crude (61.51% and 82.65%) at both doses. Results on day 7 show the presence of antimalarial principles in all the fractions. However, results on day 4 shows most of them are in the aqueous fraction.

It does not seem clear which plant chemicals are prime suspects responsible for the observed antimalarial activity in pomelo peel. However, *Citrus* peels are known to contain flavonoids, coumarins, peptides, and volatile compounds (Favela-Hernández *et al*., 2016). Pomelo peel contains citral, aldehydes, geraniol, cadinene, linalool, limonene and terpene (Bordoloi *et al*., 1999; Cheong *et al*., 2012). Even though limonene is abundant in the essential oil of the peel, any of these could be responsible for the activity (Bordoloi *et al*., 1999). The fruit peels of a member of its genus, *C. limetta* is reported to exhibit anti-malarial activity and inhibit inflammatory mediators (IFN-γ, TNF-α, IL-6) involved in malaria pathogenesis (Mohanty *et al*., 2015).

CONCLUSION

This study demonstrated that the fruit peel of *C. maxima* possess antimalarial activity. More studies are therefore suggested to identify the active compounds responsible for the antimalarial properties and the mechanisms of action involved.

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