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VALIDATION OF ETHNOPHARMACOLOGICAL USE AS ANTI-INFLAMMATORY OF A DECOCTION FROM *ANNONA MURICATA* LEAVES

A.M. Quilez, [1*] S. Montserrat-de la Paz, [1,2] R. De la Puerta, [1] M.A. Fernández-Arche,[1] M.D. García-Giménez[1]

[1]Department of Pharmacology,School of Pharmacy, University of Seville, C/ Profesor García González, 2, 41012, Seville, Spain; [2]Laboratory of Cellular and Molecular Nutrition, Instituto de la Grasa, CSIC. Av/Padre García Tejero, 4, 41012, Seville, Spain
***Corresponding autor: E-mail:** quilez@us.es

Abstract

Background: Through this work we evaluated the potential of the aqueous extract of *Annona muricata* L. leaves (AMAEL) to treat inflammatory conditions. The use of decoction or infusion of this important medicinal resource is still not scientifically validated. **Methods:** Different doses of AMAEL were assayed in carrageenan-induced inflammation and tetradecanoylphorbol acetate (TPA)-induced edema in mice, myeloperoxidase activity (MPO) in inflamed tissue, MPO released by A-23187-stimulated rat neutrophils and nitric oxide released by murine macrophages. Acute oral toxicity and cell viability of murine macrophages were also tested.
Results: A single dose of 250, 500 and 1000 mg/kg of AMAEL did not show any symptoms associated with toxicity *in vivo* and the viability of murine macrophages was of 100% at the assayed doses. AMAEL at 250mg/kg and 500mg/kg exerted a significant edema reduction in the carrageenan inflammation model (26.82±0.02%, $p<0.05$ and 52.70±0.12%, $p<0.001$ inhibition respectively, after the first hour). The TPA-induced topical edema model showed a significantly and dose-dependently inhibition (56% and 78% at doses of 2.5 mg/ear and 5 mg/ear, respectively). The decrease in (MPO) enzyme activity in the ear homogenates assayed at 5 mg/ear were highly significant (92.5% ± 1.83 inhibition, $p<0.001$) and MPO was also reduced in activated rat neutrophils at 200 µg/ml (81.98% ± 1.01 inhibition, $p<0.001$). AMAEL considerably decreased dose-dependently nitrite production in LPS-stimulated murine macrophages, the highest inhibition was achieved at 500 µg/ml (73.18% ± 2.36, $p<001$).
Conclusion: this study validates the ethnomedicinal use of the decoction of *Annona muricata* L. leaves as anti-inflammatory agent

Keywords: aqueous extract; ethnomedicinal use; inflammation; myeloperoxidase; nitric oxide

Introduction

Inflammation is a complex process that results into up-regulation of inflammatory mediator as prostaglandins, cytokines, chemokines, oxidants such as nitric oxide and superoxide and granular lytic enzymes by macrophages and neutrophils, the main phagocytic cells (Moncada et al., 1991). It is a beneficial host-response, but upon long persistence, it may result into chronic conditions such as cancer (Aggarwal et al., 2006), cardiovascular diseases (Libby, 2006), diabetes (Wellen & Hotamisligil, 2005), pulmonary disorders (Sevenoaks & Stockley, 2006), neurological diseases (Yong et al., 2001) and arthritis (Goldring & Otero, 2011). Non-steroideal anti-inflammatory drugs (NSAIDs) are commonly used to treat the symptoms of acute or chronic conditions such as pain, fever and inflammation. However, the extensive use of these drugs is associated with several side-effects. Due to such reasons, there has been a growing interest in the use of natural compounds and medicinal plants (Zeng et al., 2012).

Annona species (Annonaceae) have been used as natural remedy for a variety of illnesses, the literature is rich in studies showing the many and important pharmacological activities of this genus of medicinal plants. *Annona muricata* L. is a broadleaved, flowering, evergreen tree commonly known as soursop, graviola, guanábana or corossol among its many popular names. It is found wild and cultivated from sea level to 1,000 m elevation in the tropical areas of America, Asia, and Africa (Lim, 2012).

Traditionally, the infusion or decoction of the leaves is used internally for different pathologies: headaches, neuralgia, cystitis, liver problems, upset stomach, diabetes, cancer and as sudorific, anti-inflammatory, anti-spasmodic, anti-rheumatic, anti-dysenteric, and parasiticide. Applied topically, the cooked leaves improve rheumatism and abscesses and decoction as compresses for head lice, bedbugs, swollen feet, and inflammation (Mishra et al., 2013; Barbalho et al., 2012; Lim, 2012).

Phytochemical investigations of the leaves from *Annona muricata* revealed as the main components: alkaloids (Leboeuf et al., 1982), phenolic compounds, terpenoids, essential oils (Thang et al., 2013; Kossouoh et al., 2007) and acetogenins. These latter active compounds have been studied further, as they have demonstrated antitumor properties, presenting selective toxicity against various types of cancer cells and against different multidrug-resistant cancer cell lines (Mishra et al. 2013; Barbalho et al. 2012; Lim, 2012; Lima, 2008).
In the consulted bibliography, pharmacological studies are found supporting the popular use of *Annona muricata* in the original countries, especially related to the antitumor, anti-inflammatory, antidiabetic, antiviral, anti-bacterial, insecticidal and antiparasitic activities (Mishra et al., 2013; Ferreira et al., 2013; Vila-Nova et al., 2013; Florence et al., 2014; Grzybowsky et al., 2013; Barbalho et al., 2012; Lim, 2012; Adeyemi et al., 2010; Lima, 2008; Chan & Roslida, 2012; De Sousa et al., 2010).

The anti-inflammatory activity and the anti-arthritic effects have been demonstrated in different alcoholic extracts (Chan & Roslida, 2012; De Sousa et al., 2010) but not in the aqueous extracts. That reason and due to the interest in validating the popular use as anti-inflammatory agent, we have developed the following research work. The work started with soursop through collaboration in the TRAMIL project (a research project on the medicinal plant resources of the Caribbean). The implementation of TRAMIL´s recommendations will permit the population of this region to have a safe alternative to primary health care through the use of phytotherapy (Robineau & Soejarto, 1998).

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Materials and methods

Reagents

All the reagents were purchased from Sigma Aldrich Chem. (St. Louis, MO, USA). Stock solutions of the extracts were prepared in dimethyl sulfoxide (DMSO) and later dissolved in ethanol.

Plant Material and Extract Preparation

Young leaves of *Annona muricata* were collected in July 2004 in "Roca del Mar" area (Santo Domingo, Dominican Republic) and were identified by the botanist A. Veloz in the National Botanic Garden of Santo Domingo (JBSD). A voucher specimen was deposited in the JBSD and kept under reference Tramil-Gef Project 3142. Decoction of the leaves was prepared using water according to the Spanish Farmacopeia method (Real Farmacopea Española, 2011). The aqueous decoction was concentrated in a rotary evaporator.

Phytochemical Screening

Phytochemical screening of the aqueous extract was performed using conventional protocol. The presence of alkaloids with Mayer, Dragendorff and Bouchardat´s reagents, flavonoids with the use of Mg and HCl, tannins with 1% gelatin and 10% NaCl and $FeCl_3$ solutions, cyanogenic glycosides with picrate paper, the phenolic acids were identified by TLC, anthraquinones with the Borntranger´s reaction, saponins with the aphrometric index and phenylpropanoids with Amow reagent (Evans, 2006).

Animals

Forty-eight male Swiss albino mice of five-weeks-old, weighting 25-30 g were used for the experiments. They were randomly placed in cages (6 mice/cage) kept in a room at 22 ± 2°C, humidity 60 ± 5% and *ad libitum* feeding of a standard laboratory diet and tap water before use. All animal care and experimental procedures complied with the Guidelines of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC) and followed a protocol observed and approved by the Animal Ethics Committee of the University of Seville.

Toxicity and Viability Cell
Acute Oral Toxicity Study

The acute oral toxicity study was conducted using test guideline on acute oral toxicity test 423 according to OECD (2001). Six male Swiss albino mice of five-weeks-old per group fasted overnight, but allowed free access to water *ad libitum* were randomly assigned into the following four groups: Control group received distilled water and three groups received the extract at the doses of 250, 500 and 1000 mg/kg. Mice were not fed for 3 h following the administration. The signs of toxic effects and/or mortality were observed 3 h after administration and then, for the next 48 h.

Isolation and Culture of Murine Peritoneal Macrophages and Cell Viability

Peritoneal exudates cells ($1x10^6$ cells/well) from thioglycolate-induced mice were collected from the peritoneal cavities of Swiss mice and were suspended in culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml), and pre-cultured in 24-well plates at 37ºC in 5% CO_2 in air for 2 h. Cells, containing 1 µg/ml of LPS, were -incubated at 37°C for 24 h with 100, 250 and 500 mg/kg AMAEL doses. The viability was assayed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) (MTT) test (Mosmann et al., 1983).

Anti-inflammatory Activity:
Carrageenan-induced Mouse Hind Paw Edema

Edema was induced on the right hind paw of the mouse by subplantar injection of 0.1 ml of a solution of carrageenan, 1% w/v in saline solution (Garcia et al., 2004). A single dose by oral gavage of the aqueous extract, 250 or 500 mg/kg, was administered 1 h before the injection of carrageenan. The control group received only saline solution. Ibuprofen at 50 mg/kg was used as a reference drug. Paw volumes was measured by means of plethysmometer (LETICA-7500) before administering carrageenan (V_0), at 1, 3 and 5 h later (V_t). The percentages of inhibition were calculated for each group and each measurement, comparing with the control group, using de following ratio:

$[(V_t - V_0)_{control} - (V_t - V_0)_{treated}] \times 100/(V_t - V_0)$

TPA-Induced Mouse Ear Edema

Each mouse received 2.5 µg tetradecanoylphorbol acetate (TPA) as phlogistic agent dissolved in 20 µl acetone (Xian et al., 2011; Fernandez-Arche et al., 2010). Extracts were applied topically, at doses of 2.5 and 5 mg/ear dissolved in water, immediately after application of TPA in the right ear. Ibuprofen (0.05 mg/ear) was used as a reference compound. Inflammation was allowed to develop for 4 h and the swelling was assessed as the increase of weight of the right ear against the left ear (received only the vehicle).

Myeloperoxidase Assay (MPO)

The method of Bradley (Bradley et al., 1982) was followed, modified for lecturing in a microplate reader. MPO activity was determined on the supernatants from the homogenates of the ear biopsies prepared as it is described (Xian et al., 2011).

MPO Release by A-23187-Stimulated Rat Neutrophils

Rat neutrophils were obtained as described in Moroney et al. (1988). The cells were resuspended in complete Hank Balance Salt Solution at 0.5×10^6 cells/ml containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}. Cells were pre-incubated at 37°C for 10 min with 50, 100, 200 µg/ml of AMAEL doses. After this, calcium ionophore A23187 (1 µM) was added. The cells were pelleted by centrifugation at 2500 g for 10 min at 4°C, and the supernatants were assayed for the MPO activity (Bradley et al., 1982).

Nitric Oxide Assay

Nitrite, as index of nitric oxide (NO) generation, was determined in culture supernatants by Griess reagent (Green et al., 1982).

Statistical Analysis

All results were expressed as mean ± SEM. Statistically significant differences were evaluated by analysis of variance (ANOVA) followed by Dunnett's test. *P* value less than 0.05 was considered significant.

Results

Phytochemical Screening

Aqueous extraction yield was 24%. AMAEL showed the presence of phenol compounds (tannins, flavonoids and phenolic acids) and alkaloids in the qualitative phytochemical screening.

Toxicity and Viability Cell

A single dose of 250, 500 and 1000 mg/kg did not indicate modification of behavior in Swiss Albino mice. No mortality was recorded during the study. After sacrifice on the 2nd day, macroscopic pathology observations, revealed no visible lesions in any animal. The animals did not show any stereotypical symptoms associated with toxicity at these doses. AMAEL did not induced toxicity in the murine peritoneal macrophage when assessed by mitochondrial reduction of MTT after 24 h of treatment. Viability of cells treated with the extract was 100.3 ± 0.6%, 99.8 ± 0.7% and 100.8 ± 1.0 at concentrations 500, 250 and 100 µg/ml respectively (data not shown).

Table 1: Antiinflammatory effect of aqueous extract from the leaves of *Annona muricata* L. against mouse paw carrageenan-induced edema (**p<0.05 vs Control, *** p< 0.001vs Control, ##p<0.01 vs Ibuprofen, ###p<0,001vs Ibuprofen*).

Group	Dose (mg/kg)	Paw volume increase (ml)			Anti-inflammatory activity (%)		
		1h	3h	5h	1h	3h	5h
Control	-	188.41 ± 2.41	164.49 ± 2.5	131.16 ± 1.12	-	-	-
Annona muricata L.	250	137.88 ± 3.33 (*)	121.87 ± 2.66 (*)	121.21 ± 3.33	26.82 ± 0.02	25.85 ± 0.06	7.59 ± 0.54
Annona muricata L.	500	107.56 ± 1.53 (***)(##)	113.64 ± 1.27 (***)(###)	103.3 ± 0.95 (***)(###)	52.70 ± 0.12	37.6 ± 1.37	28.88 ± 0.08
Ibuprofen	50	113.47 ±1.87 (*)	128.17 ± 2.45 (*)	119.72 ± 1.58	50.10 ± 0.22	22.05 ± 0.38	8.72 ±0.53

Anti-inflammatory Activity
Carrageenan-induced Edema Model

The results of this test are reported in Table 1. AMAEL exerted a significant edema reduction from the first hour and remained along in the time compared to the untreated control group ($p<0.001$). Interestingly, a single dose of 500 mg/kg by oral gavage was found to be slightly more effective in reducing the paw edema as the standard Ibuprofen (50 mg/kg).

TPA-induced Edema Test

Table 2 shows the results of the topical effect of AMAEL on the TPA-induced edema in mouse ear. The edema was significantly and dose-dependently reduced (56 and 78% at doses of 2.5 and 5 mg per ear respectively).

Myeloperoxidase Activity (MPO) in Inflammed Tissue

The effect of AMAEL on MPO activity in inflamed tissue is depicted in Fig. 1. The topical administration significantly reduced MPO overproduction in a dose-dependent manner. The highest inhibition was achieved at 5 mg/ear (92.5% ± 1.83, $p<0.001$).

Table 2: Effect of aqueous extract from the leaves of *Annona muricata* L. on TPA-induced edema in mouse ear (***p<0.01 vs TPA, *** p < 0.001vsTPA*).

Group	Dose (mg/ear)	Edema (mg)	Anti-inflammatory activity (%)
Control (TPA)	-	17.11 ± 1.46	-
Annona muricata L.	2.5	7.68 ± 1.33 (**)	56.11 ± 3.88
Annona muricata L.	5	3.75 ± 1.73 (***)	78.09 ± 3.61
Ibuprofen	0.05	3.57± 0.67 (***)	79.60 ± 0.22

MPO Release by Rat Neutrophils

MPO assay in A23187-stimulated neutrophils was performed to assess possible *in vitro* effects of AMAEL on activated neutrophils. Fig. 2 shows that *Annona muricata* L. at 200 µg/ml caused an inhibition of the A23187-induced MPO expression (81.98% ± 1.01, $p<0.001$). At doses of 100 and 200µg/ml, the effect was higher than that produced by caffeic acid, the reference compound (59.66% ± 2.27).

Nitrite Production by LPS-Stimulated Murine Peritoneal Macrophages

The *in vitro* effect of AMAEL on the release of inflammatory mediators such as nitric oxide by LPS-stimulated murine peritoneal macrophages is depicted in Fig. 3. Co-incubation with *Annona muricata* significantly reduced nitrite overproduction in a dose-dependent manner. The highest inhibition was achieved at 500 µg/ml (73.18% ± 2.36, $p<.001$). AMAEL at 100, 250 and 500 µg/ml did not decrease nitrite production *in vitro* NO scavenging test, using sodium nitroprusside as a NO donator, indicating this effect was not a consequence of a direct scavenging of this radical (data not shown).

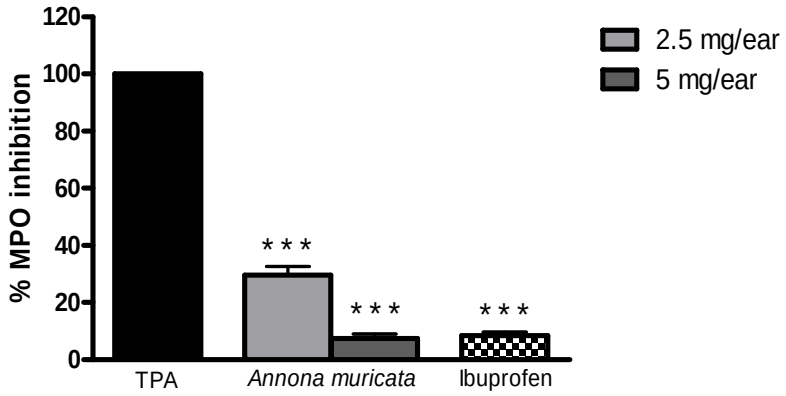

Figure 1: Effect of extract aqueous from leaves of *Annona muricata* L. on myeloperoxidase (MPO) activity in inflamed tissue. (****p < 0.001 vs TPA).*

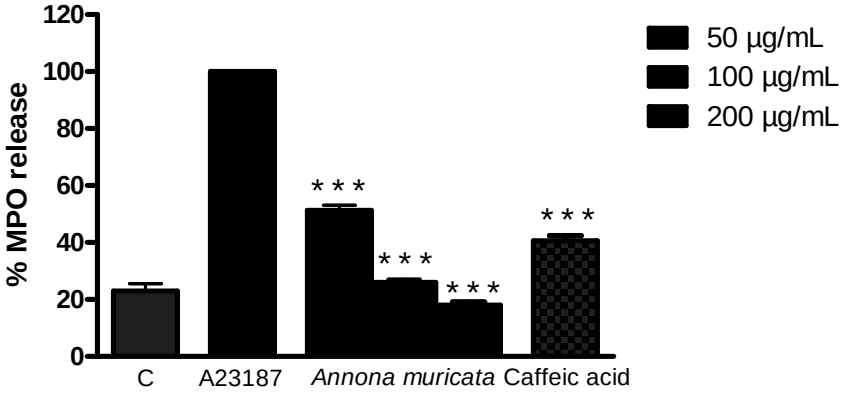

Figure 2: Effect of extract aqueous from leaves of *Annona muricata* L. on myeloperoxidase (MPO) release in activated rat neutrophils. Each value represents the mean ± SEM for three triplicate experiments (**** p < 0.001 vs A23187)*

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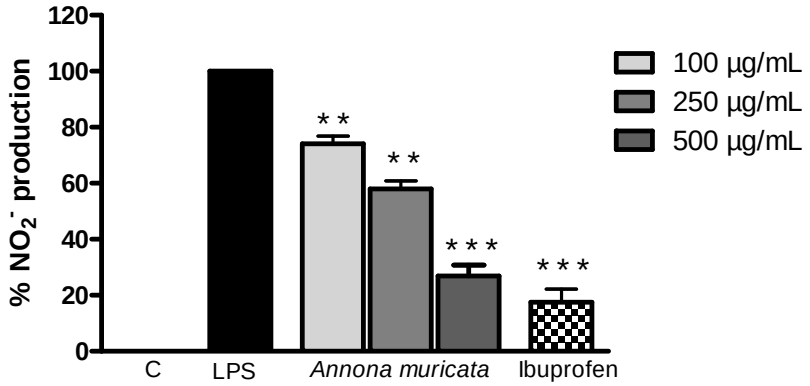

Figure 3: Effect of extract aqueous from leaves of *Annona muricata* L. on release of nitrites in LPS-activated murine peritoneal macrophages. Each value represents the mean ± SEM for three triplicate experiments (***p < 0.01 vs LPS, ***p < 0.001 vs LPS).*

Discussion

Based on the need to validate phytopharmaceutical products that allow the use of local resources in basic health systems, we have shown in this study the anti-inflammatory activity of AMAEL against two inflammation animal models and in two different cell systems, macrophages and neutrophils, the major inflammatory cells. We used the aqueous leaf extract because this is the preparation equivalent to the use in folk medicine. In the qualitative phytochemical screening we have detected phenols compounds, alkaloids having sparingly but we have not found acetogenins in detectable amounts, perhaps because of its poor water solubility (Le Ven et al., 2012). The lack of toxicity showed in the acute mice toxicity and in the MTT cell viability assays, could likely be to a poor presence in the aqueous extract of acetogenins, described by some authors as cytotoxic compounds (De Pedro et al., 2013; Grzybowski, et al., 2013; Chen et al., 2011). De Sousa et al. (2010) relate the presence of acetogenins in the composition of the ethanol extract with an acute toxicity in mice.

The anti-inflammatory activity was confirmed by the paw edema induced by carrageenan and ear edema induced by TPA in mouse. Both models are used to evaluate anti-inflammatory substances. Carrageenan induces paw edema resulting in the release of mediators such as histamine, serotonin, bradykinin, substance P and a platelet activating factor and prostaglandins (Sufredinni et al. 1999). In this study and in agreement with previous results obtained in ethanol extracts of this species by De Sousa (De Sousa, 2010) and Chan and Roslida (Chan and Roslida, 2012), oral treatment with AMAEL significantly inhibited the paw edema (Table 1) and the presence of phenol compounds (tannins, flavonoids and phenolic acids) could explain this anti-inflammatory activity and the possible synergistic effect among these compounds. This evidence suggests that the anti-inflammatory actions of the aqueous extract may be related to inhibition of one or more signaling intracellular pathways involved with the inflammatory mediators. On the TPA-induced edema, the application of AMAEL produced a reduction of about 78% compared with the control group (Table 2). This positive response on the TPA test would indicate that the inhibition of edema could be essentially due to protein Kinase C (PKC) inhibition (Nishizuka et al., 1988). TPA stimulates the actions of PKC in a manner similar to that of endogeneous diacylglycerol, liberated from membrane phospholipids (Marucha et el., 1991).

It is generally accepted that tissue injury associated with inflammation is attributed to infiltration of neutrophils and macrophages followed by the release of proinflammatory mediators such as eicosanoids, toxic radical species and lytic enzymes. Accordingly, inhibition of the function of the macrophages and neutrophils participates on the mechanism of action of a number of anti-inflammatory drugs. AMAEL significantly inhibited some of the functions of these cells, which may be implicated in the anti-inflammatory action detected in the *in vivo* assays. In this sense, the MPO activity, as an indicator of the migration of neutrophils to the inflamed tissue, was calculated and the reduction in the activity of this enzyme in both, ear homogenates and activated rat neutrophils, were highly significant (Fig.1, Fig.2, respectively). On the other hand, the inflammatory macrophages constantly express inducible nitric oxide synthase (iNOS) producing an increase amount of NO, which plays a multifaceted role in inflammation ranging from an increase in vascular permeability and edema formation to tissue cytotoxicity (Moncada et al., 1991). AMAEL exerted significant and dose-dependent inhibition of LPS-stimulated NO production in macrophages without any evidence of cytotoxic effect (Fig.3). The mechanism by which the extract inhibits NO seems to involve attenuation in induction or activity of iNOS, because of that, it was checked that AMAEL did not directly scavenged the NO radicals produced by the NO donor as the sodium nitroprusside. NO inhibition results in suppression of the inflammatory response in different models including carrageenan induced paw edema (Salvemini et al., 1996) thus NO inhibition may be implicated in AMAEL associated reduction for this inflammation model assayed.

Conclusion

The results obtained in this study demonstrated the significant anti-inflammatory activity of the decoction of *Annona muricata* L. leaves that may be related to inhibition of one or more signaling intracellular pathways involved with the inflammatory mediators. This fact validates the ethnomedicinal use of this plant in different pathologies related to inflammation and suggests that this species could be a valuable source of phytodrugs. It is important to note that plant extracts, like AMAEL, usually constitute a range of substances in different concentrations that are capable of establishing a synergism for certain biological effects. The efficacy and safety presented by AMAEL leads to our research group to make advancements to identify the active principles responsible for its anti-inflammatory effects and their possible synergistic activity.

Ethnopharmacological resources as *Annona muricata*, with a large scientific literature that validates its popular uses, represent a potential source of effective, safe, low-cost and alternatives tools to primary health care.

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