

Preclinical screening of *phyllanthus amarus* ethanolic extract for its analgesic and antimicrobial activity

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ABSTRACT

Background: To discover a new agent which possesses dual property of analgesic and antimicrobial activity, thereby reducing the burden of polypharmacy. *Phyllanthus amarus* was screened for its analgesic and antimicrobial activities. **Objectives:** The objective was to evaluate the analgesic and antimicrobial activity, of *P. amarus* ethanolic extract (PAEE). **Materials and Methods:** The ethanolic extract of *P. amarus* was prepared using Soxhlet apparatus. An *in vivo* study using Swiss albino mice was done to screen the central and peripheral analgesic activity of *P. amarus* extract. The extract was administered at a dose of 100 mg/kg body weight orally. The peripheral analgesic activity was assessed using acetic acid induced writhing test. The central analgesic activity was assessed using Eddy's hot plate apparatus. An *in vitro* study was carried out to study the antimicrobial activity of the above extract using selected species of *Streptococcus mutans*, and *S. salivarius*. The antimicrobial activities were determined using the agar well method. **Results:** The ethanolic extract of *P. amarus* showed significant ($P < 0.05$) peripheral and central analgesic activity. *In vitro* antimicrobial screening indicated that the ethanolic extract had shown a zone of inhibition against *S. mutans* and *S. salivarius* in the agar wells. **Conclusion:** This study showed that PAEE exhibited significant analgesic and antimicrobial activities.

Key words: Analgesic, antimicrobial activity, ethanolic extract, *Phyllanthus amarus*

INTRODUCTION

Dental Infections of the teeth have tormented humans constantly.^[1] Microbes play an important role in causing overall dental infections.^[2] The most common ones being *Streptococcus mutans* and *S. salivarius*, which play a major role in causing dental infections leading to caries.^[2] Carious lesions when left untreated, progress and results in severe pain.^[3] Various kind of analgesics and antimicrobials are available today in the market. However, most of them do have their own side effects.^[4,5] Hence, there is a necessity for a newer analgesic and antimicrobial agent. It will be a boon for the patients, if a new agent is discovered, which possess dual property of analgesic and antimicrobial activity, thereby reducing the burden of polypharmacy. This in turn will reduce the incidence of adverse effects and treatment cost. Here comes the importance of medicinal plants. As mentioned in the ancient literatures, a lot of herbs

are known to possess both analgesic and antimicrobial properties.^[6] Moreover they are generally safe, cost effective and nonaddictive. However, they lack proper scientific validation.^[7] Hence, the present study was conducted using and indigenous medicinal plant *Phyllanthus amarus*.

Phyllanthus amarus (*Phyllanthus niruri*) is an herbaceous plant of Euphorbiaceae family. It is commonly called as “stone breaker.” It is commonly seen in central and southern India and is found in many other countries. This herb which nurtures up to 10–60 cm tall, with elliptic leaves is used in India for curing a ailments like jaundice, urogenital problems, dysentery, dyspepsia, arthritis, ulcers, genitourinary tract infections, hemorrhoids, gonorrhea, hepatic, and urolitic diseases etc. It is against Hepatitis B and C virus. It possesses antiviral, anticancer, antitumor, antioxidant, anti-inflammatory, and diuretic activity. It is employed for treatment of nervous debility, epilepsy, and dropsy.^[8–15]

MATERIALS AND METHODS

Institutional Animal Ethical Committee clearance was obtained before the commencement of the work.

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Animals

Six months old healthy Swiss albino mice with an average weight of 25 g were selected for the study. They were maintained under standard housing conditions in the animal house of Yenepoya University.

Plant materials

Phyllanthus amarus plants were cultivated and collected from Northern Kerala. The plants were identified and authenticated at the Biological Sciences Department. They were properly washed in tap water and then rinsed in sterile distilled water and left to shade dry for several weeks. The leaves of the plants were reduced to powdered form using an electric blender. The powder was stored in air-tight containers until required.

Preparation of plant extract

Phyllanthus amarus ethanolic extract

A weighed quantity (500 g) of the coarse powder was taken and extracted with ethanol (90%) in a Soxhlet apparatus [Figure 1]. The extract was concentrated on a water bath at a temperature not exceeding 60°C (yield 20% w/w). The ethanolic extract was suspended in distilled water.

Assessment of central analgesic activity of *Phyllanthus amarus* ethanolic extract

Eddy's hot plate test

In this method, the mice in each group (6/group) was treated with vehicle (distilled water, 0.1 ml orally), *P. amarus* ethanolic extract (PAEE) (100 mg/kg body weight orally), and tramadol (5 mg/kg, intraperitoneally) serving as positive control. All the animals received the drugs for 10 days. On 10th day, after 1 h of drug administration, the mouse was placed on the Eddy's hot plate, with a temperature of 55°C [Figure 2]. Time taken by the mouse to lick its paw or to jump was noted as reaction time. The cut off time was kept as 15 s to prevent injury to the paw.^[15]

Assessment of peripheral analgesic activity of *Phyllanthus amarus* ethanolic extract

Acetic acid induced writhing reflex test in mice

Each group of mice ($n=6$), was treated with vehicle (distilled water, 0.1 ml orally), PAEE (100 mg/kg body weight orally)

and Ibuprofen (30 mg/kg orally) for 10 days. On 10th day, 1-h after the drug administration, analgesic activity was assessed by counting the number of writhes induced by 0.6% acetic acid administered intraperitoneally. Numbers of writhes per animal was counted for 10 min. A writhes was considered when animal showed contraction of abdomen with simultaneous stretching of at least one hind limb [Figure 3]. Protection against writhing was taken as an index of analgesia.^[16]

Assessment of antimicrobial activity of *Phyllanthus amarus* ethanolic extract

The pure clinical isolates were obtained from the Microbiology Department of Yenepoya University. All clinical isolates were checked for purity and maintained on nutrient and potato dextrose agar slant at 4°C in a refrigerator till required for use. Standardized inoculum (0.5 McFarland turbidity standard equivalents to 5×10^8 CFU/ml) of each test organism was spread into sterile Mueller-Hinton agar plate using sterile swab sticks so as to achieve even growth. The plates were then allowed to dry and a sterilized cork borer (9.00 mm in diameter) was used to make wells on the agar plates. A loop full of the sterile agar was dropped into the holes to prevent seepage. The ethanolic extract was introduced into the wells. The plates were allowed on the bench for 1 h for prediffusion of the extract and incubation was done at 37°C for 24 h.^[17]

Phytochemical analysis of *Phyllanthus amarus* ethanolic extract using high-performance liquid chromatography-liquid chromatography-mass spectrometry

High-performance liquid chromatography-liquid chromatography-mass spectrometry (HPLC-LCMS) analysis [Table 1] was carried out on a Shimadzu - Ultra Fast Liquid Chromatography-XR system, which is interfaced to a Mass Spectrometry (Make: AB Sciex, Model: API 4000) instrument at Sequent Laboratories, Mangalore.

Statistical analysis

Data were analyzed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test with the help of InStat -Graph Pad software [GraphPad software Inc., CA, USA].



Figure 1: Preparation of *Phyllanthus amarus* ethanolic extract



Figure 2: Eddy's hot plate test



Figure 3: Acetic acid induced writhing reflex

RESULTS

Effect of *Phyllanthus amarus* ethanolic extract on Pain using Eddy's Hot Plate

The Eddy's hot plate test [Table 2] showed that the withdrawal time of the paw was prolonged in the PAEE group when compared to the normal group thus exhibiting a central analgesic property of the extract.

Effect of *Phyllanthus amarus* ethanolic extract and *Tylophora indica* ethanolic extract on Pain in Acetic acid induced writhing

It was seen that number of writhing (stretching behavior) after acetic acid was injected intraperitoneally was reduced in the PAEE when compared to the normal group thus exhibiting peripheral analgesic property of the extract [Table 3].

Assessment of antimicrobial activity of *Phyllanthus amarus* ethanolic extract

The antimicrobial property was assessed by observing the zone of inhibition in the agar plates. *In vitro* antimicrobial screening indicated that the ethanolic extract showed a zone of inhibition [Table 4] against *S. mutans* and *S. salivarius* in the agar wells.

High-performance liquid chromatography analysis of *Phyllanthus amarus* ethanolic extract

High-performance liquid chromatography analysis of PAEE has revealed that major peaks occurred at a retention

Table 1: Chromatographic conditions

Chromatographic conditions	<i>P. amarus</i> (18)
Extract	Ethanolic extract
Mobile phase	Methanol: water (70:30)
Column	C-18
Column temperature	30°C
Injection volume	10 µL
Flow rate	0.7 ml/min
Run time	30 min
Wavelength	220 nm
Ion source	Electron spray ionization
Mode	Positive ionization
Mass scan range	100-1000 amu
Ion spray voltage	5500 V

P. amarus=*Phyllanthus amarus*

time of 14.45, 16.17, 21.00, and 22.99 min. When these peaks were subjected to LCMS analysis, the molecular weight of the compounds observed was 247.1, 261.1, and 455.3 [Table 5 and Figure 4]. On comparing with the available databases of *P. amarus*, these compounds were identified as derivatives of Phyllanthine or Nirphyllin.

DISCUSSION

In this preclinical study, it is observed that PAEE possess analgesic and antimicrobial properties.

Pain is defined by the International Association for the Study of Pain as, "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."^[17-19]

The mechanisms underlying pain involve both peripheral and central pain mechanisms. In short pain can be branded into two types, nociceptive (due to direct stimulation of peripheral nerve endings by the noxious stimuli arising from wounds, fractures, burns, angina, etc.) and neuropathic (due to dysfunction of the pain perception system within the peripheral or central nervous system as a result of injury, disease or surgical damage). These two components either or both may be involved in the pathology of pain.^[20,21]

Table 2: Eddy's hot plate test

Groups	Withdrawal of paw in seconds (day 10)
Distilled water	2.66±0.085
PAEE	11.34±0.47 ^a
Tramadol	12.28±0.81 ^a

Results are expressed as mean±SD. One-way ANOVA, followed by Tukey-Kramer multiple comparison test, n=6. ^aP<0.001 considered extremely significant on comparing group II, III with group I. PAEE=*Phyllanthus amarus* ethanolic extract

Table 3: Acetic acid induced writhing

Groups	Onset of writhing in minutes	Number of writhing
Distilled water	1.02±0.04	18
PAEE	5.34±0.47 ^a	1
Ibuprofen	3.07±0.01 ^a	1

Results are expressed as mean±SD. One-way ANOVA, followed by Tukey-Kramer multiple comparison test, n=6. ^aP<0.001 considered extremely significant on comparing group II, III with group I. PAEE=*Phyllanthus amarus* ethanolic extract

Table 4: Zones of inhibition of *P. amarus* on *S. mutans* and *S. salivarius*

<i>S. mutans</i>	<i>S. salivarius</i>
5.7±0.3	6.2±0.01

Zones of Inhibition in diameters in mm, mean±SD, n=6. *P. amarus*=*Phyllanthus amarus*; *S. mutans*=*Streptococcus mutans*; *S. salivarius*=*Streptococcus salivarius*

Table 5: Phytochemical analysis of PAEE using HPLC-LCMS

Major peaks with their retention time in minutes	Peak area %	Molecular weight observed	Possible compound
21.00	19.325	261.1	Derivative of phyllanthine
16.17	17.603	261.1	Derivative of phyllanthine
22.99	12.316	455.3	Derivative of nirphyllin
14.45	6.15	247.1	Phyllanthine

PAEE=*Phyllanthus amarus* ethanolic extract; HPLC=High-performance liquid chromatography; LCMS=Liquid chromatography-mass spectrometry

Pain originates due to the sensitization of the nociceptors by various endogenous signaling molecules, like prostaglandins, leukotrienes, histamine, bradykinines, and monoamines. Once the nociceptors are activated, an action potential is generated and transmitted through A_δ fibers and C-fibers. These fibers carry the impulses to the spinal cord and from there to the substantial gelatinosa and thalamus, the core region responsible for the assimilation of pain input. The pain impulses which are assimilated in the thalamus are transmitted to the cerebral cortex by third-order neurons and resulting in pain awareness. These areas of the brain which are involved in pain sensation possess a rich serotonergic, noradrenergic, and dopaminergic innervations thus proposing the role of monoamines in pain modulation.^[22,23]

Various studies showed that selective serotonin reuptake inhibitors (SSRIs) like citalopram fluoxetine, fluvoxamine, paroxetine and sertraline reduce acute pain in the hot plate and tail flick tests. Fascinatingly, the effects of fluoxetine are totally dampened in serotonin worn-out animals suggesting that SSRIs-induced anti-nociception comprises serotonergic pathways.^[23]

Apart from serotonin, norepinephrine also takes part in the modulation of pain. This is confirmed by the fact that repetitive administration of desipramine, a selective norepinephrine reuptake inhibitor (SNRI) produced analgesia in the tail flick and hotplate tests and potentiated the analgesic effect of morphine. This analgesic activity was reversed by naloxone strongly suggesting the involvement of the opioid system in the analgesic effect of SNRI.^[23]

Recent findings have showed a dopamine also has a modulatory role in pain sensation. This is evidenced by the clinical data which show that DA diminution by 6-hydroxydopamine in substantia nigra results in hyperalgesia. A study has shown that the systemic administration of the mixed dopamine agonist apomorphine in rodents exhibited analgesic effects at higher doses. The analgesic action of apomorphine was mediated through central D₂ receptors, as confirmed by the fact that analgesic action was blocked by central-acting D₂-like receptor antagonist sulpiride, but not by the peripheral D₂-like receptor antagonist domperidone.^[23]

Apart from monoamine neurotransmitters, gamma-aminobutyric acid (GABA) also has a prominent role in pain perception. Many GABA facilitators (mainly clonazepam and midazolam) show an analgesic effect after spinal injection in rodent models and in patients with postoperative pain. This is again confirmed by the facts that Tiagabine, a GABA reuptake inhibitor, exhibited analgesic activity in mice after intrathecal injection in different preclinical tests of pain and neuropathy associated pain in humans. Vigabatrin, a GABA transaminase inhibitor, which prevents GABA degradation, showed analgesic activity in several rodent models. Conflicting the above findings, another study reported the analgesic property of Bicuculline, a GABA A receptor antagonist. The analgesic activity of Bicuculline against a heat evoked pain stimuli in rats was observed when administered by microinjections of 40 or 400 pmol in the midbrain periaqueductal grey (PAG). This antinociceptive property of bicuculline can be due to the activation of antinociceptive output neurons in the PAG by its disinhibiting role from tonic GABAergic inhibition on these neurons.^[19,24]

Growing evidence indicates an important role of reactive oxygen species in the central nervous system that augment the sensitivity of sensory neurons and enhance pain

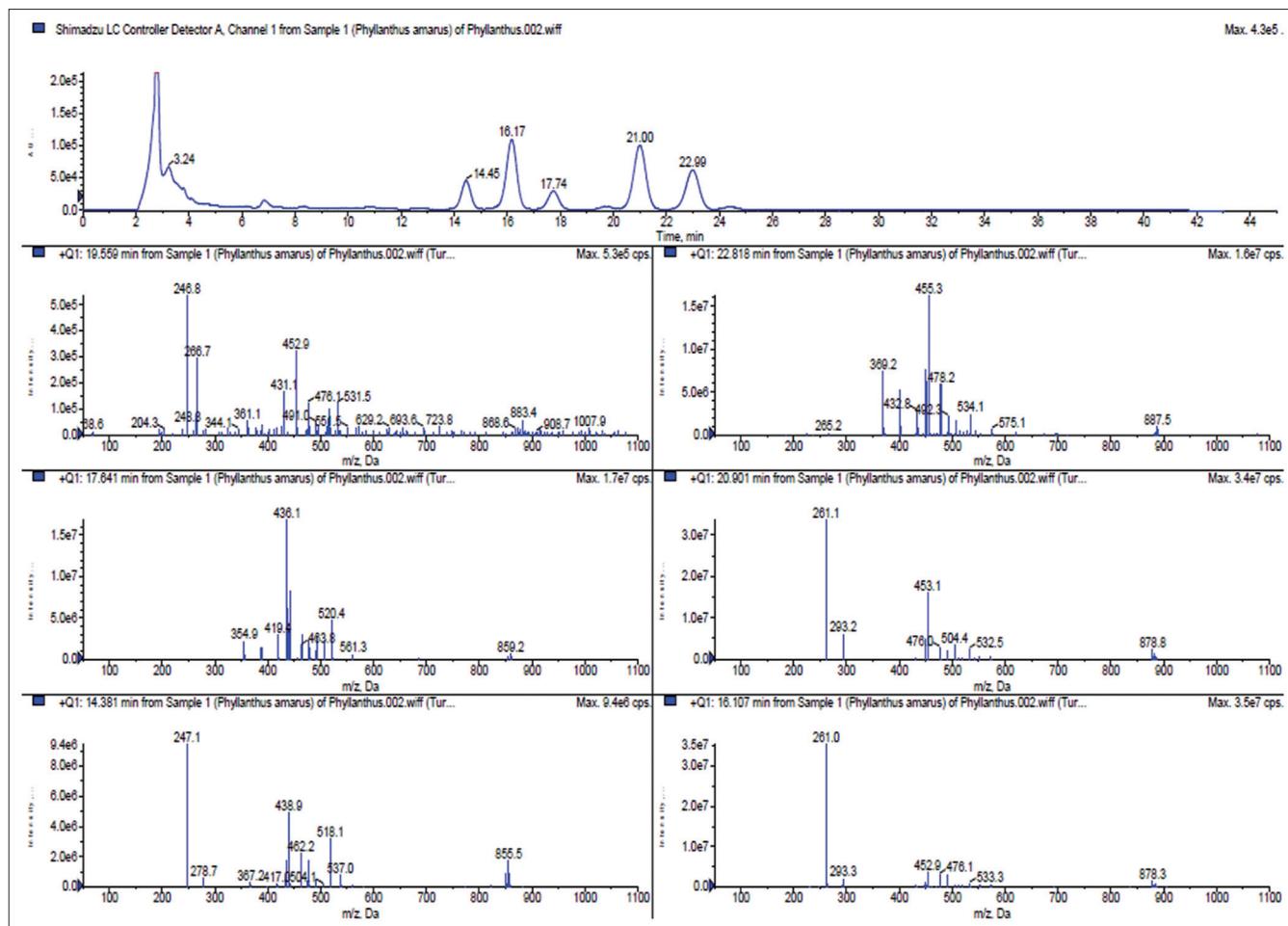


Figure 4: High-performance liquid chromatography-liquid chromatography-mass spectrometry analysis of *Phyllanthus amarus* ethanolic extract

perception. The number of neurons showing mitochondrial reactive oxygen species production is significantly increased in the spinal dorsal horn of rats with neuropathy.^[25,26]

The treatment of pain has improved with the discovery of potent analgesics like aspirin and morphine that interact with the transmitters and modulators of the pain. However, none of currently used centrally and peripherally acting analgesic agents are devoid of adverse effects.^[17-20]

A lot of technical glitches are associated with the screening for analgesic activity of an agent in animals. Main obstacle is pain cannot be checked directly in animals, but can only be measured by noting their reactions to nociceptive stimuli. Usually, thermal, mechanical, electrical, and chemical stimuli are employed in the animal models of pain. The neuronal basis of the above models is not known properly. However, they are helpful in predicting the analgesic activity of a newer agent.^[18]

In this study, the ethanolic extract of *P. amarus* showed significant analgesic activity in the Eddy's hot plate test as well as in acetic acid induced writhing test. The

Eddy's hot plate test is a commonly accepted model for revealing the activity like opioid analgesics.^[18] The acetic acid induced writhing test is mainly used for revealing the activity of peripheral acting analgesics.^[27] The result of our study agrees with the findings of Chopade *et al.*, who observed that the ethanolic extract of *P. amarus* demonstrated central antinociceptive activity in the tail flick model at a dose of 100 mg/kg body weight administered intraperitoneally. The same study also showed the peripheral analgesic activity of PAEE in acetic acid induced writhing model.^[28]

The analgesic activity of *P. amarus* can be attributed to the various phytochemicals present in their ethanolic extracts. There are abundant studies showing that phytochemicals like alkaloids, phytosterols, saponins, triterpenoids, carbohydrates, flavonoids, tannins, phenolic compounds possess analgesic property.^[29]

From HPLC-LCMS analysis carried out on the ethanolic extracts of *P. amarus*, it is postulated that the active components of PAEE are Phyllanthine and Nirphyllin or their derivatives.

Phyllanthine, an indolizidine alkaloid is a derivative of securinine. Securinine compound is found to possess GABA-A antagonistic property. This agent might have shown analgesic activity by the blockade of GABA-A receptors in the midbrain PAG and thereby activating the antinociceptive output neurons in the PAG like Bicuculline, another GABA-A antagonist which is proven to have analgesic property. Or like any other alkaloid, the analgesic property of this component may be due its modulating role on other neurotransmitters, which is not yet proved.^[19,30-32]

The analgesic property of PAEE may be due to the presence of phytochemicals other than alkaloids. Nirphyllin the second active component as seen by the HPLC-LCMS analysis belongs to the group of Lignans. Lignans are known to have antioxidant property. Nirphyllin being a lignan might have shown analgesic property by virtue of its free radical scavenging activity as reactive oxygen species have a role in pain.^[33,34]

Several studies have reported the antimicrobial property of *P. amarus*. As per our knowledge, this is the first study reporting on the antimicrobial activity of *P. amarus* against the common oral pathogens *S. mutans* and *S. salivarius*. This activity of this indigenous medicinal is due to the presence of phytoconstituents like tannins, saponins, cardiac glycosides, and alkaloids.^[35,36]

This study has given an insight about a component, which might possess dual pharmacological properties. That is, analgesic and antimicrobial properties. If the compound is identified and scientifically validated for its dual property, it will be a benefit for the patients suffering from toothache due to microbial infections.

Even though in our study, we were able to show the analgesic and antimicrobial properties of *P. amarus*, further extensive studies are required to establish the analgesic and antimicrobial activities of the key constituents namely phyllanthine and nirphyllin.

CONCLUSION

The results from this study revealed the efficacy of the analgesic and antimicrobial properties of *P. amarus*. The phytoconstituents of this indigenous medicinal plant have a role in battling out pain and microbial infections.

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