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Antibacterial Activity Screening and HPLC Analysis of Crude Extract from *Diacure* a polyherbal formulation

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ABSTRACT

The antibacterial activity of methanolic crude extract of Diacure, a polyherbal formulation was investigated against *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Micrococcus luteus, Escherichia coli and Candida albicans as test strains* at different concentrations (80, 90 and 100µl/disc). Better antibacterial activity was observed with the extracts of *diacure*, that showed excellent inhibitory activity against *Klebsiella pneumoniae, Escherichia coli and Candida albicans* at concentration of 100 µl/disc. Among different bacteria tested *Klebsiella pneumoniae, Escherichia coli and Candida albicans* were found to be more sensitive to crude extract when compared to others. HPLC analysis of the crude extract of diacure, a polyherbal formulation of 11 medicinal plants showed four different Phenolic acids (Tannic acid, Gallic acid, Ferulic acid and Caffeic acid). The results of the study provide scientific basis for the use of the plant extract in the future development as antioxidant, antibacterial, antifungal and anti-inflammatory agent.

Introduction

Medicinal plants as a group comprise approximately 8000 species and account for around 50% of all the higher flowering plant species of India. Over one and a half million practitioners of the Indian System of Medicine use medicinal plants in preventive, promotive and curative applications. In recent years, secondary plant metabolities (Phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents [1] . The World Health Organization (WHO) has given guidelines to the member states to ensure about genuine use of plants and their parts before their use for human health. [2]

Valerian is a well known and frequently used medicinal herb that has a long and proven history of efficacy. It is note specially for its effect as a tranquilliser and nervine, particularly for those people suffering from nervous overstrain [6] . Valeriana has been shown to encourage sleep, improve sleep. It is also used internally in the treatment of painful menstruation, cramps, hypertension, irritable bowel syndrome etc. [7] . It shoud not be prescribed for patients with liver problem. Externally it is used to treat eczema, ulcers and minor injuries [8] . The root is antispasmodic, carminative, diuretic, hypnotic, powerfully nervine sedative and stimulant [9] .

Various extract of medicinal plants have shown inhibitory effects against phytopathogenic fungi *in vitro*[10] Diverse pharmacological activities have been accredited to Phenolic acids by HPLC for instance gallic acid has inflammatory [11] , antibacterial [12] ; caffeic acid with anti-inflammatory [13] ; ferulic acid with anti-inflammatory [13] and antifungal [14] ; tannic acid with antioxidant and astringent property [15, 16] .

The objective of this research was to auntheticate the antibacterial sensitivity and HPLC analysis of methanolic extracts of phenolic acid present in diacure a polyherbal formulation to lengthen the queue of antimicrobial herbs.

Materials and methods

Collection and extraction of medicinal plant material

Plant materials

Ethnobotanicals survey:

Plants were selected for this study based on their medicinal use.Fresh plant parts were collected from the tribal villages in palani hills of tamilnadu, india.

The voucher specimens in duplicate were deposited in herbarium department of botany, Annamalai University (india).Here we are using Diacure a polyherbal formulation of 11 medicinal plants as given below in table.

Preparation of plant extract

500g of dried, powdered plant material diacure, a polyherbal formulation a combination of 11 medicinal plants are mixed 1:1 ratio,the medicinal plants are listed in the table given above, were extracted successively with methanol using soxhelt apparatus. The residual extract was suspended in water for overnight and filtered. The filtrate was dried and was stored at 40C until used.

Antibacterial activity:

Antimicrobial tests. The disc diffusion method was used to determine the antimicrobial activity of the spices. A volume of 0.1 ml of the tested microorganisms gro wn in liquid growth media (at 37– C for 24 hrs, 108-109 cells/ml), was inoculated on Mueller- Hinton growth media, and then spread on the entire surface of the dish using a sterile spatula. Then, sterile paper discs (Whatman: 1.6 mm) with absorbed diacure methanolic extract (80µl, 90 µl ,100 µl/ disc) were placed onto the agar at certain intervals by pressing

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gently. After the plates were incubated at 35ῗ 2−C for 48 hours, the inhibition zones around the discs where no growth occurred were measured in millimeters. The experiments were repeated in duplicate for all of the test strains.

Sample Preparation of phenolic compounds

The phenolic acids were extracted as per the method of Singh et al.17. One gram of each extract was macerated and suspended in 5 ml methanol-water (80:20; v/v). The collected samples were subjected to ultrasonication (Branson Sonifier, Danbury, CT, USA) for 15 min at 4°C followed by centrifugation at 12 500 x g for 15 min. The clear supernatant was subjected to charcoal treatment. The residue was re-extracted twice with the same extracting solution and the supernatant was pooled prior to evaporation under vacuum (Buchi Rotavapor Re Type, Labco, India; Ambala Cantt. India). Dried extract were resuspended in 1.0 ml high-performance liquid chromatography (HPLC)-grade methanol by vortexing and filtered through ultra membrane filter (pore size 0.45 µm: Millipore) before HPLC analysis.

HPLC analysis

Quantitative analysis of the sample was performed according to the method of Singh et al.17 . The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 µl. The peak area was calculated with a Winchrom integrator. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6 mm i.d., particle size 5 µm, Luna 5µ C-18(2); phenomenex, Torrance, CA, USA) at 25°C. Running conditions included: injection volume, 5µl; mobile phase, methanol: 0.4% acetic acid (80: 20 v/v); flow rate, 1 ml/min; and detection at 290 mm. Samples were filtered through an ultra membrane filter (pore size 0.45 µm; E-Merck, Darmstadt, Germany) prior to injection in the sample loop. Tannic, gallic, caffeic, ferulic, benzoic, cinnamic, capachin and salicylic acids were used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards. The amount of each phenolic acid is expressed as micrograms per gram of fresh weight unless otherwise stated.

Results and Discussion

Comparative analysis of antibacterial activity

HPLC analysis

Recent researches indicate that the polyphenols, being secondary metabolites, are present in rich amount in several plants. Many of them possess antioxidant, anti-inflammatory and several others therapeutic properties. The HPLC fingerprints of the crude extracts of diacure showed four types of the Phenolic acids i.e. Tannic acid, gallic acid, Ferulic acid and caffeic acid that are present in varying amount (Fig. 2). Although a primary objective of carrying out HPLC may be to standardize dosage, more information may be obtained during the course of a run, if appropriate detection hardware and software are used.

Results and discussion

Table 1 provides the botanical name, family, plant parts used together mixed in 1:1 ratio forming a polyherbal formulation of 11 medicinal plants. Methanolic extract of diacure showed antimicrobial activity by inhibiting one or more microorganisms. The results of the antimicrobial screening of the methanolic extract were shown in figure 1 below.The tested plant extracts were most active against gram positive microorganisms than the gram negative microorganisms. This is in agreement with the previous reports by several workers (Gupta MP etal).

Methanol extracts of diacure, a polyherbal formulation were tested for antimicrobial activity with 80, 90 and 100µl/disc concentrations. The antibacterial activity may be indicative of the presence of some metabolic toxins or broad spectrum antibiotic compounds (ref20). Many medicinal plants as Syzigium cumini showed good activity against many microbes (Kloucek P,etal) as reported by Rajakaruna etal also. In previous findings flower, roots, and stem of some medicinal plants showed a range of activity against several bacteria and protozoa.(Diallo D etal).

In this study methanolic extract of diacure a polyherbal formulation of 11 medicinal plants showed antibacterial activity against Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Micrococcus luteus, Escherichia coli and Candida albicans, of concentrations 80, 90 and 100µl/disc. Of which methanolic extract of 100µl/disc concentration showed greatest activity against Pseudomonas aeruginosa, Escherichia coli as compared with other organisms tested. Then 80µl/disc concentration showed greatest activity against Klebsiella pneumoniae, Micrococcus luteus, Escherichia coli and Candida albicans, as compared with other organisms tested. Finally 90µl/disc concentration showed greatest activity against Klebsiella pneumoniae, Escherichia coli and Candida albicans.Overall the methanolic extract showed greatest activity against Klebsiella pneumoniae, Escherichia coli and Candida albicans, This is in agreement with the previous reporters of same microbes with different medicinal plants(Diallo D etal).

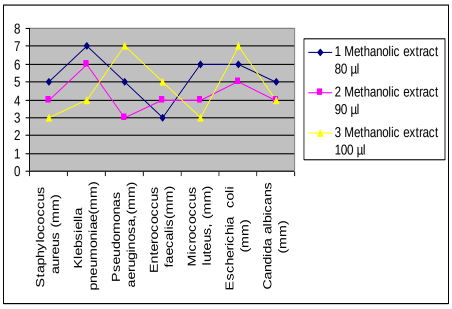

Figure 1 showing the antimicrobial activity of diacure a polyherbal formulation

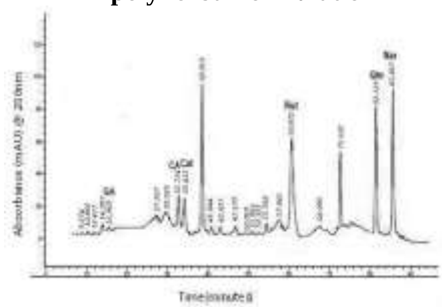

Fig 2a : Hplc of standard samples

Peak identities: GA, gallic acid; Cat, catechin; CA, caffeic acid; ChA, chlorogenic acid; Rut, rutin; Pzn, phloridzin; Qtn, quercetin; Nar, naringenin.

The HPLC 'fingerprint' (Fig. 2b) of the methanolic extract of diacure show major peaks at the retention times (min.) of 2.61 at a wavelength of 280 nm. Out of the the extracts, diacure

showed maximum amount of naringenin(400 µg/g), tannic acid (319.33 µg/g) followed by gallic acid (20.76 µg/g) and ferulic acid (37.55 µg/g) (Fig. 2b). Diacure also revealed three types of Phenolic acids in which tannic acid (285.90 µg/g) was present in maximal amount whereas gallic acid and caffeic acid were in trace (Fig. 2b) HPLC analysis of the samples revealed wide-variability in their Phenolic acid content (Fig. 2a). The results of the antibacterial activity of the various crude extracts were in agreement with the uses of the extract of Diacure in traditional medicine. The rhizome and aerial parts of the plants appeared to be a potential source of broad spectrum antibiotics.

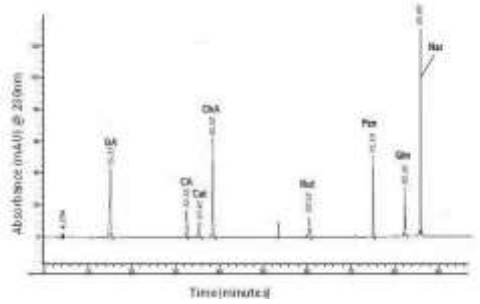

Fig2b: HPLC of methanolic extract of diacure a polyherbal formulation

According to Bauer and Tittel [21] and Springfield *et al.*, [22] , HPLC fingerprinting is the best way for chemical characterization, and therefore this study also established HPLC fingerprint for the active phenolic acids that can act as antioxidant, antifungal, antibacterial and anti-inflammatory. diverse pharmacological activities have been accredited to phenolic acids for instance, gallic acid has anti-inflammatory [23] , antibacterial [24] , caffeic acid with anti inflammatory [13] , antibacterial, antifungal [24] ; ferulic acid with anti-inflammatory [13] , antifungal [25] ; cinnamic acid with antifungal [25] , anthelmintic [15] ,natural protection against infections by pathogenic microorganisms [26] ; salicylic acid with antipyretic and antiinflammatory [27] , externally used as antiseptic, antifungal and for various skin conditions [15] .

Naringenin are considered as one of the main groups of compounds responsible for the sedative activity of *Valeriana*. For the quantitative determination of valepotriates a direct spectrophotometric scanning on TLC plates was compared with HPLC [30]. In *Tectonagrandis*, centrifugal partition chromatography was used to isolate the active compound such as deoxylapachol and tectoquinine that indicated fungal cell wall stress [4]. The actively ingredients are called valepotriates, research has confirmed that these have a calming effect on agitated people, but are also a stimulant in cause of fatique [31] . The leaves of the swietenia macrophylla plant possess antidiabetic, anti-inflammatory, antipyretic, analgesic, antispasmodic, antimicrobial, cathartia and expectorant activities [32] .

Conclusion

In conclusion, this study provides new scientific information aboutdiacure, based on its antimicrobial potential and chemical profiling that has never been reported. The antibacterial activity of *diacure* may be attributed to the various phytochemical constituents present in the crude extract. The purified components may have even more potency with respect to inhibition of microbes. Further work on the types of phytoconstituents and purification of individual groups of bioactive components can reveal the exact potential of the plant to inhibit several pathogenic microbes and encourage in developing a noval broad spectrum antimicrobial herbal formulation in future.

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