Antinociceptive and anti-inflammatory effects of roots extracts from *Actinidia arguta* (Sieb.et Zucc.) Planch

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ABSTRACT

Aims: The roots of *Actinidia arguta* (Sieb. et Zucc.) Planch (also named *tengligen*) have medicinal uses as anti-tumour, antinociceptive, and anti-inflammatory agents. In this study, we evaluated the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch *(Tengligen)*. ***Methods:*** Three conventional methods were used to carry out the antinociceptive effect: *acetic acid*-induced abdominal writhing, formalin induced hind paw licking, and hot plate test. In addition, the anti-inflammatory effect was investigated by carrageenan-induced paw edema in rats. ***Conclusion:*** From the obtained results, we found that the total ethanol extract, ethyl acetate fraction, and n-butanol fraction all significantly inhibited acetic acid-induced writhing and both phases of the formalin induced pain response, increased the time of response to thermal stimulation in hot plate test, and exhibited significant dose-related inhibition of carrageenan induced paw edema volumes when compared with the control group. Based on our findings, we conclude that the flavonoid and saponin contents of *tengligen* are responsible for the antinociception and anti-inflammatory effects of *Actinidia arguta* (Sieb. et Zucc.) Planch, respectively.

Keywords: *Actinidia arguta* (Sieb.et Zucc.) Planch; antinociceptive; anti-inflammatory.

INTRODUCTION

Inflammation and pain are two kinds of defense reactions of living systems in reply to any invasive factor. Considering the frequent occurrence of adverse side effects of current drugs, the screening and development of new agents with more powerful analgesic and anti-inflammatory effects and with lesser side effects is still in progress.

Actinidia arguta (Sieb.et Zucc.) Planch belongs to Actinidia genus in the family Actinidiaceae, which is a family composed of large, deciduous vines. The genus *Actinidia* contains 54 species. Most species in this genus are found in the mountains of South China, but some species are also found in Siberia, Japan, Indochina, Malaysia, Indonesia, and New Zealand.[1–3]

The fruits of *A. arguta* (Sieb.et Zucc.) Planch (kiwi fruit) are edible, while the roots of the plant, which are called *Tengligen*. *Tengligen* is normally used as an ingredient in folk medicine, different preparations of this plant such as, decoctions, infusions and powders, are used in traditional Chinese medicine to treat several diseases such as inflammatory, ache, tumors, diabetes, hyperlipidemia, hepatitis, and so on. Especially, *Tengligen* has been used to treat alimentary canal tumors, such as those characterizing gastric, esophagus, and liver cancer.[4–6] Phytochemical investigation previous resulted in the isolation and identification of many metabolites including flavonoids, saponins, terpenoids, polysaccharide, and essential oils from *Tengligen*.[7]

To the best of our knowledge, there is no study to date evaluating the antinociceptive and anti-inflammatory effects of the major fractions of *Tengligen*, despite the prevalent historical use of these plant parts in traditional

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Chinese medicine for more than 2000 years. Here, we filled this gap in knowledge by evaluating the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch *(Tengligen)*. The antinociceptive effect was examined on chemically and thermally induced nociceptive pain in mice through *acetic acid*-induced abdominal writhing, formalin induced hind paw licking, and hot plate test. In addition, the anti-inflammatory effect was investigated by carrageenan-induced paw edema in rats.

MATERIALS AND METHODS

The roots of *A. arguta* (Sieb.et Zucc.) Planch were collected in the Changbai Mountains, Jilin Province, China, in March of 2012, and identified by Dr. Yue-Chun Sun. A voucher specimen was deposited at Life Science and Technology College, Heilongjiang Bayi Agricultural University (No120326).

Phytochemical screening

Approxmiately 5kg of dried *Tengligen* was extracted with 95% ethanol(2×10L) under heat reflux for 2h in duplicate, and the solvent was removed under reduced pressure. The residue of the ethanol extract (525.3g) was suspended in 1000mL of distilled water and was partitioned sequentially with cyclohexane, chloroform, ethyl acetate, and then n-butanol at room temperature. In total, 5 major fractions were collected and concentrated under reduced pressure until all the solvent had been removed to give an extract sample. The chemical constituents of the extract were analyzed qualitatively and screened to detect saponins, flavonoids, alkaloids, and terpenoids by thin layer chromatography.[8,9] The extract was stored at –20ºC until pharmacological tests.

Animals

Wistar rats (aged 8–12 weeks; weight, 180–200g) and ICR(Institute of Cancer Research,USA) mice (aged 2–3 weeks; weight, 18–22g) of either sex were used for experiments described here. Animals were bought from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and used once each. All animal treatments were strictly in accordance with international ethical guidelines concerning the care and use of laboratory animals, and all the experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University (number CEAAU-189). All animals were kept in standard laboratory conditions (relative humidity 55–60%, room temperature 25±2ºC, 12h light/dark cycle), and had free access to standard diet and water *ad libitum* during the duration of the experiment. All animals were acclimated to the laboratory environment for a period of 7 days prior to performing the experiments.

Writhing test

Writhing test was carried out as described by Pinheiro et al.,(2010) and Mariana et al., (2012).[10,11] In brief, the mice were pre-treated with positive drug and test drug for 3 days; approximately a half hour after the final administration on the third day, 0.6% acetic acid (0.1mL/10g) was given by intraperitoneal injection. The number of writhing movements during the next 15 minutes was recorded. The number of writhes in each treated group was compared with the number observed in the control group, which received only a saline injection instead of acetic acid, but was otherwise treated identically. The inhibition rate of writhes $[(\bar{x}$ control-$\bar{x}$ test$)/\bar{x}$ control$]$ × 100, was then calculated.

In the writhing test, mice were randomly divided into 8 groups. These groups were (1) control group with intragastrical administration (i.g.) isometrical physiological saline, the test drug groups at dosages of (2–6) 50mg/kg of 5 major fractions, (7) total ethanol extract (TEE, 200mg/kg), (8) a positive drug control group (i.g. ibuprofen [50mg/kg]).

Formalin induced nociception

The procedure we followed for formalin-induced nociception was similar to the method described by Hunskaar and Hole (1987),[12] but with modifications by Gomes et al., (2007).[13] In short, animals were divided into 7 groups of 10 mice each. Each mouse was pretreated with an oral dose of 1 of the 5 major fractions (50mg/kg, respectively), TEE (200mg/kg), or ibuprofen (50mg/kg) (positive drug). The mice were pre-treated with the positive drug and the test drugs for 3 days; a half hour after the final administration on the third day, 20μl of 5% v/v formalin was injected subcutaneously into the right hind paw of the mouse. The time that the animal spent on licking or biting of the injected paw was recorded as the animal's pain response. Based on previous studies of this response pattern,[11] we measured responses up until 5 min after formalin injection (early phase, neurogenic pain response), and from 15–30 min after formalin injection (later phase, inflammatory pain response).

Hot plate test

Hot plate test was conducted as described by Eddy and Leimbach (1953).[14] In this test, female mice were placed in a 24cm diameter glass cylinder on a heated metal plate that was maintained at 55±1ºC. Each animal was habituated twice to the hot plate prior to the experiment. The response was defined as licking or biting of a paw, or jumping. The time in seconds between the placing of the animal on the platform and the first reaction observed was recorded as the response latency time. The mice exhibiting latency times greater than 30s or less than 5 s was excluded from analyses. Animals were divided into 8 groups of 10 mice each and pretreated with 1 of the 8 treatments described above. Mice were tested at 30 min, 60 min, 90 min, and 120 min intervals after oral administration of the extracts or ibuprofen.

Carrageenan induced paw edema

Carrageenan induced hind paw edema model was used to determine the anti-inflammatory effect.[15] Rats were orally treated with 1 of the 7 previously described treatments approximately 30 min prior to injection of 1% carrageenan (0.1mL) in the right hind paw (sub-plantar region) of each rat. Hind paw edema volumes were measured using the plethysmometer at 0.5h, 1h, 2h, and 3h intervals post injection. The percentage inhibition was calculated according to the hind paw volume.

Statistical analysis

The experimental data was expressed as mean±standard error of the mean (SEM). The statistical analysis was carried out using a one way analysis of variance (ANOVA) followed by Tukey's t-test. The differences with $p<0.05$ were considered statistically significant, $p<0.01$ were considered highly significant.

RESULTS

Fractionation and phytochemical screening

Extraction yield was measured and comprised about 42.7% of the final product, from which 5 major fractions were separated. As shown in Table 1, during phytochemical screening, the fractions of ethyl acetate and n-butanol exhibited strong positive reactions for flavonoids and saponins, respectively.

Writhing test

In the acetic-acid-induced writhing test (shown in Table 2), the TEE, EAF (ethyl acetate fraction), and NBF (n-butanol fraction) exhibited significant analgesic effects after oral administration in mice that were subjected to acetic acid-induced writhing compared with the control ($p<0.01$). In addition, the highest analgesic activity was observed with the TEE (200mg/kg), which was lower than the analgesic activity observed with 50mg/kg ibuprofen alone. The maximum inhibition of the nociceptive response (81.37%) was achieved with the 50mg/kg ibuprofen dose (alone). However, no significant analgesic effects were observed for any of the doses of CYF (Cyclohexane fraction) group, CHY (Chloroform fraction) group or the aqueous fraction group.

Formalin induced pain

Treatment with TEE (200mg/kg), EAF (50mg/kg), NBF (50mg/kg), and ibuprofen alone (50mg/kg) each caused significant increases in the percentage pain inhibition in both the early and later phases of the formalin test (both $p<0.01$) (Figure 1). Moreover, in both early and late phases of the experiment, the percentage of pain inhibition observed during treatment with TEE, EAF, and NBF was as good as with ibuprofen alone. However the

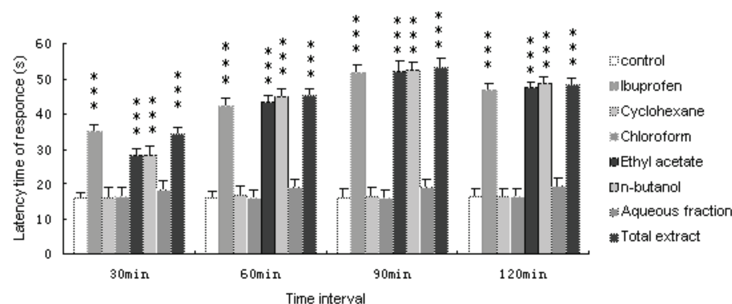

Figure 1. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in hot plate test. The effect has been calculated on the basis of latency time of response. Each point is the mean±SEM of ten animals.*** $p < 0.01$ in comparison to normal saline group.

percentage pain inhibition observed during treatment with TEE, EAF, NBF, and ibuprofen alone was greater in the later phase than in the early phase.

Hot plate test in mice

In the hot-plate test, the TEE, EAF, NBF, and ibuprofen treatments produced a significant increase in the response time from 30 min to 120 min (both $p<0.01$) (Figure 2). At the same time, the antinociceptive response observed with the TEE, EAF, and NBF were considerably more pronounced than that obtained with ibuprofen at all the tested doses from 60 min to 120 min. Lastly, the analgesic activity observed with the TEE, EAF, and NBF was lower than the analgesic activity of ibuprofen alone group from 0 min to 30 min.

Carrageenan-induced paw edema

In the carrageenan-induced hind-paw edema experiment we conducted the treatments we evaluated all demonstrated significant anti-inflammatory activity at all tested doses in comparison with the negative control 3h after carrageenan administration ($p<0.01$) (Table 3). Amongst the

Table 1. The result of phytochemical screening of total ethanol extract and separated fractions from Tengligen.

Sample	color	Saponin	Flavonoid	Alkaloid	Terpenoids
TEE	yellowish	+++	+++	–	++
CYF	whitish	–	–	–	++
CHF	whitish	–	–	–	–
EAF	yellowish	+	+++	–	–
NBF	yellowish	+++	+	–	–
AQF	slight yellowish	+	+	–	–

TEE (total ethanol extract); CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction).
+++: high content; ++: medium content; +: low content; -: no content (content was evaluated as the sediment or the intensity of color by thin layer chromatography).

Table 2. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in acetic acid-induced nociception (per 15 min).

Group	Dose (mg/kg)	Number of writhing	Inhibition(%)
Control	–	56.27 ± 2.03	–
Ibuprofen	50	10.48 ± 2.46***	81.37***
CYF	50	54.12 ± 2.49	3.82
CHF	50	52.27 ± 2.12	7.11
EAF	50	12.59 ± 2.26***	77.63***
NBF	50	16.41 ± 2.57***	70.84***
AQF	50	53.43 ± 2.64	5.47
TEE	200	11.73 ± 2.87***	79.15***

CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction) ;TEE (total ethanol extract).
*** $p < 0.01$ significantly different from control.

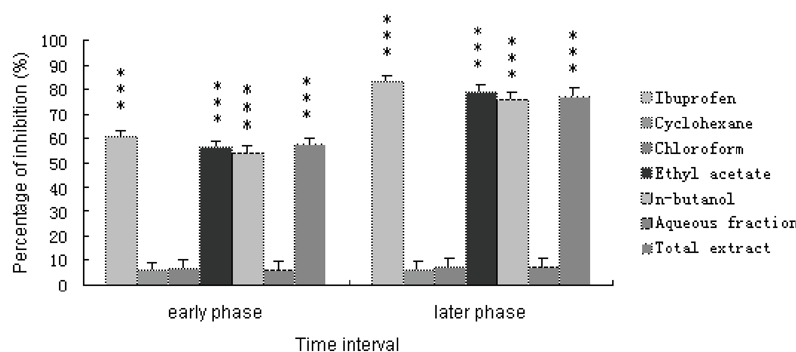

Figure 2. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in formalin-induced nociception. The effect has been calculated on the basis of percentage of pain inhibition. Each point is the mean±SEM of ten animals.*** $p < 0.01$ in comparison to normal saline group.

Table 3. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in carrageenan-induced hind paw edema.

Group	Percent of inhibition (%)			
	0.5h	1h	2h	3h
Ibuprofen (50mg/kg)	8.21 ± 1.68	43.73 ± 2.51**	71.79 ± 2.49**	96.61 ± 2.16***
CYF (50mg/kg)	3.15 ± 2.62	19.82 ± 2.85	36.82 ± 2.57	45.07 ± 3.93**
CHF (50mg/kg)	4.41 ± 2.31	16.58 ± 2.03	45.73 ± 2.71**	49.79 ± 2.17**
EAF (50mg/kg)	16.57 ± 1.87	46.45 ± 1.73**	78.96 ± 2.63**	95.31 ± 2.82***
NBF (50mg/kg)	17.15 ± 2.69	44.82 ± 2.36**	74.898 ± 2.27**	93.63 ± 2.93***
AQF (50mg/kg)	11.47 ± 2.52	19.87 ± 2.93	45.25 ± 3.09**	54.46 ± 2.97**
TEE (200mg/kg)	15.36 ± 1.82	42.76 ± 2.09**	79.74 ± 2.94**	97.62 ± 1.88***

CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction) ;TEE (total ethanol extract).

** $p < 0.05$ significantly different from control

*** $p < 0.01$ significantly different from control

treatments we examined, the TEE (200mg/kg), EAF (50 mg/kg), NBF (50mg/kg), and ibuprofen alone (50mg/kg) all significantly reduced paw edema volume from 1h to 3h of the experiment (p<0.05 and p<0.01).The TEE resulted in the highest inhibition of paw edema volume at 3h in comparison with the negative control group (p< 0.01).

DISCUSSION

Pain is the most common motivating factor to seek medical attention. Although adequate pain relief is achieved with the currently available analgesic agents like opioids or NSAIDs, some of their serious side effects are major limitations to their routine use in therapy. Recently, many natural medicines derived from plants, soil microbes, marine organisms,etc, were considered as the effective and safer for the treatment of various diseaces including inflammation and pain.

The roots of *Actinidia arguta* (Sieb. et Zucc.) Planch (*Tengligen*) is a traditional herb, which has been used for treatment of several diseases such as inflammatory, ache, tumors, diabetes, hyperlipidemia, hepatitis, and so on. In this study, we evaluated the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch (*Tengligen*).

Here, we investigated the antinociceptive and anti-inflammatory effects of *Tengligen* in three analgesic models: the acetic acid-induced writhing model, the formalin induced licking model, and the hot-plate test. All of these were employed with the goal of assessing the antinociceptive effects of the total ethanol extract and its sub-fractions. In addition, the carrageenan-induced hind paw edema model was used to assess anti-inflammatory properties of the total ethanol extract and its sub-fractions.

The acetic acid-induced writhing reaction in mice has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents, and is described as a typical peripheral analgesic model for visceral inflammatory pain.[16] The hot-plate test, which uses thermal stimulus to induce pain, is frequently used to evaluate the centrally mediated antinociceptive effect.[17] The acetic acid-induced writhing test and hot plate test are both regarded as gold standards for determining the peripheral- and central-acting analgesic effects of drugs, respectively.[12,18] The peripheral analgesic effect may be mediated via the inhibition of cyclooxygense and/or lipoxygenases(and/or inflammatory mediators), while the central analgesic action may be mediated via inhibition of central mediators. For this reason, we selected the acetic acid-induced writhing test and the hot plate test to test analgesic potential. In order to explore the analgesic activity pathway of 95% ethanol extract and different fractions of *Tengligen*, ibuprofen was selected as the reference drug. Ibuprofen is a nonselective COX inhibitor, in that it inhibits two isoforms of cyclooxygenase, COX-1 and COX-2. The analgesic, antipyretic, and anti-inflammatory activity of NSAIDs appears to operate mainly through inhibition of COX-2.

Our results indicated that the TEE, EAF, and NBF all exhibited significant analgesic properties, apparent by their statistically significant inhibition of writing, and the increased latency of response to chemical and thermal stimulation, respectively, in comparison with control groups (Table 2 and Figure 1). Overall, the results of our study show that at all dose levels examined, the TEE, EAF, and NBF treatments significantly reduced the number of acetic acid-induced writhes, which suggests that the analgesic effects of TEE and some fractions may be mediated via peripheral pathways of pain perception. The increase in reaction time, as evidenced by the latency measured in the hot plate test, indicates

that the TEE, EAF, and NBF may also possess a central analgesic effect.

Formalin induced nociception is a well-described model for evaluating the mechanism of pain and analgesia.[13] The nociceptive behavior after formalin injection was distinctly recorded in two phases. The early phase of paw licking/biting response starts immediately after injection and is considered probably due to direct stimulation of nociceptors.[19] The later phase which appears little later is considered to be due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing.[20] In formalin test, the central antinociceptive agents can inhibit both phases of formalin-induced pain while peripherally active ones inhibit the later period of pain.[21]

Our study here demonstrates that TEE, EAF, and NBF of *Tengligen* can inhibit both phases of formalin induced pain with a more potent effect on the later phase than the early phase (Figure 2). Based on the results we observed, we tentatively suggest that the analgesic activity of the extract is dependent upon both central and peripheral sites of action. This conclusion is congruent with that reported by Shibata et al in 1989.[22] Taken together, our data suggest that the ability of the TEE, EAF, and NBF of *Tengligen* to suppress pain perception may be mediated via both the peripheral and central pathways of pain perception.

The carrageenan test is highly sensitive to non-steroidal anti-inflammatory drugs and has long been used as a phlogistic tool for evaluating new anti-inflammatory drugs. Carrageenan-induced inflammation has been used previously to detect orally active anti-inflammatory agents, and therefore has a significant predictive value for anti-inflammatory agents that act by inhibiting the mediators of acute inflammation.[23] The results we obtained here show that the *Tengligen* extract does indeed possess anti-inflammatory activity (Table 3).

Previous investigations have reported that saponins, flavonoids, phenylpropanoids, quinines, and steroid compounds have all been separated and structurally identified from *Tengligen*. Flavonoids, saponins, tannins, phenolic compounds, and glycosides have all been associated with various degrees of anti-inflammatory and analgesic activities.[24–28] Our results indicate that the mechanism of antinociceptive and anti-inflammatory effects of *Tengligen* extracts may be related to flavonoids and saponins. Flavonoids inhibit cyclooxygenase and lipoxygenase which are involved in initiation stage of inflammation reactions, but the precise mechanism of flavonoids in inhibition of these enzymes is not known. In addition, flavonoids are putative antioxidant with high activity of free radical scavenging. Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response.[29]

In the past, pharmacological studies have focused mainly on crude extracts, and many of the constituents responsible for different pharmacological activities remain unknown. The antinociceptive and anti-inflammatory effects observed in this study may be in part due to the activity(s) of one or a combination of some of the classes of compounds we identified and examined here. However, more studies are needed to firmly establish the clinical efficacy of the TEE and fractions of *Tengligen* we examined, as well as to reveal the exact mechanism of action that characterizes the response to these substances.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Standardization of a polyherbal formulation (HC9) and comparative analysis of its cytotoxic activity with the individual herbs present in the composition in breast cancer cell lines

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ABSTRACT

Introduction: The present study aims to standardize a polyherbal formulation (HC9) that was previously shown to exhibit excellent antioxidant and cytotoxic activity in breast cancer cells. Here, we have compared the cytotoxic activity of HC9 with its individual components in breast cancer and non-cancerous cells. **Methods:** Physico-chemical and phytochemical evaluation of HC9 was performed. Qualitative and quantitative HPTLC analysis of component herbs and HC9 was done by using specific markers. The cytotoxic activity of HC9 with its individual components was evaluated in breast cancer (MCF-7 and MDA MB-231) and non-cancerous cell lines (HEK-293, HaCaT and MCF-10A) by MTT dye uptake. Results: Physico-chemical results revealed that HC9 contained 7.24% total ash content, 9.52% of alcohol-soluble extractive, 0.801 specific gravity, 0.50g/ml bulk density and exhibited 7.18% loss on drying. Phytochemical results revealed the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds, and absence of terpenoids. The individual herbs of HC9 and the formulation showed the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, swertiamarin, berberine, connesine and 2-hydroxy-4-methoxybenzaldehyde. At 160μg/ml concentration, HC9 exhibited cytotoxicity in both MCF7 and MDA MB231 with no cytotoxicity in MCF-10A, HaCaT and HEK-293. In contrast, at this concentration, the individual herbs of HC9 exhibited cytotoxicity not only in cancerous cells, but also in non-cancerous cells. **Conclusion:** These results suggest that the standardized HC9 formulation was safe to non-cancerous cells and exhibited significant antineoplastic potential in breast cancer cells. Thus, HC9 could be a potential drug candidate in breast cancer.

Keywords: Cytotoxicity, HPTLC, physicochemical, polyherbal formulation HC9, phytochemical, standardization.

INTRODUCTION

Herbal medicines have gained global importance over the past few decades.[1–6] Their medicinal and economic benefits have been accepted in both developing and industrialized nations, particularly in developing countries, where they are being traditionally used against various disease complications.[7–9] Herbal remedies, in the form of a single herb or polyherbal formulations, play a prime role in the healthcare system because of their wide biological activity, easy accessibility, cost effectiveness and safe usage.[10–12] However, these medicines have not yet integrated into the modern clinical practice due to lack of experimental and clinical evidence on their quality.[13,14]

The complexity of polyherbal formulations impose a greater challenge in establishment of their quality, efficacy and safety, compared to single herbal counterparts.[15–17] Thus, it becomes important to standardize the herbal drugs by various parameters and sophisticated techniques to ensure their quality, safety and efficacy.[18–20] Various

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regulatory bodies such as World Health Organization (WHO), European Agency for the Evaluation of Medicinal Products (EMEA), United States Pharmacopeia (USP), and Department of AYUSH, Government of India, have provided the standardization guidelines for development of herbal preparations.[21,22]

In the present work, we have standardized a polyherbal formulation (HC9) that we previously reported to possess antioxidant and cytotoxic activity.[1] It is composed of nine medicinal herbs that include *Picrorhiza kurroa*, *Cyperus rotundus, Zingiber officinale*, *Cedrus deodara*, *Tinospora cordifolia*, *Holarrhena antidysenterica*, *Swertia chirata*, *Cissampelos pareira* and *Hemidesmus indicus* (Table 1). The formulation was prepared based on the reported anticancer and immunomodulatory activity of the individual herbs present in it (Table 2). We have standardized each herbal component of HC9 and the composite formulation with respect to their marker compounds. We have also evaluated the physicochemical and phytochemical parameters of HC9. After standardizing HC9, we have compared the cytotoxic activity of HC9 with its individual components in human breast cancer (MCF-7 and MDA MB-231) and non-cancerous transformed (HEK-293 and HaCaT) cell lines. This was done to evaluate whether the individual components of HC9 were more active than the whole formulation.

MATERIALS AND METHODS

Chemicals and reagents

Tissue culture plasticware was purchased from BD Biosciences (CA, USA) and Axygen Scientific Inc (CA, USA). Dulbecco's Modified Eagles Medium (DMEM) powder, penicillin and streptomycin were obtained from Invitrogen/Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). All other common solvents were procured from Qualigen Fine Chemicals (Mumbai, India) and HPTLC grade solvents were purchased from Merck (Mumbai, India). Reference marker compounds for High Performance Thin Layer Chromatography (HPTLC) analysis were obtained from the Natural Remedies Pvt Ltd (Bangalore, Karnataka-India) and Sigma-Aldrich (St. Louis, MO, USA). The solvents used for high-performance thin-layer chromatography (HPTLC) analysis were obtained from MERCK (Mumbai, India).

Collection, identification and authentication of plant materials

The whole/parts of all nine component herbs of HC9 were purchased from Shri Shailya Medi-Pharms (Solapur, Maharashtra, India). The individual bulk herb samples were stored in air-tight containers and kept in air-conditioned environment until further use. The samples were authenticated and validated macroscopically and microscopically in Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India). Voucher specimens of herbs have been deposited at the Department of Botany, Agharkar Research Institute and Herbaria of Medicinal Plant Conservation Centre (MPCC), Pune (Table 1).

Extract preparation

All nine herbs of HC9 were washed, dried and fine powdered separately. Ethanolic extracts of individual herbs were prepared by soxhlet extraction method.[1] For the preparation of HC9 formulation, equal parts of each powdered plant material of HC9 were mixed in 1:1 ratio and subjected to soxhlet extraction method using ethanol. The resulting extracts were centrifuged at 13000rpm for 15 min to remove the particulate matter. The supernatants were filter-sterilized using Swiney filter (pore size, 0.45 μm) and the resultant filtrates were stored in aliquots at -80°C until further use.

Organoleptic evaluation of HC9

The organoleptic characters of the powdered HC9 were evaluated by appearance, size, shape, color, texture, odor and taste according to the guidelines of Indian Pharmacopoeia.[47]

Table 1. Composition of polyherbal formulation (HC9).

Plant materials in HC9	Family	Parts used	Voucher specimen no.
Picrorhiza kurroa	Plantaginaceae	Root	R-120
Cyperus rotundus	Cyperaceae	Rhizome	R-121
Zingiber officinale	Zingiberaceae	Bark	R-122
Cedrus deodara	Pinaceae	Root	S/B-096
Tinospora cordifolia	Menispermaceae	Stem	S/B-097
Holarrhena antidysenterica	Apocynaceae	Seed	S-119
Swertia chirata	Gentianaceae	Whole plant	WP-078
Cissampelos pareira	Menispermaceae	Root	MPCC 290
Hemidesmus indicus	Apocynaceae	Root	MPCC 2354

This table shows nine plant materials, their families, voucher specimen numbers alongwith the parts used in the preparation of polyherbal formulation (HC9).

Table 2. Properties of individual herbs of HC9.

Herbs in HC9	Reported anticancer activity	Medicinal properties	References
Picrorhiza kurroa	Breast and skin cancer Protect against Adrynomycin induced cardomyopathy	Antioxidant, immunomodulatory, antibacterial, antiperiodic, hepatoprotective, antiasthamatic, gastrointestinal, anti-urinary activity	23–26
Cyperus rotundus	Gastric cancer, lymphoma, leukemia, cytotoxic and apoptotic role	Appetizer, antioxidant, immunomodulatory, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, high estrogens reducer, breast pain inhibitory activity	27–29
Zingiber officinale	Breast, cervical, colon, lung, ovary, prostate cancer protect against Doxorubicin induced acute nephrotoxicity	Antioxidant, appetizer, anti-inflammatory, antiangiogenic, cardiotonic, antiplatelet, antihepatotoxic, antifungal activity	30–32
Cedrus deodara	Breast, cervical, skin, leukemia, colon, lung and ovary cancer	Anti-inflammatory, immunomodulator, anti-ulcer, anti-fungal, anti-arthritic, anti-allergic, anti-oxidant activity	33–35
Tinospora cordifolia	Prostate, liver, skin and breast cancer Protect against chemo induced leucopenia	Immunomodulator, antioxidant, anti-inflammatory, anti-stress, gastrointestinal and hepatoprotection, anti-allergic activity	36–38
Holarrhena antidysenterica	Anticancer	Appetizer, anti-diabetic, diarrhea, anti-oxidant activity	39
Swertia chirata	Skin cancer	Blood purifier, appetizer, Antioxidant, , anti-inflammatory, immunomodulator, anti-hepatotoxic, antidiabetic, antimicrobial activity	40–41
Cissampelos pareira	Lung, leukemia, lymphoma	Antioxidant, , immunomodulator, anti-inflamatory activity, skin diseases, gastric ulcers, cardiac and abdominal pain reducer	42–43
Hemidesmus indicus	Hepatocancer	Blood purifier, appetizer, antioxidant , anti-inflammatory, anti-microbial, anti-hepatotoxic activity and used to treat kidney, urinary and skin diseases	44–46

This table shows the reported anticancer, immunomodulatory as well as other medicinal properties of component herbs of HC9.

Determination of physicochemical parameters of HC9

Physico-chemical parameters such as total ash content, total viable count, loss on drying of extract, ethanol-extractable matter in the mixture of air-dried powder material, determination of pH, bulk density as well as specific gravity of HC9 extract were carried out at Indian Drug Research Institute (IDRI), Pune (Maharashtra, India) according to the prescribed standard methods in Indian Pharmacopoeia.[47]

Preliminary phytochemical analysis of HC9

The preliminary phytochemical analysis of HC9 was done by Indian Drug Research Institute (IDRI), Pune (Maharashtra, India). The extract was screened to detect the presence of secondary metabolites such as alkaloids, carbohydrates, flavanoids, saponins, terpenoids, tannins and phenolic compounds.[47]

HPTLC finger printing profile

Identity of individual herbs and HC9 formulation was confirmed by detecting the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde in *P. kurroa*, *C. rotundus, Z.officinale*, *C. deodara*, *T. cordifolia*, *H. antidysenterica*, *S. chirata*, *C. pareira* and *H. indicus,* respectively. Stocks and working solutions of different marker compounds were prepared in respective diluents (Table 3). Standard (marker compounds) and samples (HC9 and nine individual herbs) were applied onto a thin layer chromatography (TLC) plate, using an automatic TLC sampler (Linomat 5) as described previously.[5]

In brief, the samples (standards and test samples) were spotted as bands (8mm width) with a Camag (Muttenz, Switzerland) Hemilton microlitre syringe onto a precoated aluminum-backed silica gel 60F-254 plate (20 × 10cm; layer thickness 250µm; Merck, Darmstadt, Germany) using a Camag high-performance thin-layer chromatography (HPTLC) system equipped with an automatic TLC sampler (Linomat 5), TLC scanner 3, and integrated software Win-Cats version 4. A constant application rate (0.1µL/s) was employed and the space between the two bands was 6 mm. The respective working solutions of standards (Table 3) were applied to the TLC plate along with the test solution. Linear ascending development was carried out in 20cm × 10cm twin trough glass

Table 3. Solvents of marker compounds along with their concentrations.

Marker compounds	Solvents	Stock solution (mg/ml)	Working solution (mg/ml)
Picroside-I	Methanol	2	0.2
Nootkatone	Methanol	10	0.1
6-Gingerol	Methanol	10	2
Matairesinol	Methanol	1	0.1
Berberine	Methanol	2	0.02
Conesine	Methanol	1	1
Swertiamarin	Methanol	2.3	0.02
2-hydroxy-4-methoxybenzaldehyde	n-Hexane	10	0.2

This table shows respective marker compounds of individual herbs of HC9 along with their diluents, stock and working solutions.

chamber pre-saturated with the respective mobile phase. The optimum chamber saturation time for the mobile phase was 20 min at room temperature. The chromatoplates were developed up to 80mm under chambersaturation conditions to get good resolution of phytochemical contents. Subsequent to development, TLC plates were dried in a current of air with the help of an air-dryer to evaporate solvents from the plates. The plates were examined using A Camag model III TLC scanner with CATS 4.0 integration software. Densitometric scanning was performed in the appropriate absorbance mode with a slit dimension of 6 × 0.45mm and ascanning speed of 10mm/s. A deuterium lamp was used as source of radiation. The amount of marker compounds present in HC9 was determined from the calibration curve obtained by plotting the concentration of standard against the peak area of test samples.

Cell lines

The human breast carcinoma cell lines, MCF-7 and MDA MB-231 and non-cancerous transformed cell lines, HEK-293 (Human Embryonic kidney) and HaCaT (Human Keratinocyte) used in the study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2mM L-glutamine supplemented with 10% fetal bovine serum and 100U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO2 incubator at 37°C. Non-tumorigenic normal mammary epithelial cell lineMCF-10A was a kind gift from Dr. Milind Vaidya (ACTREC, Mumbai). The cells were grown in DMEM:Ham's F12 (1:1) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100U/ml of penicillin-streptomycin, 10μg/ml insulin, 20ng/ml EGF and 0.5 mg/ml hydrocortisone. The cells were incubated in a humidified 5% CO2 incubator at 37°C.[48]

Cytotoxic Assay

Cytotoxicity of nine component herbs of HC9 and the whole formulation was determined in the cancerous and non-cancerous cell lines by MTT dye uptake.[49] Briefly, MCF-7, MDA-MB-231, HEK-293 HaCaT and MCF-10A cells were seeded at 1×10^5/ml density in 96-well plates. Next day, the cells were incubated with various concentrations of HC9 and ethanolic extracts of individual herbs (0–160μg/ml) for 24 h and incubated in 5% CO2 incubator at 37°C. Next day, the MTT solution (5mg/ml) was added to each well and the cells were cultured for another 4 h at 37°C in 5% CO2 incubator. The formazan crystals formed were dissolved by addition of 90μl of SDS-DMF (20% SDS in 50% DMF). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) with the ELISA microplate reader (Biorad, Hercules, CA) at OD 570–630nm. The percentage viability was calculated as:

$$\% \text{ Viability} = [\text{OD of treated cells}/\text{ OD of control cells}] \times 100$$

Statistical analysis

IC_{50} values were calculated by using Kyplot software. All the assays were performed in triplicates and repeated at least three times at different time points. The data has been presented as IC_{50} values and mean ± SD.

RESULTS AND DISCUSSION

Organoleptic evaluation

The powdered HC9 was evaluated for its organoleptic properties. The results revealed that HC9 was dark green in color with characteristic odor, bitter taste and fine texture. These parameters form the basic criteria for selecting a raw drug.[54] Fine texture of powdered HC9 indicated the smoothness and surface uniformity that forms the primary character to assess the quality of a herbal drug.[50]

Physico-chemical analysis

HC9 was evaluated for total ash content, ethanol soluble extractive, loss on drying at 105ºC, pH, specific gravity and bulk density. All the values have been summarized in Table 4a. Physico-chemical analysis of HC9 revealed that the total ash content present in HC9 was 7.24%. The total

Table 4 (a) Physio-chemical characteristics of HC9.

Parameters	Values
Total ash content	7.24%
Ethanol extractives	9.52%
Loss on drying	7.18%
pH	6.1 ± 0.2
Bulk density	0.50g/ml
Specific gravity	0.801

Table 4(b) Permissible limits of physico-chemical parameters of individual herbs in HC9.

Plant materials in HC9	Total ash content (%)[a]	Ethanol extractives (%)[b]	Loss on drying (%)	References
Picrorhiza kurroa	NMT 7	NLT 10	NMT 13	Ayurvedic Pharmacopoeia of India; Volumes I,III and IV
Cyperus rotundus	NMT 8	NLT 5	–	
Zingiber officinale	NMT 6	NLT 4.5	NMT 7.13	
Cedrus deodara	NMT 2	NLT 7	–	
Tinospora cordifolia	NMT 7	NLT 6	NMT 7.5	
Holarrhena antidysenterica	NMT 7	NLT 18	–	
Swertia chirata	NMT 6	NLT 10	–	
Cissampelos pareira	NMT 7	NLT 11	–	
Hemidesmus indicus	NMT 4.3	NLT 15	–	

[a]NMT: not more than; [b]NLT: not less than, –: not available

This table shows shows permissible limits of physico-chemical parameters of individual herbs in HC9 according to Ayurvedic pharmacopoeia of India; Volume I, III and IV

ash values of the individual plant materials of HC9 have been reported to be in the range of 2–8% (Table 4b). Determination of total ash value is an important criteria to judge the authenticity and purity of the crude drug.[50] It indicates total amount of inorganic material present in the drug after its complete incineration. A high ash value indicates contamination, substitution, adulteration during the preparation of drug.[51] The results indicated that HC9 has low inorganic material.

The percentage yield of alcohol-soluble extractive of HC9 was found to be 9.52% w/w. The alcohol-soluble extractive values of the individual plant materials in HC9 have been reported to be in the range of 4.5–18% (Table 4b). The extractive value indicates the amount of active ingredient present in the given amount of plant material when extracted with respective solvent.[50] Less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying, storage or formulation preparation. The alcohol-soluble extractive of HC9 was found to be within the acceptable range.

Loss on drying at 105ºC of HC9 was found to be 7.18%. This value is indicative of amount of moisture content present in the drug.[51] The test for loss on drying actually determines water as well as volatile matter content in drug when subjected to heat. The high moisture content in herbal drugs endorses microbial as well as insect contamination. The low moisture content is always desirable for higher stability of drugs. Our results showed that the formulation could be stored for a long period and would not be easily contaminated with microbes.[51]

The pH conventionally represents the acidity or alkalinity. HC9 (1% w/v solution) showed a pH of 6.1 indicating that the formulation was acidic in nature.

Bulk density, a measure used to describe packing of particles or granules, of HC9 was found to be around 0.50 g/ml. Lower value of density indicates good flow and higher value indicates poor flow properties of formulation.[50] The specific gravity of HC9 was found to be 0.801. All these values indicated that HC9 exhibited good flow properties.

Preliminary phytochemical evaluation

The preliminary phytochemical screening of HC9 demonstrated the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds, and absence of terpenoids. These qualitative tests are used to detect the presence of functional groups, which play an important role in the biological activity of the drug.[49]

HPTLC analysis of ethanolic extracts of individual components of HC9 and the composite formulation

TLC fingerprinting profile followed by HPTLC analysis of component herb present in HC9 and the formulation was performed by using respective marker compounds

Table 5. Major chemical compounds present in HC9 along with the markers used in the study.

Plant materials	Major chemical compounds	Selected marker compounds in the study	References
Picrorhiza kurroa	Picroside I, Picroside II, Picroside IV and 6-ferulloylcatalpol	Picroside-I	23–24, 26
Cyperus rotundus	α-copaene , cyperene, β-selinene, β-cyperone, nootkatone,valerenal, caryophyllene oxide, α-selinene	Nootkatone	27, 52
Zingiber officinale	6-gingerol, 8-gingerol 10-gingerol and, 6-shogaol	6-Gingerol	30–31
Cedrus deodara	Wikstromol, matairesinol, dibenzylbutyrolactol	Matairesinol	33–34
Tinospora cordifolia	Cordifolioside A, tinocordifolin, berberine, tinosporadine, tinocordifolioside, makisterone,cordifol	Berberine	53–54
Holarrhena antidysenterica	Conesine, antidysentericine	Conesine	39,55
Swertia chirata	Mangiferin, swertiamarin, sweroside,amarogentin, Swertinin, swertianin, swerchirin	Swertiamarin	40, 56–57
Cissampelos pareira	Mensmine, pareirine, hayatinine, bebeerine, beberine, tetrandrine	Berberine	42, 58–59
Hemidesmus indicus	2-hydroxy-4methoxy-benzaldehyde, Hemidesmin 1 and 2, α-amyrin, β-amyrin, lupeol	2-hydroxy-4-methoxybenzaldehyde	44–46

This table shows major chemical compounds present in individual herbs of HC9 along with the markers used in the study for HPTLC analysis of HC9.

Table 6. HPTLC analysis of HC9 and individual plant materials.

Marker compounds	Mobile phase	λ_{max} (nm)	Rf Value	Amount (%) of marker compound in HC9	Amount (%) of marker compound in individual herbs
Picroside-I	Choloform: ethanol [8.8:1.2]	282	0.22	18.41	27.76
Nootkatone	N-hexane: EtOAc [3:7]	249	0.97	4.59	6.59
6-Gingerol	N-hexane: EtOAc [6:4]	282	0.6	12.04	46.03
Matairesinol	EtOAc:MeOH:FA:H2O [7:1.5:0.5:1]	284	0.83	8.89	30.01
Berberine	n-but: EtOAc: GAA:H2O [3:5:1:1]	350	0.29	4.06	10.36
Conesine	Toulene: EtOAc: diethylamine [6.5:2.5:1]	520	0.68	1.66	6.58
Swertiamarin	EtOAc:MeOH:H2O [7.5:1.5:1.2]	244	0.66	4.41	12.43
Berberine	n-but: EtOAc: GAA: H2O [3:5:1:1]	350	0.29	4.2	5.95
2-hydroxy-4-methoxybenzaldehyde	Toulene: EtOAc: GAA [7:2:1]	282	0.91	12.72	13.78

EtOAc: ethyl acetate; MeOH: methanol; FA: formic acid; H2O: water; n-but: n-butanol; GAA: glacial acetic acid
This table summarizes the marker compounds, mobile phases, wavelength (λ_{max}) as well as Rf values of spots visible in the HPTLC profiles of the each herb.

(Table 5). The finger printing profiles were developed in respective solvent systems as given in Table 3 and their corresponding peaks were recorded at respective Rf values. Table 6 summarizes the marker compounds, mobile phases, wavelength (λ_{max}) and R_f values of spots visible in the HPTLC profiles of each herb.

The individual plant extracts showed Rf values of 0.22, 0.97, 0.6, 0.83, 0.29, 0.68, 0.66 and 0.91 corresponding to their marker compounds picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin and 2-hydroxy-4-methoxybenzaldehyde in the extracts (Table 6). Similarly, HC9 showed Rf values corresponding to the presence of respective marker compounds in the formulation. Thus, all the component herbs of HC9 were authenticated and found to be present in the the formulation based on HPTLC analysis.

The amount of marker compounds present in the extracts of individual plants of HC9 and the formulation was also evaluated. *P. kurroa*, *C. rotundus, Z. officinale*, *C. deodara*, *T. cordifolia*, *H. antidysenterica*, *S. chirata*, *C. pareira*

and *H. indicus* was found to have 27.76, 6.59, 46.03, 30.01, 10.36, 6.58, 12.43, 5.95 and 13.78% of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 6). on the other hand, HC9 was found to have 18.41, 4.59, 12.04, 8.89, 4.06, 1.66, 4.41, 4.2 and 12.72% w/w of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 6). The overall results indicated that HC9 contained more amount of picroside-I, 6-gingerol, matairesinol and 2-hydroxy-4-methoxybenzaldehyde compared to the other marker compounds. Thus, the activity of HC9 could be due to the presence of high amounts of picroside-I, 2-hydroxy-4-methoxybenzaldehyde, 6-gingerol and matairesinol.

Effect of individual extracts and HC9 formulation on cell viability

After standardization of HC9, we wanted to compare its cytotoxic activity with the component herbs to know whether the individual components of HC9 were more active than the whole formulation. Thus, cytotoxicity was evaluated in human breast cancer cell lines, MCF-7 and MDA MB-231 as well as in non-cancerous cell lines, MCF-10A, HaCaT and HEK-293.

The cytotoxicity results showed that IC_{50} value of HC9 in MCF-7 was lower (150.29μgml^{-1}) than individual plant extracts except for *S. chirata* showing IC_{50} value of 109.35μgml^{-1} (Table VII). However, *S. chirata* was cytotoxic to non-cancerous cells at lower concentrations with IC_{50} values of 65.87, 24.63 and 71.10μgml^{-1} in MCF-10A, HaCaT and HEK-293, respectively (Table 7).

In MDA MB-231, IC_{50} value of HC9 was lower (184.50 μgml^{-1}) than individual plant extracts except for *Z. officinale, C. deodara,* and *H. indicus* showing IC_{50} values of 176.38, 158.62 and 130.88μgml^{-1}, respectively. However, these herbs were cytotoxic to the non-cancerous cells and showed IC_{50} values of 166.67, 84.33 and 217.23μgml^{-1}, respectively in MCF-10A; 62.36, 58.15, 107.19μgml^{-1}, respectively in HaCaT and 48.71, 50.64 and 105.61, respectively in HEK-293 (Table 7). Interestingly, HC9 showed higher IC_{50} values in MCF-10A (>640μgml^{-1}), HaCaT (>640μgml^{-1}) and HEK-293 (586.10μgml^{-1}) compared to the component herbs (Table 7). HC9 was non-cytotoxic up to 160µg/ml concentration in non-cancerous cell lines and exhibited significant cytotoxicity in MCF-7 and MDA MB-231 at the same concentration (Supplementary data S1–S5). These results suggest that the standardized HC9 formulation was safe to non-cancerous cells and exhibited significant anticancer potential for breast cancer cells compared to the component herbs.

CONCLUSION

Standardization of the polyherbal formulation (HC9) was done according to Ayurvedic Pharmacopoeia of India guidelines (Department of AYUSH, Government of India). The present study may be used as a reference standard for quality control and standardization of polyherbal formulations that could help in strengthening the use of medicinal herbs. Our study suggests that compared to the component herbs, HC9 exhibited significant cytotoxicity in breast cancer cells without killing the non-cancerous cells. Further investigations are underway to identify the underlying mechanisms of antineoplastic activity of HC9 in breast cancer.

Table 7. IC_{50} values of HC9 and individual herbs in breast cancer and non-cancerous cell lines.

Plant materials in HC9	IC50 (μgml^{-1})				
	MCF-10A	HaCaT	HEK-293	MCF-7	MDA MB-231
P.kurroa	351.55	497.61	336.92	320.76	374.60
C. rotundus	193.01	131.42	144.23	180.65	331.64
Z. officinale	166.67	62.36	48.71	186.29	176.38
C. deodara	84.33	58.15	50.64	157.50	158.62
T.cordifolia	102.00	211.78	162.47	384.95	471.15
H. antidysenterica	299.95	457.38	188.82	>640	>640
S. chirata	65.87	24.63	71.10	109.35	233.95
C. pareira	89.07	137.04	72.59	291.84	492.92
H. indicus	217.23	107.19	105.61	182.99	130.88
HC9	>640	>640	586.10	150.29	184.50

This table summarizes the IC_{50} values of HC9 and individual herbs that were dosed at different concentrations (0–640 µg/ml) in non-cancerous and breast cancer cell lines.

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PHCOG J ORIGINAL ARTICLE

Effect of Digoxigenin-3-O-rutin isolated from *Trigonella foenum graecum* on T_4-induced hyperthyroidism and serum lipid concentrations

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ABSTRACT

In this study, effect of digoxigenin-3-O-rutin isolated from the seeds of *Trigonella foenum graecum* on thyroid hormones and serum lipid concentrations was evaluated in L-thyroxine (L-T_4)-induced hyperthyroidism in rats. Digoxigenin-3-O-rutin was administered (10mg/kg) to L-thyroxine (L-T_4)-induced hyperthyroidic rats and alterations in the concentrations of serum thyroid hormones, insulin, glucose, hepatic 5′-monodeiodinase (5′DI) and glucose-6-phosphatase (G-6-Pase) activity were analyzed. Antioxidant status was estimated by determining the levels of antioxidative enzymes and lipidperoxidation. L-T_4 (500μg/kg, s.c./d) administration increased the serum levels of thyroxine (T4), triidothyronine (T3), glucose, insulin, different lipids, activity of hepatic 5′-DI and G-6-Pase. High lipidperoxidation level was observed both in liver and cardiac tissues with a depletion in cellular antioxidants. On the contrary, test drug (10mg/kg) treatment improved the alterations with respect to hormonal levels, lipid concentrations and lipid peroxidation towards normalcy and enhanced the antioxidant activities. Rats treated with PTU generally gave lower results compared to groups treated with the test drug. The antithyroidic role of the test compound is mediated possibly through the inhibition in 5′DI activity. Improvement in lipid profile by the test drug might have protective effect on cardiovascular health in vivo.

Keywords Digoxigenin-3-O-rutin, hyperthyroidism, 5″DI, serum lipids, insulin.

INTRODUCTION

Cardiac glycosides have a long history of therapeutic use for the treatment of heart-diseases. The relationship between thyroid hormone and the cardiovascular system has been extensively demonstrated in numerous experimental and clinical studies.[1,2] Heart is one of the main target organs for the action of thyroid hormone, and any change in the thyroid hormone status indirectly affects the cardiac function.[3]

Thyrotoxicosis is a term given for the clinical manifestation of hyperthyroidism which can invoke heart and vascular abnormalities through the mechanism at heart muscle cells nuclear level. Also the cardiac contractility, resting heart rate and cardiac output are increased. Hyperthyroidism may cause cardiac complications because of the increase in heart rate, myocardial contractility, oxygen demand and give rise to conditions silent coronary artery disease or compensated heart failure.[4]

Therefore, amelioration of hyperthyroidism is sometimes considered as an important aspect in controlling cardiovascular problems. For its regulation some conventional medicines such as neomarcazole, methimazole and propyl-thiouracil are prescribed. Despite the fact that many patients give preference to herbal drugs that are known to be safe and economic, nothing much has been investigated on cardiac glycoside which can ameliorate hyperthyroidism. Most of the investigations made so far on cardiac glycosides are in relation to the regulation of ischemic stroke, cancer and neurodegenerative diseases.[5,6] Since natural products are considered as a major source of potential drugs, in the present investigation I isolated and evaluated a novel phytochemical for its potential to

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ameliorate hyperthyroidism and related cardiovascular abnormalities.

Trigonella foenum graceium (TFG) seeds have been reported to protect against dyslipidemia, cardiac problems, hypercholesterolemia, and hyperthyroidism.[7–12] Recently we isolated and reported the cardio-protective potential of, digoxigenin-3-O-rutin a novel compound of TFG in isoproterenol induced myocardial infarction.[12] In the present investigation, an attempt has been made to evaluate the effect of digoxigenin-3-O-rutin on T_4-induced hyperthyroidism with reference to changes in serum thyroid hormones, hepatic 5'DI activity, lipids, antioxidative enzymes, blood glucose and insulin levels, keeping in mind the association of CVD and thyroid problems.

EXPERIMENTAL

Chemicals

L-thyroxine (L-T_4) was purchased from Sigma Chemical Co. Ltd. St. Louise, USA. Radioimmunoassay (RIA) kits for the estimation of serum T_4, T_3 and Insulin were supplied by Bhaba Atomic Research Center. Assay kits for different lipids and glucose were purchased from Ranbaxy Pvt. Ltd., Mumbai, India, thiobarbituric acid, sodium dodecyl sulphate, sulphuric acid and ethylene diamine tetra-acetic acid were obtained from E. Merck (India) Ltd., Mumbai, India.

Plant material and extraction

Dried fenugreek seeds (1kg) were finely powdered and subjected to 70% ethanolic extraction in a soxhlet apparatus at 60°C for 12h. The extract was vacuum dried to obtain it in ethanol-free powdered and was then processed for the isolation of cardenolide following as done in our laboratory previously and the modified method of Lei *et al.*[12,13] In brief, it was partitioned between n-hexane and water and the aqueous layer (110g) was subjected to silica gel chromatography followed by elution with water and 40% MeOH. The elute (14g) was subjected to column chromatography using sephadex LH-20 and silica gel to obtain the final compound (25mg).

Animals

Experiment was carried out in Wistar albino rats, weighing 190–200gm, housed in a standard photoperiod (14h light: 10h dark) and temperature (27 ± 1°C) controlled room with the provision of laboratory feed (Gold Mohur feed, Hindustan Lever Limited, Mumbai, India) and water ad libitum. Ethical guidelines of the Committee for the Purpose of Control and Supervision on Experiments in Animals, Ministry of Social Justice and Empowerment, Government of India, (Regd. No. 779/2012-13) were followed.

Experimental design

Thirty five healthy rats were divided in to five groups of seven each. While Group I animals receiving distilled water (0.1ml/day/animal) served as control, animals of group II, III, IV and V were made hyperthyroidic by administering L-T_4 (500μg/kg, s.c.) for 12 consecutive days as done earlier.[14] After 12 days of thyroxine treatment, group II animals, serving as hyperthyroidic control were administered with distilled water while animals of group III and IV received two different concentrations of digoxigenin-3-O-rutin dissolved in distilled water (5.0 and 10.0mg/kg, p.o) respectively. Group V was administered with 10mg/kg of PTU.[15] The administered doses of digoxigenin-3-O-rutin and L-T_4 and PTU were taken from our earlier studies.[11,12,14] Experiment was continued for 4 weeks and then terminated. On the day of termination over night fasted animals were sacrificed by cervical decapitation and blood from each animal was collected, and serum was separated for the estimations of thyroid hormones and lipids.

Biochemical estimations

After exsanguinations, heart and liver of each animal were removed quickly, washed and homogenized with phosphate buffered saline (PBS, pH 7.4). The homogenates were centrifuged at 17,000g for 30 min at 4°C and the supernatant was used for biochemical estimations including lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) and glutathione peroxidase (GPx).

Radioimmunoassay of thyroid hormones and insulin

Total circulating T_3 and T_4, hepatic 5′-DI and insulin were estimated by radioimmunoassay (RIA) in serum samples following the protocol provided in the RIA kits supplied by Bhabha Atomic Research Centre (BARC), Mumbai, India, as done routinely in our laboratory.[16,17] Intra-assay variations for T_4 and T_3 were <5% and <1%, respectively, and for insulin assay it was <4.9%.

Estimation of marker enzymes, lipids and glucose

Different serum lipids were estimated using standard commercial kits. Low- density lipoprotein

cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated using the formula of Friedwald et al.[18] Serum glucose concentration was measured by the glucose oxidase/peroxidase method of Trinder.[19]

Study of lipid peroxidation (LPO)

Tissue lipid peroxide level in heart was measured by the method of Ohkawa et al.[20] and finally LPO was expressed as nM of MDA formed/h/mg protein. The levels of lipid hydro peroxide (LOOH) were measured by the method of Jiang et al.[21]

The activities of antioxidants such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) levels were assayed by the methods of Marklund and Marklund,[22] Aebi,[23] Rotruck et al.[24] and Ellman[25] respectively. G-6-Pase was assayed by the inorganic phosphate release method as described by Baginski et al.[26] Protein content was determined following our routine method of Lowry et al.[27]

Statistical analysis

All values were expressed as mean±S.E.M. Differences in mean values were compared using version Prism 4 software for windows, Inc., La jolla, CA, USA and by one way analysis of variance (ANOVA) followed by post hoc Newman-Keuls multiple comparison tests. $P<0.05$ was considered as statistically significant.

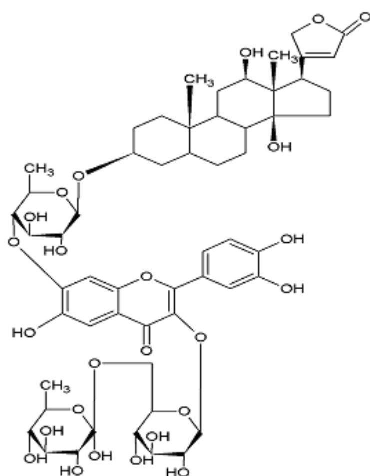

Figure 1. The structure of the isolated compound, derived from UV, IR, NMR and Mass spectra, which was identified as digoxigenin-3-O-rutin.

RESULTS

Digoxigenin-3-O-rutin, obtained as yellow crystalline powder and the molecular formula was assigned as $C_{56}H_{72}O_{25}$ which was deduced from ESI. The isolated compound revealed its UV, IR and NMR data consistent with our earlier report[10] (Fig 1).

Effects on serum concentration of thyroid hormones

The effects of L-T_4 in serum T_3, T_4, hepatic 5′DI, G-6-pase activity and body weight are shown in Fig. 2. In T_4 treated rats T_3, T_4, hepatic 5′DI, G-6-pase activity enhanced significantly $P<0.001$ as compared to control values. A significant decrease $P<0.001$ in body weight was observed in T_4-induced animals as compared to control. Administration of the test drug 5mg/kg to hyperthyroid animals significantly decreased the T_3, hepatic 5′DI, G-6-pase activity ($P<0.001$, $P<0.01$ and $P<0.05$ respectively) as compared to T_4 treated animals. However, at 10mg/kg the test drug administration significantly decreased T_3, T_4, hepatic 5′DI, G-6-pase activity with an increase in body weight ($P<0.001$ or $P<0.01$) as compared to T_4-induced animals. In PTU+T_4 treated group also thyroid hormones decreased significantly ($P<0.001$ or $P<0.01$ as compared to T_4-induced animals).

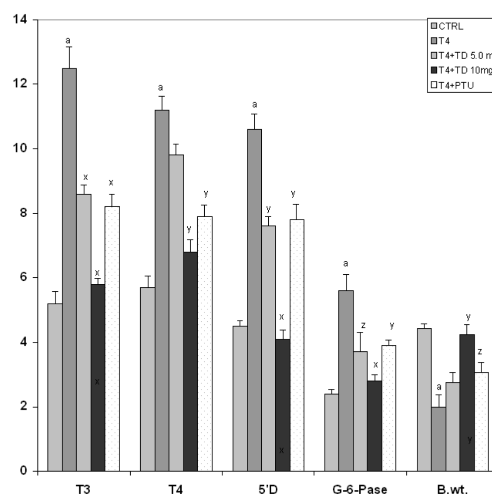

Figure 2. Changes in concentrations of serum T_3 (ng/ml), T_4 (ng/ml X10), 5′D-I (ng/ml), G-6-Pase (μM of inorganic phosphate liberated/h/mg protein×10^{-1}), % increase in body wt. following the administration of digoxigenin-3-O-rutin (5.0 and 10.0mg/kg/d) to the L-T_4-induced animals. Each vertical bar represents the mean±S.E.M. (n=7); $^aP<0.001$ as compared to the respective control value, whereas $^xP<0.001$, $^yP<0.01$ and $^zP<0.05$ as compared to the respective value of thyroxine treated animals.

Effects on serum glucose, insulin and lipid levels

T_4-induced rats revealed a significant decrease in TG, TC, LDL-C, HDL-C and VLDL-C *P*<0.001 or *P*<0.01 or *P*<0.05 as compared to control animals (Table 1). Treatment with of 10.0mg/kg the test drug to hyperthyroid animals significantly *P*<0.001 increased HDL-C and rest of the lipids were nearly to normal values. At the dose of 5.0mg/kg only cholesterol and triglycerides levels were increased significantly (*P*<0.001 and *P*<0.05 as compared to T_4-induced animals).

The test drug at 5.0mg/kg and 10mg/kg decreased both serum glucose and insulin levels significantly (*P*<0.001, *P*<0.01; *P*<0.001 respectively, as compared to T_4-induced animals). In T_4+PTU treated group TC and TG increased significantly (*P*<0.001, *P*<0.01 respectively as compared to T_4-treated animals). Serum glucose and insulin levels were also decreased significantly (*P*<0.001, *P*<0.01 respectively as compared to T_4-treated animals) (Table 1).

Effects on LPO and antioxidants

As shown in table 2, the amount of MDA and LOOH was significantly increased in cardiac tissue in T_4-treated animals (*P*<0.01 as compared to control animals) and a significant decrease in SOD, CAT, GPx and total GSH content *P*<0.001 as compared to control animals was observed. In hepatic tissues also a significant increase in LPO and LOOH levels with a decrease in antioxidants were observed in T_4-induced animals (*P*<0.001 or *P*<0.01 or *P*<0.05) as compared to normal controls. The antioxidant levels in hepatic tissues were increased significantly following the administration of 10mg/kg of the test drug (P<0.001 or *P*<0.01 or *P*<0.05) as compared to T_4 alone treated animals (Table 3). In 5mg/kg of digoxigenin-3-O-rutin and PTU+T_4-treated group GSH remain unaltered in cardiac and hepatic tissues other antioxidative enzymes were enhanced significantly (*P*<0.001 or *P*<0.05). The dose of 10mg/kg found to be highly effective in decreasing MDA and LOOH levels and increasing SOD, CAT, GPx and total GSH (*P*<0.001 or *P*<0.01 as compared to T_4-induced animals).

DISCUSSION

A significant increase in serum T_3, T_4 concentrations and 5′DI activity in L-T_4 induced animals indicates clearly the hyperthyroidic condition as has been observed by us previously.[16,17] Interestingly, following the administration of digoxigenin-3-O-rutin in hyperthyroid

Table 1. Effects of digoxigenin-3-O-rutin (TD-5, 10mg/kg and T_4+PTU) in the alterations in serum concentrations of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), very low density lipoprotein cholesterol (VLDL-C), and triglyceride (TG), all expressed in mg/dl, serum insulin (IU/ml) and serum glucose concentration (mg/dl) in thyroxine (T_4) treated rats.

	CHOL	HDL	TG	VLDL	LDL	Glucose	Insulin
CTRL	98.97±3.85	40.29±2.16	85.96±4.39	17.09±1.14	41.12±2.99	96.36±3.48	6.85±0.27
T_4	59.35±3.19^{a}	24.54±1.71^{a}	61.17±3.94	12.23±1.99^{c}	23.14±1.94^{a}	168.00±4.35^{a}	12.26±0.69^{b}
T_4 + (TD-5.0mg)	92.03±4.63^{x}	30.96±3.68	89.02±5.16^{z}	17.80±1.76	45.28±3.11^{x}	132.24±5.67^{x}	8.02±0.37^{y}
T_4 + (TD-10mg)	99.34±5.52^{x}	63.78±5.02^{x}	80.46±3.69^{y}	16.09±1.09^{c}	20.92±0.79	112.24±4.33^{x}	6.52±0.44^{x}
T_4 + (PTU-10mg)	97.07±6.16^{x}	38.67±3.99	83.78±5.01^{y}	16.75±1.46	43.66±2.17	102.68±5.08^{x}	7.99±0.76^{y}

Data are mean±S.E.M. (n=7). ^{a}***P***<0.001, ^{b}***P***<0.01 and ^{a}***P***<0.05 as compared to their respective control values; whereas ^{x}***P***<0.001, ^{y}***P***<0.01 and ^{z}***P***<0.05 as compared to the respective value of thyroxine treated animals. TD=test drug.

Table 2. Alterations in lipid peroxidation and antioxidants following T_4 (500µg/kg), T_4+TD(5mg/kg), T_4+TD (10mg/kg) in the cardiac tissues of rats.

	Control	T_4	T_4+TD 5.0mg	T_4+TD 10.0mg	T_4+PTU
LPO (nmol MDA formed/h/mg protein)	0.87±0.06	1.94±0.08^{a}	1.29±0.09^{x}	0.67±0.05^{x}	1.16±0.04^{x}
LOOH (nmol/mg protein)	3.02±0.67	7.62±1.05^{b}	5.67±0.44^{z}	3.10±0.78^{y}	5.99±0.57
SOD (U/mg protein)	6.16±0.32	2.29±0.23^{a}	4.76±0.34^{x}	7.49±0.52^{x}	3.03±0.46
CAT (µmoles of H_2O_2 decomposed/ min/mg protein)	21.21±3.50	12.70±2.30^{a}	19.72±2.11^{z}	26.14±3.69^{y}	20.14±1.74^{z}
GSH (µmoles GSH/mg protein)	6.99±0.57	3.06±0.47^{a}	3.85±0.26	6.09±0.42^{x}	3.64±0.51
GPx (µmoles of GSH oxidized/mg protein)	6.27±0.37	3.14±0.23^{a}	5.97±0.48^{z}	7.93±0.96^{x}	6.01±0.55^{x}

Data are means±SEM, n-7. ^{a}***P***<0.001 and ^{b}***P***<0.01, as compared to the respective control values. ^{x}***P***<0.001, ^{y}***P***<0.01, ^{z}***P***<0.05, as compared to the respective values of the T_4-induced animals.

Table 3. Alterations in lipid peroxidation and antioxidants following T_4 (500µg/kg), T_4+TD (5mg/kg), T_4+TD (10mg/kg) and T_4+PTU (10mg/kg) in the hepatic tissues of rats.

	Control	T_4	T_4+TD 5.0mg	T_4+TD 10.0mg	T_4+PTU 10.0mg
LPO (nmol MDA formed/h/mg protein)	0.57±0.06	2.63±0.18^a	1.79±0.12^x	0.35±0.04^x	0.79±0.07^x
LOOH (nmol/mg protein)	2.02±0.67	9.22±2.05^b	6.60±0.52^z	2.49±0.32^y	3.99±0.86^z
SOD (U/mg protein)	5.46±0.32	2.38±0.33^a	4.99±0.14^x	6.77±0.56^x	3.67±0.21^y
CAT (µmoles of H_2O_2 decomposed/min/mg protein)	55.21±3.87	29.30±3.40^a	48.43±3.21^z	59.98±4.77^y	30.76±3.99
GSH (µmoles GSH/mg protein)	4.99±0.57	2.12±0.23^a	3.66±0.30	5.78±0.60^x	3.44±0.41
GPx (µmoles of GSH oxidized/mg protein)	8.57±0.62	4.16±0.73^a	6.22±0.55^z	9.76±0.89^x	5.99±0.84

Data are means ± SEM, n-7. ^{a}***P***<0.001, as compared to the respective control values. ^{x}***P***<0.001, ^{y}***P***<0.01, ^{z}***P***<0.05, as compared to the respective values of the T_4-induced animals. TD = test drug.

animals could decrease the T_3 concentration and 5′DI activity, suggesting the inhibition of peripheral monodeiodination of T_4, the principal source of generation of T_3. Thus it is presumed that inhibition of 5′DI activity is likely to be responsible for hypothyroidic action of the test drug. This finding corroborate with our earlier observation on crude seed extract of *Trigonella foenum garecium* that had indicated its thyro-inhibitory action in mice.[11]

Hyperthyroidism is characterized by decreased body weight, increased insulin and glucose indicative of insulin resistance as well as decreases in plasma lipids such as plasma cholesterol and triglycerides.[28,29]

Thyroxine (T_4) stimulates hepatic gluconeogenesis, and even a small change in T_4 levels can affect glucose metabolism.[30] In the present study, following exogenous L-T_4 administration leads to enhanced glucose levels in this condition may be explained by increased endogenous glucose production through more rapid glycogenolysis and gluconeogenesis.[31] The hyperthyroid rats were also found to be insulin resistant, with increased insulin levels. Interestingly, the administration of test compound to hyperthyroid animals normalized the serum insulin and glucose levels. This reduction could be the result of the direct effects of thyroid hormones on the β-cells for insulin secretion as suggested earlier.[32]

A significant reduction in hepatic-glucose-6-phosphatase activity following the digoxigenin-3-O-rutin administration further supports the inhibitory role in T_3 formation as the activity of this enzyme is commonly related to the alteration of the thyroid hormone levels.

Hyperthyroidism is generally associated with a loss in body weight .In the present study a decrease in body weight in hyperthyroid animals and an increase in test drug treated animals were observed. Since body weight changes are very often associated with the alterations in T_3 level, these observations further support the T_3 inhibitory nature of the test drug.[33]

Thyroid hormones play an important role in regulating lipid metabolism, and thyroid dysfunction can result in lipid abnormalities which increase the risk of endothelial dysfunction, hypertension and cardiovascular disease. Thyroid dysfunction has a great impact on lipids as well as a number of other cardiovascular risk factors. A significant decrease in the level of TC, TG, HDL-C, LDL-C and VLDL-C was observed in L-T_4-induced rats.[32] Reduction in total cholesterol due to thyroxine treatment was due to decrease in HDL and LDL cholesterol.[33,34]

However, treatment with the isolated compound normalized most of these changes induced by hyperthyroidism. Interestingly, 10mg/kg, of digoxigenin-3-O-rutin increased HDL-C levels significantly as compared to that of hyperthyroidic animals maintaining the LDL-C nearly to normal values .This increase in HDL-C might also be the result of its antithyroidic property, as T_4 is believed to reduce serum HDL levels.[35,36] Thus an increase in HDL-C following the treatment with digoxigenin-3-O-rutin, suggests its beneficial role in the regulation of hyperthyroid- induced cardiovascular problems.

Most of the available data indicates that an experimentally induced hyperthyroid state causes an increased radical production.[37,38]

It has been reported that the increase in reactive oxygen species induced by thyroid hormone leads to an oxidative stress condition in liver, cardiac and some skeletal muscles with a consequent lipid peroxidative response.[39,40] Hyperthyroidism is a hyper metabolic state accompanied by increased oxygen utilization, increased production of reactive oxygen species and consequently measurable changes in anti oxidative factors.[41,42]

In this study also T_4 administration enhanced MDA level in both the hepatic and cardiac tissues and antioxidants such as SOD, CAT, GPx and total GSH were found to be decreased. The increase in lipid peroxidation may be due to increased free radical production. These observations are also in agreement with previous findings.[43–45] Interestingly, the isolated compound at the dose of 10mg/kg prevented the oxidative damage in both the cardiac and hepatic tissues with an increase in antioxidants in hyperthyroid rats suggesting its free radical scavenging activity.

CONCLUSIONS

In conclusion the present investigation suggests that the isolated compound, digoxigenin-3-O-rutin from fenugreek seeds appears to attenuate oxidative stress, not only by fortifying antioxidant defense capacity but also by lowering lipid peroxidation and helped to maintain the levels of lipids in the serum. The antithyroidal activity exhibited by the compound is through the inhibition of 5′DI activity.

CONFLICT OF INTEREST

Author declares no conflict of interest.

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PHCOG J ORIGINAL ARTICLE

Evaluation of Anti-Diabetic Activity of Methanolic Extract from the Leaves of *Rotula Aquatica Lour* in Alloxan-Induced Diabetic Rats

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ABSTRACT

Objective: The objective of the present study was to evaluate the anti-diabetic activity of methanolic extract from the leaves of *Rotula aquatica lour* in Alloxan-induced diabetic rats. **Materials and Methods:** Diabetes was induced in rat by injection of Alloxan (120mg/kg, i.p.). Diabetic rats were divided into different groups and methanolic leaves extract of *Rotula aquatica lour* (RA-ME) was administered at dose ranges of 100–400mg/kg, p.o for 21 days. Control group received normal saline (0.9%) for 21 days. Glibenclamide (5mg/kg, p.o) was used as standard drug. Blood samples were collected from all the groups and analyzed for serum glucose and lipid levels such as total cholesterol (TC), triglyceride (TG), proteins (TP). RA-ME was also tested for oral glucose tolerance test (OGTT) in normal fasted rats. **Results:** RA-ME (400mg/kg, p.o) showed a significant ($P<0.01$) reduction of serum glucose level in Alloxan-induced diabetic mice as compared with diabetic control. RA-ME (200 and 400mg/kg) also showed a significant reduction in serum TC, TG, and TP levels in Alloxan-induced diabetic rats. RA-ME (200 and 400mg/kg, p.o) significantly ($P<0.01$) increased the glucose tolerance in OGTT. **Conclusion**: The results obtained from the present study revealed the potential anti-diabetic activity of methanolic extract from the leaves of *Rotula aquatica lour*.

Keywords: Alloxan, Anti-diabetic, Glibenclamide, *Rotula aquatica lour.*

INTRODUCTION

Diabetes mellitus is a leading metabolic disorder characterized by fasting and/or postprandial state hyperglycemia, resulting from defects in insulin secretion or action. It is well reported that diabetes mellitus is associated with a large number of macro vascular and micro vascular complications such as obesity, hypertension, hyperlipidaemia, nephropathy and neuropathy.[1,2] A growing body of research has suggested that diabetes mellitus is increasing in an epidemic proportion throughout the globe, especially in India. Moreover, the prevalence of diabetes is expected to increase by more than two-fold worldwide and approximately 57 million Indians would be affected by this disorder in the year 2025, illustrating the severity and impact of the disorder on the quality of life.[3,4] Despite the steady increase in the number of anti-diabetic agents, the prevalence of the disorder remains stable may be due to the inconsistent efficacy of currently available drugs. In addition, the currently available anti-diabetic drugs have a large number of adverse effects and high rates of secondary failure[5]. Therefore, this remains a grave need to develop and discover new therapy with a proper balance of risk to benefit that could be fruitful for the treatment of diabetes mellitus. In recent decades, many researchers have sought new plant products to treat diabetes mellitus, as they contain many bioactive substances with therapeutic potential.[4]

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Rotula aquatica lour is species of aromatic flowering shrub belonging to family *Boraginaceae*. It is mostly present in aquatic region. It is a rare rheophyte native to India, where it is a member of the lotic ecosystem of streams.[6] *Rotula aquatic lour* is also called as pashanbed; it is widely distributed in India, Sri Lanka, tropical south-eastern Asia and Latin America. In India from kumaun to Assam and

western to southern India. The plant contain Baunerol, steroid, alkaloid, and rhabdiol and allantoin. The medicinal values of plant lie in their component phytochemical such as alkaloids, flavonoid, phenolic compounds and other nutrients like as amino acid, proteins, which produce a definite physiological action on the human body. A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical and drug research. It is Ayurvedic plant which is an important traditional medicine for kidney and bladder stones. In Ayurveda, plant used for cancer, piles, diabetes, venereal diseases, kidney and bladder stones, cough, heart problems, blood disorders, fever, poisonings, ulcers and uterine diseases. It is used as laxative, diuretic, antioxidant, anthelmintic, astringent, bitter etc. The different parts of plant extract have been reported to acquire above activities. But so far, there are no reports made on the comparison of antioxidant activity and phytochemical properties of stems of *Rotula aquatica lour* in various organic and aqueous extracts.[7]

MATERIALS AND METHOD

Animals

Wistar rats, of either sex, weighing 150–250g were used. They were housed under standard conditions of temperature (23±2°C), humidity and dark–light cycle (lights on from 6:00 am to 6:00 pm). Tap water was available at libitum. All the animals were carefully monitored and maintained in accordance with CPCSEA guidelines on control and supervision of experimental animals. The ethical clearance was obtained from the Institutional Animal Ethics Committee (Approval no. 651/02/c/CPCSEA) before the experiment.

Chemicals

All chemicals and solvents used were of analytical grade, from S.D Fine Chemicals Ltd, Mumbai, India. Alloxan was purchased from Sigma Chemical Co. (St Louis, MO, USA) and Glibenclamide (Aventis Pharma Limited, Verna, Goa). Diagnostic kits of total cholesterol, triglycerides, and total proteins (Agappe Diagnostics Ltd., Pattimattom, Ernakulum), and rest all other reagents and chemicals were of analytical grade.

Plant Material and Preparation of Plant Extract

The leaves of *Rotula aquatica lour* was collected from Sawantwadi, Maharashtra. The collected leaves were authenticated at botanical survey of India, Ministry of Environment and Forests, Government of India. The collected leaves of *Rotula aquatica lour* was dried under shade for 10 days and then made into a coarse powder. Initially, 400g of dried bark was defatted with petroleum ether (60–80°C) in soxhlet apparatus (continuous hot percolation process) and after complete extraction (46h), the solvent was removed by distillation under reduced pressure and resulting liquid was dried using heating plate at 50°C to get semisolid residue. After the extraction with petroleum ether, the same plant material was dried and further extracted with chloroform (36h) followed by methanol (75h) until the extraction was complete. The methanolic bark extract was concentrated under reduced pressure and dried using heating plate at 60°C to get semisolid residue or respective residue.

Acute oral toxicity studies

The acute oral toxicity studies[8] of extracts were carried out as per the OECD guidelines, draft guidelines 423 adopted on 17 December 2001 received from CPCSEA, Ministry of Social Justice and Empowerment, Government of India. Administration of the stepwise doses of the methanolic extract of *Rotula aquatica lour* from 50mg/kg b. wt. up to a dose of 4000mg/kg b. wt. caused no considerable signs of toxicity in the tested animals. One-tenth of the upper limit dose was selected as the level for examination of Anti-Diabetic activity.

Oral glucose tolerance test

OGTT was performed in non-diabetic rats. The fasted rats were divided into 4 groups (*n*=6/group). Group I: glucose load control group. Group II, III and IV rats received RA-ME at a dose of 100, 200 & 400mg/kg body weight, respectively. The rats of treatment groups were loaded with glucose (2g/kg, p.o.) 30min after the administration of the RA-ME. Blood samples (100–200µL) were collected at 0min before the glucose load and 30, 60 and 120 min after the glucose load by retro-orbital vein plexus puncture under mild ether anaesthesia. The serum was separated and the glucose concentration was estimated by glucose oxidase-peroxidase (GOD-POD) method.[9]

Study of the *Rotula aquatica lour* leaves extract on the Alloxan-induced hyperglycemia

The cytoprotective effects of extract were studied in Alloxan-induced diabetic rats according to the method reported.[10] The diabetes was induced by administration of 120mg/kg Alloxan monohydrate (Sigma). The diabetic rats (glucose level >275mg/100ml) were divided into six groups of six rats each. Group I served as negative control and received distilled water. Groups II served as the

diabetic control, Group III, IV, V received the RA-ME at doses of 100, 200 and 400mg/kg as an aqueous solution, p.o. and Group-VI received the Glibenclamide 5mg/kg as standard drug. The administration of the extract was continued for 21 days, once daily. Blood samples were collected from the retro-orbital plexus on days 1, 15 and 21 of extract administration. The blood glucose levels, triglyceride level, total cholesterol and total proteins were determined for all the samples by the glucose oxidase method.

Blood collection

The blood samples (500–750μL) were collected by retro-orbital vein plexus puncture of anaesthetized mice. Blood samples were collected at the time of grouping of animals (basal reading) and at 1st, 15th, and 21st day of treatment. Blood was centrifuged at 3500 r.p.m. for 20 min and serum was separated for biochemical estimation.

Estimation of serum glucose

The glucose concentration was estimated by (GOD-POD) method[11] using commercially available kit. The absorbance and concentration of test and standard samples were noted against blank at 505nm with an auto-analyser.

Estimation of cholesterol

Serum total cholesterol was estimated by cholesterol oxidase-peroxidase (CHOD-POD) method[12] using commercially available kit.

Estimation of triglycerides

Serum triglyceride was estimated by glycerophosphate oxidase-peroxidase (GPO-PAP) method by the addition of enzyme present in reagent kit.[13] The absorbance and concentration of test and standard samples were noted against blank at 505nm with an autoanalyser.

Estimation of total proteins

Serum proteins were estimated by Biuret method using commercially available kit.[14] The absorbance and concentration of test and standard samples were noted against blank at 546 nm with an autoanalyser.

Statistical analysis

Data were expressed as the mean±S.E.M. The significance of the results was calculated using ANOVA and post hoc Dennett's t-test and the results were considered statistically significant when P<0.05.

RESULTS

Oral glucose tolerance test

The effects of RA-ME (100–400mg/kg, p.o) on OGTT are summarized in Table 1 & Figure 1. Maximum serum glucose level was found at 30 min. in all groups after glucose load. The control group had a significant elevation in serum glucose level throughout the total measurement period, i.e., for 120 min, with respect to RA-ME treatment group as shown in Figure 1. However, in the RA-ME extract treated groups, blood glucose level although it reached the peak level within 30 min of administration of glucose but it almost resettled to the normal level by 120 min. The glucose level significantly (*P*<0.01) resettled close to the normal value in RA-ME (200mg/kg and 400mg/kg) treated group. Moreover, at the doses of 200mg/kg & 400mg/kg, the glucose level was significantly (*P*<0.01) less as compared with glucose loaded control rats throughout at 120 min. However, no significant affect was observed at a dose of 100mg/kg.

Table 1. Effect of extract on oral glucose tolerance test in rats.

Groups	0 min	30 min	60 min	120 min
Vehicle control	78.33 ± 2.3	146.43 ± 1.7	122.87 ± 3.6	95.76 ± 1.9
Extract dose I	80.5 ± 1.8	141.56 ± 2.5 **	125.98 ± 1.4 ns	89.87 ± 3.1***
Extract dose II	81.42 ± 3.2	132.86 ± 1.6 ***	116.65 ± 2.7 **	86.75 ± 1.9 ***
Extract dose III	79.35 ± 2.7	125.86 ± 3.2 ***	108.72 ± 2.1 ***	82.73 ± 1.6 ***

Values are presented as mean ± SEM. (n=5), ANOVA followed by Dunnett test. *p<0.05, **p<0.01 when compared with Control group.

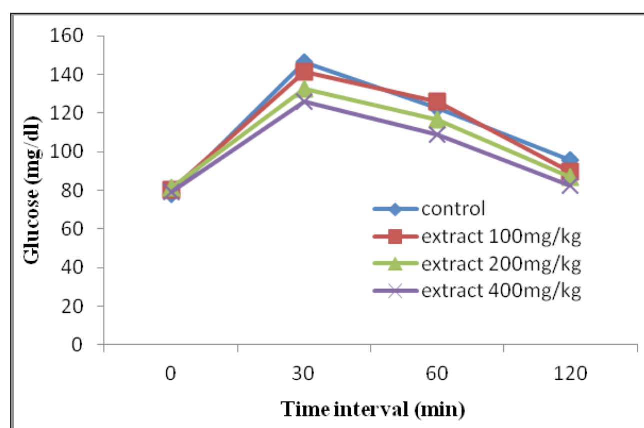

Figure 1. Effect of extract on blood glucose concentrations in fasting conditions at 0 min (pre-treatment) and 30, 60 and 120 min after oral glucose load in normal control rats, and rats treated with various doses of the methanolic leaves extract.

Alloxan-induced diabetic rats

Effect of RA-ME on serum glucose level

Table 2 summarizes the serum glucose levels in normal and diabetic rats. RA-ME at a dose range of 100–400mg/kg decreased serum glucose levels in diabetic rats. The significant (P<0.01) effect on serum glucose level was found at a dose of 400mg/kg body weight, observed on 15th and 21st day of treatment. Moreover, the most pronounced decrease in serum glucose was observed on Day 21st at a dose of 400mg/kg. In addition, the positive control Glibenclamide also significantly decreased the serum glucose level in diabetic rats as compared with diabetic control rats. However, RA-ME at a dose of 100mg/kg failed to reach the level of significance as compared with diabetic control rats.

Effect of RA-ME on serum triglyceride, cholesterol and protein levels

Table 3 illustrates the serum triglyceride, cholesterol and protein levels in normal and diabetic rats. Treatment of diabetic rats with RA-ME (200–400mg/kg) produced a significant reduction in serum levels of triglyceride, cholesterol. The effect observed in our study was dose dependent and time dependent. Moreover, Glibenclamide treatment also significantly decreased the serum levels of triglyceride, cholesterol.

DISCUSSION

Diabetes mellitus has been recognized as one of the most common metabolic disorders associated with common

Table 2. Effect of methanolic extract of *Rotula aquatica lour* on serum glucose levels in alloxan-induced diabetic rats.

Groups	Serum glucose (mg/dl)		
	1st day	15th day	21st day
Normal	89.41±5.42	91.15±3.89	85.12±5.71
Diabetic	308.8±5.27$^{##}$	299.5±6.55$^{##}$	302.64±8.54$^{##}$
Glibenclamide (mg/kg)	299.74±8.9	159.67±7.87**	152.3±9.67**
Extract (100mg/kg)	297.1±6.13	287.98±11.56	288.56±6.78*
Extract (200mg/kg)	288.67±7.55	238.67±9.88**	231.43±5.87**
Extract (400mg/kg)	299.5±9.2	198.56±7.63**	186.67±9.82**

Values are presented as mean ± SEM. (n=5), ANOVA followed by Dunnett test. *p<0.05, **p<0.01 when compared with Control group. $^{##}$p<0.01 when Diabetic control compared with Normal group.

Table 3. Effect of methanolic extract of *Rotula aquatica lour* on Triglyceride, cholesterol and protein levels in alloxan-induced diabetic rats.

Groups	Days	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	Total proteins (g/dl)
Normal group	0	83.17 ± 4.63	79.17 ± 4.62	6.33 ± 0.21
	15	89.69 ± 5.31	82.31 ± 5.19	6.38 ± 0.32
	21	87.71 ± 5.42	81.45 ± 3.72	6.22 ± 0.21
Diabetic control	0	84.75 ± 6.69	75.63 ± 4.56	6.45 ± 0.32
	15	175.67 ± 7.76$^{##}$	177.45 ± 7.75$^{##}$	5.33 ± 0.29$^{##}$
	21	187.51 ± 8.46$^{##}$	183.66 ± 5.77$^{##}$	5.12 ± 0.29$^{##}$
Glibenclamide	0	83.43 ± 5.87	74.89 ± 6.73	5.78 ± 0.67
mg/kg	15	138.56 ± 6.75**	143.64 ± 6.39**	5.72 ± 0.64
	21	133.59 ± 5.37**	136.45 ± 6.91**	6.3 ± 0.43**
Extract	0	84.74 ± 8.15	75.98 ± 5.38	6.38 ± 0.58
(100mg/kg)	15	167.55 ± 7.6	162.41 ± 7.71**	5.42 ± 0.55
	21	174.01 ± 8.43*	158.91 ± 5.78**	5.36 ± 0.3
Extract	0	85.02 ± 5.84	75.51 ± 6.71	6.23 ± 0.49
(200mg/kg)	15	159.55 ± 5.88**	163.06 ± 6.9**	5.46 ± 0.41
	21	156.63 ± 7.51**	151.11 ± 6.8**	5.58 ± 0.56
Extract	0	84.35 ± 6.54	74.88 ± 5.95	6.15 ± 0.6
(400mg/kg)	15	151.17 ± 6.13**	149.66 ± 6.67**	5.59 ± 0.52
	21	145.67 ± 5.84**	145.86 ± 7.53**	5.89 ± 0.47*

Values are presented as mean ± SEM. (n=5), ANOVA followed by Dunnett test. *p<0.05, **p<0.01 when compared with Control group. $^{##}$p<0.01 when Diabetic control compared with Normal group.

features such as hyperglycemia and hyperlipidaemia. Alloxan is a β-cytotoxin diabetogenic agent, which induces diabetes by destructing the β-cells of the islets of pancreas, leading to a decreased insulin release and increased blood glucose level.[15] In accordance with the previous findings, the present study reports the significant increase in serum glucose level in Alloxan-induced diabetic rats.

The chronic administration (21 days) of the RA-ME produced a decrease in serum glucose levels of diabetic rats. This effect may be due to regeneration of the β-cell following destruction by Alloxan. The growing body of data suggested that to achieve maximum effect, therapy with plant products should be continued for a longer duration.[16] Considering this, RA-ME was administered daily for 21 days, the period which may be produced a significant reduction in all the diabetic markers, and this effect was potent as compared to acute dosing.

In the present study we also investigated glucose tolerance test in normal rats. The RA-ME significantly decreased the serum glucose levels in glucose loaded rats, and this information could be endorsed to the potentiating of the insulin effect of blood by increasing the pancreatic secretion of insulin from existing β-cells or its release from bound insulin.[17] In this context, a number of other plants have been observed to have similar pattern of hypoglycemic effects. Results on the insulin release from pancreas directly indicate that the anti-diabetic activity of *Rotula aquatica lour* may be through the release of insulin from the pancreas.

CONCLUSION

Herbal hypoglycemic agents can provide better option to avoid harmful side effects caused by prolong intake of synthetic ones. From present preclinical studies, *Rotula aquatica lour* proved to be hypoglycemic in action. But one can speculate that in clinical trials, the drug may act as safe and effective hypoglycemic agent. The remarkable hypoglycemic potential of *Rotula aquatica lour* was quite competent with standard drug. Although the test drug could not correct deranged levels of serum metabolites, it can be used in polyherbal formulations. Further studies are necessary to elucidate details of active phytochemical and their mechanism of hypoglycemic action. Isolation and study of active principles are under process.

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PHCOG J | ORIGINAL ARTICLE

Hydrodistilled volatile constituents obtained from the roots of *Operculina turpethum*

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Sir, *Operculina turpethum* (basionym: *Convolvulus turpethum* L.; homotypic synonym: *Ipomoea turpethum* L., *Merremia turpethum* L.) is a perennial climber from Convolvulaceae. The plant roots, which are called Turbad in Persian folk medicine, are a strong purgative and have also been used for phlegmatic disorders, burns, fevers, jaundice, cough, uterine problems, joints and muscles pain, paralysis and sciatica.[1] The plant has a wide range of applications in Ayurvedic formulations. Extracts of Turbad exhibited antioxidant, antihyperglycemic, antiulcer, anti-inflammatory, antimicrobial, cytotoxic, immunodulatory and anti-edema activities.[2]

Several secondary metabolites including, glycoside resin, alkaloids, steroids and saponins have been isolated from the plant.[3,4] The aim of this study was to investigate the volatile constituents of Turbad.

Roots of *O. turpethum* were purchased from Shiraz herbal market and authenticated by Miss Sedigheh Khademyan (taxonomist) and the voucher specimen was preserved with the code PM-160 in the Department of Pharmacognosy, Shiraz School of Pharmacy. The essential oil was extracted for 3 hours by hydrodistillation clevenger–type apparatus. The GC/MS analyses were carried out using a Hewlett-Packard 6890. The gas chromatograph was equipped with a HP-5MS capillary column (phenylmethylsiloxane, 25m´ 0.25mm i.d.). The oven temperature was programmed from 60°C (4 min) to 250°C at a rate of 3°C/min and increased at a rate of 5°C/min to 280 and held for 10min. The carrier gas was helium with a flow rate of 1.2 ml/min. The mass spectrometer was operating in the EI mode at 70 eV. The interface temperature was 250°C; mass range was 30–600 *m/z*. The Kovats index (KI) of components was calculated using a homologous series of n-alkanes under the same operational conditions of analysis. Identification of components was based on a comparison of their KI and mass spectra with Wiley (275), Adams libraries spectra and Pherobase Kovats Index Database.[5]

The volatile constituents of Turbad were trapped in hexane and gave a colorless solution with characteristic odor. Nineteen compounds were identified which represented about 92% of the total detected constituents. The identified compounds, their percentage and Kovats index are summarized in table 1.

Carvacrol (37%), caryophyllene oxide (14.7%), and thymol (11.3%) were the major components. The main class of the compounds was found to be oxygen containing monoterpenes (65.17%), sesquiterpene hydrocarbons (19.83%) and monoterpene hydrocarbons (0.15%). Oxygen containing sesquiterpenes were not identified.

The authors of this study could not find any reports on essential oil components of the *O. turpethum* but Wang and Kays[6] evaluated the volatile constituents of *Ipomoea batatas* (another species of this genus) and reported linalool, palmitic acid, and beta caryophyllene as aromatic constituents of the plants root although the amount of these components were different from the results of this study. The presence of linalool, α- and β-caryophyllene, β- and γ-elemene, and β-cubebene in *Ipomoea batatas* tips during its fermentation was reported by Cue et al.[7] In another report, p-cymene and carvone have been reported as the major constituents of *Ipomoea cairica* (another species of this genus) volatile oil which have larvicidal and repellent activity against malarial fever mosquitoes.[8]

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Table 1. Volatile constituents of *O. turpethum*.

	Compound	KI	Percentage
1	n-Decane	1000	0.16
2	*p*-Cymene	1024	0.15
3	1,8-Cineole	1032	0.54
4	Fenchone	1088	0.12
5	Linalool oxide trans	1088	0.24
6	n-Undecane	1099	0.20
7	Linalool	1104	5.96
8	Bornyl acetate	1285	0.41
9	Anethole	1291	8.15
10	1-Methoxy-4-(1-propenyl)-benzene	1299	1.66
11	Thymol	1312	11.30
12	Carvacrol	1322	36.95
13	Unknown	1352	2.19
14	Carvacrol acetate	1375	1.50
15	β-Caryophylene	1414	3.54
16	Aromadendrene	1434	0.57
17	α-Humulene	1449	0.46
18	Ledene	1490	0.55
19	Caryophyllene oxide	1579	14.71
20	Unknown	1660	1.57
21	Palmitic acid	2056	4.81
22	Unknown	2225	1.97

Antimicrobial activity of carvacrol, caryophyllene, thymol and linalool, the major volatile components of the root in this analysis, has been reported several times.[9] These volatile components exhibited some other biological activities including anti inflammatory, anti spasmodic, analgesic, anti oxidant and insect repellent properties.[10]

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Bioactivity of *Viscum album* extracts from Olive and Almond host plants in Palestine

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ABSTRACT

Introduction: *Viscum album* is a semi-parasitic medicinal plant which has been used for many years as a remedy in traditional medicine. The plant is widely used in folk medicine in Palestine; mainly in the treatment of cancer, diabetes and heart disease. Since no previous reports on bioactivity of this plant in association with host plant specificity in Palestine, the current study aimed at evaluating bioactivity of almond and olive variants of this plant. **Method:** Methanolic extract of viscum *album* cultivated from almond and olive host plants were tested for antioxidant, antimicrobial . Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were also used to assess anticoagulant activities of plant extracts. **Result:** The result demonstrated that *Viscum album* have an IC_{50} of $25.34 \pm 3.8 \mu g/ml$ when hosted by olive while the IC_{50} was $15.37 \pm 2.2 \mu g/ml$ when hosted by almond. Crude extracts of both *Viscum album* plants showed strong inhibition effects on the growth of the studied *Staphylococcus aureus* strains (ATCC 25923 and MRSA) with a pronounced effect when extracts of almond host was used. However, the effects of both host extracts were very limited or absent when tested against Gram-negative reference and clinical strains. Plant extracts of both host showed prolonged PT and PTT compared to phosphate buffered saline control solution. **Conclusion:** In conclusion, variations in the bioactivity of *Viscum album* is clearly influenced by host type and further studies required to illustrate such variations using other host plants.

Keywords: Viscum album, Antioxidant, Prothrombin time, Antimicrobial.

INTRODUCTION

Complementary and alternative medicine (CAM) has become increasingly popular for various conditions and diseases over the last decades. Most of these complementary treatments are herbal remedies and among these is *Viscum album* (Mistletoe) extracts.[1] A number of biological effects were reported for *Viscum album* including anticancer, apoptosis–inducing, antimycotic, antibacterial, antiviral, antidiabetic, and immunomodulatory activities have been reported.[2]

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Viscum album is a small, dioecious and shrubby semi-parasitic plant that grows wild on trees, bushes and other plants.[3] It has an oblong evergreen leathery leaves, clear dichasial branching and four-part flowers which form white sticky berries with a faint but characteristic odor and a bitter taste.[4] Mistletoe is considered a semiparasitic plant because it synthesizes its own chlorophyll but depends on the host for its supply of water and minerals.[5]

Recent scientific research has confirmed that *Viscum album* extract induced apoptotic killing of cultured human tumor cells and lymphocytes, and stimulated the immune system[6–9] so that, it affects positively on the lifespan of individuals respectively.[10]

The phytochemical profile of mistletoe depends of the host trees of this plant.[11] The main bioactive compounds found in mistletoe are lectins, viscotoxin, flavonoids, as well as acidic arabinogalactan.[12–14] The alkaloid

concentrations also depends on the host tree type.[15] These constituents suggesting that this plant may be an important source of natural products with chemopreventive and chemotherapeutic activities.[16]

The antioxidant molecules found in mistletoe are represented by flavonoids quercetin and quercetin methyl ethers.[14] Quercetin and flavonol, has been demonstrated to display a very strong antioxidant activity, often accompanied by antiviral and antibacterial activity.[17] Sengul et al. showed that *Viscum album* had the highest antioxidant activity (82.23%) among some medicinal plants.[18] Phenolic acids in mistletoe plants, such as digallic acid and *o*-coumaric acid in the free or glycosilated forms are also considered to be compounds with antioxidant activity,[11] they readily forms a resonance stabilized phenoxy radical which accounts for their potent antioxidant potential.[19]

To evaluate the antioxidant capacities of plant extracts, numerous in vitro methods have been developed.[20] In the current study, DPPH method, which relies on the reduction of 2, 2-diphenylpicrylhydrazyl (DPPH) radical was used. This method is simple, fast and inexpensive for measuring the antioxidant capacity. Furthermore, it is not specific to any particular antioxidant component and could be applied to either solid or liquid samples. The DPPH with free radical has a purple color and a strong absorption maximum at 517nm. As shown in Figure 1, when the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant it will be reduced to DPPH-H, the color becomes yellow and the molar absorptivity of the DPPH radical at 517nm reduces from 9660 to 1640.[21]

The anti antioxidants activity is usually compared with a reference standard and a common example is Trolox.[21,22] Trolox is a (Hoffman-La Roche) trade name for (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid); a water soluble vitamin E analogue used in this research as an antioxidant standard.[23]

Anticoagulants play a role in the prevention and treatment of thromboembolic disorders.[24,25] Anticoagulant drugs consisting of heparin and its derivatives, and vitamin K antagonists, have been the main anticoagulants in clinical practice. Despite their efficacy, major and life-threatening side effects of these agents have also been reported.[26,27] Plants may serve as an alternative source for the development of new anticoagulants due to their biological activities. There is compelling evidence demonstrating that the consumption of dietary anticoagulants or phytochemicals with anticoagulant properties can ultimately minimize the risks of thromboembolic diseases.[28,29]

In Palestine, mistletoe is widely used in the folk medicine as antimicrobial, antidiabetic and anticancer. The plant mainly cultivated from almond and olive hosts grown in northern Palestine. No previous bioactivity studies have been reported on *Viscum album* in the country. This study aimed to investigate bioactivity of this plant and search for differences in activity that might be influenced by host type.

METHODOLOGY

Materials and reagents

Trolox ((S)-(-)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. Methanol analytical grade was used for extraction purposes. Other chemical reagents were purchased from reliable commercial sources.

Microorganisms used in the current study were reference strains obtained from the American Type Culture

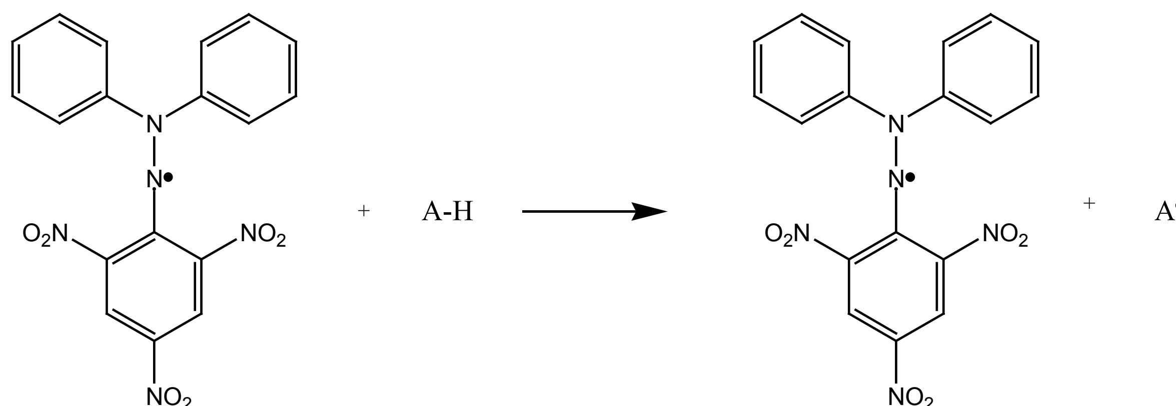

Figure 1. DPPH reduces to DPPH by the antioxidant agent.

Collection (ATCC), including *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MRSA Positive), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). In addition, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Proteus mirabilis* clinical isolates were included. Isolates were identified by Gram stain, growth on MacConkey, and API20E (BioMericux, France).

Instrumentation

Shaker device (LabTech Shaking Incubator) was used in extraction of the plants, rotatory evaporator [Heidolph OB2000 (the heater) and Heidolph VV2000 (the rotator)] was used for condensation purpose, Spectrophotometer (Jenway 6505 UV/Vis Spectrophotometer) was used to measure the optical density.

Anticoagulant activity tests were done using HumaClot Duo plus Hemostasis Analyzers, HUMAN, Germany. The samples were collected in sodium citrated blood tubes (Vacutainer, BD) and centrifuged by Hettich Zentrifugen, Germany.

Plant material

Leaves of *Viscum album* were collected from olive and almond trees in spring from places in north Palestine. The leaves of plants were dried in dark and stored in dry place for the research until it was started in summer.

Extract preparation

Leaves of *Viscum album* hosted by olive and almond were powdered separately using a grinder. The extraction was performed at room temperature. About 100g of the grounded leaves were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours and stored in refrigerator for 4 days. The extracts were then filtered using filter papers. The extract was then concentrated under vacuum on a rotatory evaporator. The crude extract was stored at 4ºC for further use.

Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of the host plants and Trolox standard at different concentration were plotted and tabulated and the IC_{50} for each of them was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the adapted model.

The antimicrobial activity of the plant was compared to different standards by measuring the inhibition zone, the tests were carried out in duplicates for each concentration and inhibition zones of bacterial growth were measured and reported in mm.

For PT and PTT tests the plant extracts of varying concentrations was carried out in duplicate and the average clotting time in seconds were measured and reported.

Anti oxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/1ml in methanol was firstly prepared for the two plant extracts and trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100μg/ml) were prepared by suitable dilution with methanol from the stock solution.

Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ration of 1:1:1 respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 minute at room temperature before the absorbance readings were recorded at 517nm.

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plants and the Trolox standard were calculated using the following formula:

$$\text{Percentage of inhibition of DPPH activity (\%)} = \frac{A-B}{A} \times 100\%$$

where: A = optical density of the blank,

B = optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant samples and the standard were calculated using BioDataFit edition 1.2 (data fit for biologist).[30]

Antimicrobial activity assays

Preparation of inoculum

Stock cultures were maintained at 4ºC on slant of nutrient agar. Active cultures prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient

broth and incubated at 37°C for 24 hrs. Cultures were then diluted with fresh nutrient broth to achieve optical densities corresponding to 10^8 CFU/ml (turbidity = McFarland barium sulfate standard 0.5) as described by Smânia *et al.*, 1999.

Antimicrobial susceptibility test

Agar diffusion well-variant was performed as described by Smânia *et al.,* 1999.[31] Bacterial inoculums were uniformly spread using sterile cotton swab on a Petri dish of Mueller Hinton Agar (MHA). Six wells (6mm in diameter) were made on each plate. Three concentrations (10, 20, and 30%) of each plant extract were prepared using DMSO and methanol separately. A sample of 50µl of each extract was loaded into each well and plates were then incubated at 37°C for 24 hours under aerobic conditions. Cultures were carried out in duplicates and inhibition zones of bacterial growth were measured in mm.

Agar diffusion disc-variant was performed using selected staphylococcal reference strains and the wells were replaced by 6mm sterile disc loaded with 20µl of sample of plant extract. Reference commercial antibiotic discs were used as positive or negative controls. These included Vancomycin (30µg), Gentamicin (10µg), Oxacillin (5µg), and Cefotaxime (30µg). The inoculation of MHA and measurements of inhibition zones were performed as shown above in the well method. Negative controls were made by replacing plant extracts with DMSO solution.

Anticoagulant activities

Blood sample collection

Blood samples from three healthy volunteers were collected in sodium citrated blood tubes (Vacutainer, BD) and centrifuged for 15 minutes at 1500g to prepare platelet poor citrated plasma.

PT and a PTT tests

Tests were performed using HumaClot Duo plus Hemostasis Analyzers, HUMAN, Germany.

Equal volumes of plant extracts of varying concentrations and citrated platelet poor plasma were incubated for 5 min at 37°C.

For PT test, 100µl of tissue thromboplastin (HUMAN) was added to 50µl to the pre-warmed mixture and clotting time was measured.

For aPTT test, 50µl of rabbit brain extract was added to equal volume of the pre-warmed platelet poor plasma-extract mixture, incubated for 1 minute after which 50µl of 0.025M calcium chloride (HUMAN) was added and clotting time was measured PBS was used as a control.

RESULTS AND DISCUSSION

Antioxidant activity

The free radical scavenging activity of the methanolic extract of *Viscum album* hosted by olive and almond trees has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100µg/ml. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The results are shown in Table 1. The table readings are presented in Figure 2.

The results showed that the anti-oxidant activity reaches a platuea at a concentration more than 100µg/ml for

Table 1. Absorbance and the calculated percentage inhibition activity for Trolox standard and *Viscum album* hosted on almond and olive.

Concentration µg/ml	%inhibition Trolox	%inhibition Olive	%inhibition Almond
1	37.705	1.098	5.738
2	54.098	1.639	6.557
3	81.148	4.918	11.475
5	86.066	5.639	13.115
7	91.803	9.836	17.213
10	92.614	17.614	31.25
20	93.75	43.182	55.682
30	94.886	59.091	85.227
40	94.886	71.591	89.773
50	95.455	82.955	91.477
80	96.023	89.773	92.614
100	97.159	90.341	92.773

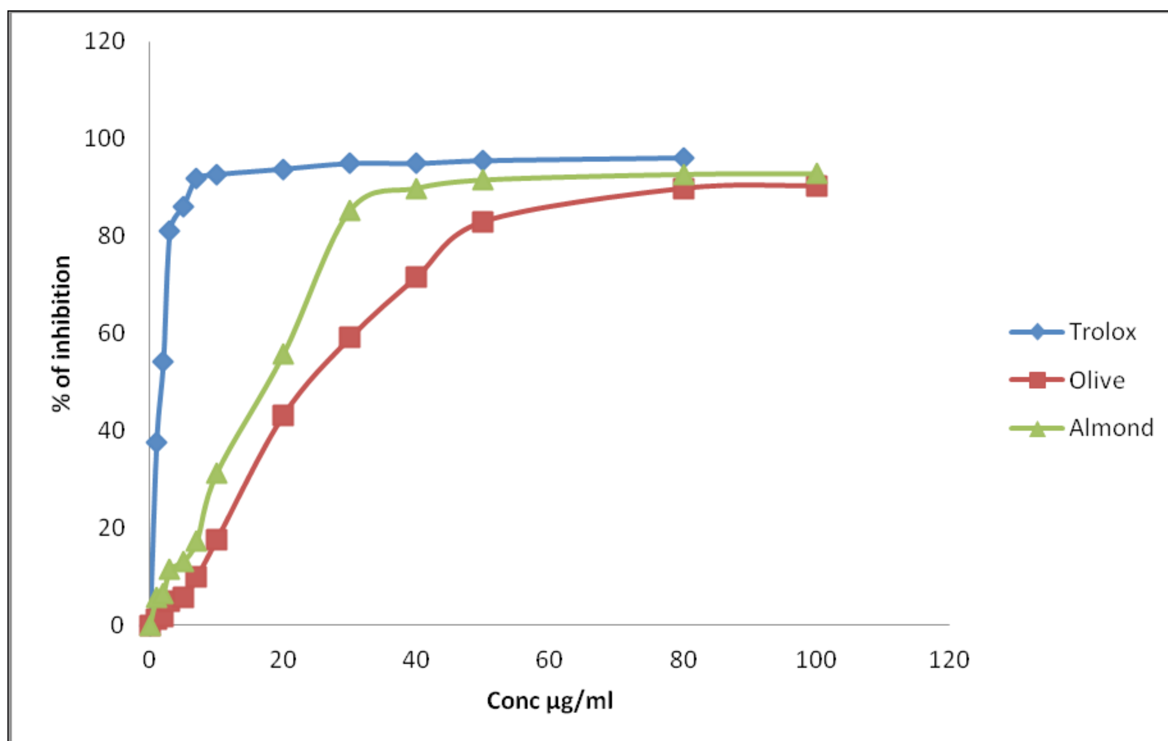

Figure 2. Inhibition activity of Trolox standard and *Viscum album* hosted on almond and olive.

Trolox standard as well as both *Viscum album* hosted by both of the plants. The Graphs show a difference in anti oxidant activity for two host plants. The more potent activity was for *Viscum album* hosted on almond. The Calculated antioxidant IC_{50} for olive *Viscum album* was 25.34±3.8µg/ml and for almond *Viscum album* 15.37± 2.2µg/ml, while it was 1.52±0.05µg/ml for the trolox standard. The results clearly demonstrate a difference in antioxidant activity for *Viscum album* cultivated from different plants. The antioxidant activity was comparatively lower compared to the Trolox reference standard which is known to have a huge antioxidant activity.

Antimicrobial activity

Antimicrobial activity of *Viscum album* methonolic and DMSO crude extracts was investigated against several reference and clinical isolates. The activity was assayed using different extract concentrations on agar plates. Data presented in Table 2 show the results of agar diffusion well-variant using DMSO. Crude DMSO extracts of both variants of *Viscum album* showed strong inhibition effects on the growth of *Staphylococcus aureus* (ATCC 25923) and MRSA. However, the inhibition effect of almond variant was more pronounced compared to that of olive in all examined concentrations. Agar diffusion disc-variant method showed similar effects on bacterial growth. Metabolic extracts of both variants showed similar effect on the growth of Gram positive tested strains (data not shown).

The effect of crude DMSO extracts of both variants showed limited growth inhibition activity in all tested Gram-negative reference and clinical isolates except *Proteus mirabilis*. These extracts failed to show any effect on the growth of *Proteus mirabilis*. No clear differences in growth inhibition zones of both variants on Gram-negative bacteria were observed.

Methanolic extracts of both variants of *Viscum album* showed limited effect on the growth of *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus mirabilis*, however, almond extracts showed some inhibitory effects on the growth of *Escherichia coli* (ATCC 25922) (data not shown). Antibiotic susceptibility testing results of the reference and clinical strains are shown in Table 2.

Anti coagulant activity

The PT and aPTT are indicators of coagulation and are used to determine the clotting of the tissue factor and contact pathways respectively (Table 3).

A significant prolongation of PT was observed with the extract of the *Viscum album* hosted on almond and olive

Table 2. Antimicrobial activity of DMSO extracts of *Viscum album* hosts.

Bacterial Strains	Viscum. album-Olive (%)			Viscum. album-Almond (%)			Antibiotics			
	30	20	10	30	20	10	OX	VA	CN	CTX
Gram-positive strains										
Staphylococcus aureus (ATCC 25923)	32	26	20	35	30	25	20	19	25	30
Staphylococcus aureus (MRSA Positive)	26	24	20	32	30	24	0	25	25	0
Gram-negative strains										
Escherichia coli (ATCC 25922)	12	15	16	16	16	14	–	–	24	35
Pseudomonas aeruginosa (ATCC 27853)	15	14	13	13	14	14	–	–	21	22
Klebsiella pneumoniae	11	12	12	11	11	10	–	–	21	32
Enterobacter cloacae	10	13	14	12	11	10	–	–	21	33
Proteus mirabilis	0	0	0	0	0	0	–	–	0	35

Abbreviations: OX, Oxacillin; VA, Vancomycin; CN, Gentamicin; CTX, Cefotaxime.

Table 3. Viscum album on prothrombin time and Partial Thromboblastin Time.

Effect of organic extract of *Viscum album* on prothrombin time (s)				
	Almond		Olive	
	%	Mean + SD	%	Mean + SD
Control 22.5 + 0.3	10%	*	10%	*
	5%	*	5%	195 + 3.2
	2.50%	31.6 + 0.6	2.50%	26.9 + 1.1
	1.25%	22.9 + 0.5	1.25%	21.6 + 1.2
Effect of organic extract of *Viscum album* on Activated Partial Thromboblastin Time(s)				
	Almond		Olive	
	%	Mean + SD	%	Mean + SD
Control 32.6 + 2.1	10%	*	10%	*
	5%	*	5%	*
	2.50%	55.7 + 3.5	2.50%	67 + 1.5
	1.25%	27.3 + 2.1	1.25%	54.3 + 3.1
	0.75%	25 + 1.7	0.75%	+ 0.6

(*) Clot detection time was greater than 200 seconds.

trees at a concentration of 2.5%. The extract of the *Viscum album* hosted on almond trees prolonged aPTT at a concentration of 2.5% while the extract of the *Viscum album* hosted on olive trees were noticed at a concentration as low as 0.75%.

CONCLUSION AND FUTURE WORK

The observed variations in bioactivity of the tested variants strongly indicate that bioactivity is influenced by host plant. This suggests a careful consideration of the host plant when administering this plant in to folkloric medicine. Further research is required to determine active ingredient involved in each bioactivity.

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Pharmacokinetic study of phyllanthin and hypophyllanthin after oral administration to rats

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ABSTRACT

Objective: The present study was carried out to develop a sensitive and cost effective HPLC method for the determination of bioactive lignans (phyllanthin and hypophyllanthin) and its application in a pharmacokinetic study. **Methods:** Identification of lignan compounds on C−18 column was monitored at a range of 199−400nm using photodiode array detector (PDA) with methanol-water (66:34, v/v) as mobile phase at a flow rate of 1ml/min. Carbamazepine was used as internal standard. **Results:** From the developed method LOD and LOQ values were found to be 56.14ng/ml and 169.99ng/ml for phyllanthin, and 56.04ng/ml and 169.82ng/ml for hypophyllanthin. The validated RP−HPLC method herein was applied for pharmacokinetic studies and C_{max} (ng/ml) values for administered three oral doses (2.5, 5 and 10mg/kg) of phyllanthin and hypophyllanthin were 0.28 ± 0.06, 0.53 ± 0.16, 0.98 ± 0.22 and 0.68 ± 0.76, 1.35 ± 0.23, 2.45 ± 0.33, respectively. **Conclusion:** In conclusion, developed HPLC−PDA method effectively determined the phyllanthin and hypophyllanthin in various solvent and plasma samples. This method was successfully applied in conducting their oral pharmacokinetic studies.

Keywords: *Phyllanthus amarus,* phyllanthin, hypophyllanthin, HPLC−PDA, pharmacokinetics.

INTRODUCTION

Phyllanthus amarus Schum and Thonn (Euphorbiaceae) is widely disturbed in tropical areas of world such as China, Java, Southern Florida, Bahmas, West Indies and Tropical America.[1] Phyllanthus genus is having significant ethnomedical importance[2,3] and is officially listed in Indian herbal medicine system of Ayurveda. *P.amarus* was used for the treatment of inflammation,[4] liver ailments,[5] free radical scavenging activity,[6] kidney stones[7] and also acts as immunomodulatory agent,[8] anticancer agent,[9] antiviral agent[10] and HIV replication and reverse transcriptase inhibitor agent.[11] *P.amarus* is well known for its biologically active compounds. The main active constituents of the herb are lignans, terpenes, sterols, flavonoids, alkaloids, volatile oils and tannins.[12] Lignans like phyllanthin and hypophyllanthin (Fig. 1) from *P.amarus* found to have potent bioactive nature in the treatment of several diseases like cancer,[13] hepatic disorders,[14] free radical scavenging activity[15] and inflammation.[16]

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Pharmacokinetic studies are essential for fixing the dose and for studying the bioavailability. However, till now, research on phyllanthin and hypophyllanthin was focused on pharmacological effects while their pharmacokinetic studies were unclear. Therefore, the aim of the present paper was to investigate the pharmacokinetic properties of phyllanthin and hypophyllanthin at different doses.

High performance liquid chromatography (HPLC) is a powerful tool for natural product chemists in the separation and identification of very close structurally related compounds. Many analytical methods including gas chromatography (GC), GC–mass spectrometry (GC–MS), HPLC–ultraviolet detection (HPLC–UV), HPLC–fluorescence detection and HPLC–MS have been described for the measurements of lignans in plants.[17,18] Though HPLC–UV is regularly used, this method is less sensitive because of poor detection nature of the prisms used in UV detector. HPLC–fluorescence detection, HPLC–MS and GC–MS are highly sensitive

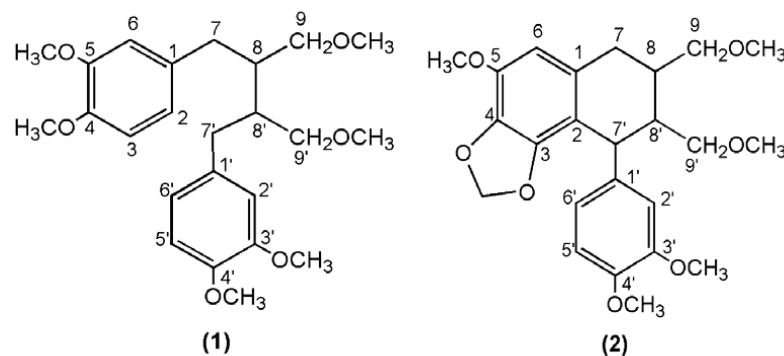

Figure 1. Chemical structures of phyllanthin (1) and hypophyllanthin (2).

with very low levels of detection of sample in plasma, but these methods are very expensive because of high cost of the equipment and laborious sample processing methods. These methods also include additional steps like prefiltration and derivatization of sample before injection. It is not possible for small research groups and academic departments to purchase and maintain these expensive instruments. Hence, there is need for the development of simple, reliable, sensitive and cost effective method for the analysis of bioactive lignans. In the present paper, reverse phase (RP) HPLC method with photo-diode-array (PDA) detection was developed for systematic identificationand pharmacokinetic studies of the two bioactive lignans, phyllanthin and hypophyllanthin in solvent system and also in blood plasma.

EXPERIMENTAL

Chemicals and reagents

Silica gel 60–120 mesh was purchased from ACME Synthetic Chemicals (Mumbai, India), HPLC grade methanol was purchased from Merck (Germany). Deionized water for HPLC was obtained from Milli–Q instrument (Millipore, USA). EDTAcentrifugation tubes were obtained from CML Biotech (P) Ltd (India).

Extraction and isolation

The leaves of *P.amarus* were collected from Paderu, Visakhapatnam District, Andhra Pradesh and authenticated by Dr. M.Venkayya, Taxonomist. Voucher specimen (BG/PMK/PA-10-10) was deposited in the herbarium, College of Pharmaceutical Sciences, Andhra University. Freshly collected aerial parts of the plant were shade dried and pulverized. The powdered material (5kg) was then subjected to successive soxhlet extraction with hexane, ethyl acetate and methanol. Solvent thus obtained was separately concentrated under vacuum at 40ºC by using rotary evaporator (Buchi, Switzerland). 25g of hexane extract was then fractionated over a column (100cm length × 35mm diameter) of silica gel (60–120 mesh size). Gradient elution was done in the following sequence, hexane (100, v/v)→hexane-ethylacetate (95:5, v/v)→hexane-ethylacetate (90:10, v/v). Fractions (Fr150-189) collected for hexane-ethylacetate (90:10 v/v) showed the presence of lignans on TLC and these fractions were pooled together and concentrated. The lignan fraction was subjected to preparative separation using RP-HPLC (Waters Delta Prep-PDA, USA) for obtaining the pure (>95%) phyllanthin and hypophyllanthin.[15,16]

Instrumentation

HPLC system consists of a quaternary gradient HPLC (Waters Delta Prep HPLC system, USA) with a rheodyne injector for loading the sample. A reversed phase C–18 column (250m×4.0mm;Waters associates, USA) was used with the following analytical conditions: mobile phase of methanol-water (66:34, v/v) with a flowrate of 1 ml/min at room temperature (27°C). Sample injection volume was 20µl. Chromatograms were recorded at 199–400nm range using a photodiode array detector (Waters PDA 2998). HPLC system was equipped with EMPOWER 2 software (Waters, Milford, MA, USA) for data acquisition and processing.

Animals

Healthy Sprague Dawley rats of either sex weighing about 220–250gm were supplied by National institute of nutrition, Hyderabad. Animals were acclimatized under standard environment conditions (23±2ºC, 55±5% relative humidity, 12h light/dark cycle) for one week. Pharmacokinetic studies were carried out in NAIP/ICAR Project laboratory, University college of Pharmaceutical Sciences, Andhra University and were approved

by institutional animal ethics committee (Regd. No. 516/PO/c/01/CPCSEA) for experimentation on animals.

Preparation of stock solutions and calibration standards

Stock solutions (1mg/ml) of compounds phyllanthin and hypophyllanthin were prepared in methanol (HPLC grade) and were further diluted with methanol to prepare different concentrations of their standard solutions. Calibration curves were established on twelve data points covering a concentration range of 0.5 to 100µg/ml for both compounds. Aliquots (20µl) were used for the HPLC injections. Linear regression of peak area of compounds of interest versus compound concentration was performed in order to estimate the slope, intercept and correlation coefficient of each calibration curve.

Plasma sample preparation

Blood samples were collected into micro-centrifuge tubes containing approximately 10mg heparin; centrifuged at 4000×*g* for 5 min at 15ºC. To 125µl of plasma, 25µl of internal standard (carbamazepine stock solution 10µg/ml in methanol) was added and vortexed (Vortex mixer, Genei, Mumbai) for 60 seconds. Then 500µl of methanol was added to precipitate proteins and vortexed for 5 min and centrifuged at 5000×*g* for 10 min. Supernatant was taken and dried in vacuum oven at 40ºC. Dried samples were then redispersed in 100µl methanol and vortexed.[19] Supernatant was transferred in to a micro- centrifuge tube and from this 20µl was injected for HPLC analysis.

Assay validation

Accuracy and precision

Samples were analyzed against calibration curves. Meanwhile, standard deviation and relative standards deviation (RSD) were calculated from samples and used to estimate their intra and inter-day precision. Accuracy was assessed by comparison of calculated mean concentrations with their known concentrations. The intra-day accuracy and precision was determined for each compound at four concentrations with six replicates each carried out in a single day, whilst the inter-day values were carried out over 6 consecutive days. The accuracy was expressed in terms of recovery, and calculated by multiplying the ratio of measured drug concentration to its theoretical concentration with 100, so as to give percent recovery, whereas precision was expressed as coefficient of variation.

Recovery studies

The recovery efficiency was determined by adding measured amounts (2, 6, 10 and 40µg) of phyllanthin and hypophyllanthin to their known concentrations (10µg). Accuracy was expressed in terms of the recovery and calculated by multiplying the ratio of measured drug concentration to its theoretical concentration with 100, so as to give percent recovery. The study was replicated 6 times and values were expressed as mean ± standard deviation (SD).

Limit of detection (LOD), limit of quantification (LOQ) and linearity

LOD was defined as the lowest concentration that analytical system can reliably differentiate from the background level, whilst LOQ was defined as lowest quantifiable concentration that can be measured with a stated level of confidence. Stock (1mg/ml) solutions of phyllanthin and hypophyllanthin were prepared in pooled blank rat plasma. LOD and LOQ were determined by injections of successive two-fold dilution of stock solutions in blank rat plasma. Standard calibration curves were constructed as plots of mean peak area ratio against the corresponding concentration at a range of 0.1–100µg/ml for both the lignans. Linearity of the curve was evaluated by linear regression analysis.

Pharmacokinetic study

Animals were divided into six groups of 4 each. First three groups (Groups I–III) received phyllanthin orally at doses of 2.5, 5 and 10mg/kg, respectively. The next three groups (Groups IV–VI) received hypophyllanthin orally at doses of 2.5, 5 and 10mg/kg, respectively. Lignans were given orally in aqueous solution of Tween 20 and the volume was made to 5ml/kg. Blood samples (0.4ml) were withdrawn from retro-orbital plexus at 0, 30 min, 1, 2, 4, 8, 12, 24 and 36 h after oral administration and were collected into micro centrifuge tubes containing approximately 10 mg heparin; centrifuged at 4000 × *g* for 5 min at 15ºC and resulting plasma was kept at –20ºC before HPLC analysis.

Data analysis

The mean plasma concentration versus time data was analyzed by non-compartmental extravascular method and the following pharmacokinetic parameters were estimated: Peak plasma concentration (C_{max}) and time to reach peak concentration (T_{max}), elimination rate constant (K_{el}), terminal elimination half-life ($t_{1/2}$), area under

the plasma concentration–time curve (AUC), volume of distribution of terminal phase (V_z) and total body clearance (Cl) were calculated by taking plasma drug concentration (ng/ml) on y-axis and time (h) on x-axis using Kinetica software (Thermo scientific, USA) by log-linear trapezoidal rule.

Statistical analysis

Pharmacokinetic parameters of the three doses after oral administration of phyllanthin and hypophyllanthin were compared by one-way analysis of variance (ANOVA) model, respectively. The results were expressed as mean ± SD. Statistical significance was determined at the level of $P<0.05$.

RESULTS AND DISCUSSION

Assay validation

The objective of this study was to develop a sensitive HPLC method for the analysis of phyllanthin and hypophyllanthin in solvent system and plasma samples using most commonly employed C–18 column with PDA detection. Retention times (R_t) for phyllanthin and hypophyllanthin were observed as 27.69 and 24.16 minutes. No interfering peaks were found in the chromatogram.

Figs. 2A and 2B demonstrate the chromatograms in which the analytes were eluted without interference. The recovery, within-day and between-day precision and accuracy for measurements of different lignan concentrations are depicted in Table 1. Calibration curves of the both compounds showed linearity in the range of 0.1–100µg/ml. The linear regression equation for calibration curves of phyllanthin and hypophyllanthin were Y=22601x+25070 and Y= 16339x+28982, respectively. The intra and inter-day precisions of phyllanthin and hypophyllanthin were in the range of 1.97–39.89 and 1.969–39.91. In accuracy assessment, recovery for phyllanthin and hypophyllanthin was found to be 99.08–99.82% and 97.98–100.52%, respectively. Thus, this HPLC method is simple, sensitive, precise and highly accurate.

Pharmacokinetic study

Chromatograms of plasma sample from a rat after 1 h of administration of 5mg/kg of phyllanthin and hypophyllanthin spiked with carbamazepine (Rt-6.4 min) were shown in Figs. 2A and 2B, respectively. The total run time was 40 minutes.

Calibration curves over a concentration range of 0.25–100µg/ml was linear for phyllanthin and hypophyllanthin with regression equations of Y=0.539x + 0.017 and Y=0.450x – 0.087, respectively. The correlation values of both compounds was greater than 0.999. Because of large difference in plasma level of lignans, a wider analytical range was used in present study to cover the entire range of expected concentrations. LOD and LOQ values of the tested lignans were approximately 56.14 ng/ml and 169.99ng/ml for phyllanthin; 56.04ng/ml and 169.82ng/ml for hypophyllanthin at signal to noise ratio of 5:1.

The reported HPLC–MS method for analysis of plasma lignans has limits of detection between 0.021–0.2ng/ml.[20] It was reported that analysis of lignans in biological fluids by GC–MS method had LOD values between 0.04 and 10nM, it was less than 5nM for HPLC–MS method. Vikneswaran and Chan reported the HPLC–fluorescence method with detection limits between 2.91–14.19nM for lignan analysis in plasma.[21] Furthermore, Nurmi et al reported a HPLC method using coulometric electrode

Table 1. Recovery, Intra-day and Inter-day precision values of phyllanthin and hypophyllanthin in solvent system.

Drug concentration (µg/ml)	Recovery		Precision	
	Mean (± SD) amount (µg) recovered (n=6)	Mean (± SD) % of recovery (n=6)	Intra-day (% CV), n=6	Inter-day (%CV), n=6
Phyllanthin				
2	1.98 ± 0.14	99.08 ± 0.38	1.97 (0.46)	1.969 (0.27)
6	5.95 ± 0.11	99.11 ± 1.78	6.02 (3.69)	5.98 (1.51)
10	9.94 ± 0.028	99.34 ± 0.28	9.91 (0.76)	9.84 (1.17)
40	39.92 ± 0.67	99.82 ± 0.17	39.89 (0.14)	39.91 (0.52)
Hypophyllanthin				
2	1.959 ± 0.11	97.98 ± 0.56	1.95 (1.26)	1.952 (1.12)
6	6.03 ± 0.19	100.52 ± 1.44	5.84 (1.03)	5.94 (0.52)
10	9.94 ± 0.042	99.36 ± 0.42	9.94 (0.41)	9.87 (0.71)
40	39.76 ± 0.27	99.41 ± 0.67	39.77 (2.62)	39.29 (2.5)

array detection with detection limits between 1.9–3.9nM for lignan analysis in human urine.[22] The present HPLC–PDA method has comparable detection and quantification limits.

The recovery, intra and inter-day precision and accuracy measurements of different concentrations are revealed in Table 2. The present method produced a satisfactory recovery of lignans phyllanthin and hypophyllanthin from 97.12–99.74% and 98.34–99.76% respectively, thus implying that deproteinization of plasma with methanol did not result in any substantial loss of chemical constituents. The intra-day and inter-day precision and accuracy were between 97.12–99.74 for phyllanthin and 98.34–99.76 for hypophyllanthin, respectively, indicating that the developed method was reliable and reproducible. Figs. 3A and 3B reveals mean plasma concentration versus time profiles up on oral administration of 2.5, 5 and 10mg/kg of phyllanthin and hypophyllanthin, respectively.

The present HPLC–PDA method makes it possible to measure lignan concentrations in rat plasma even up to 12h after oral administration. Pharmacokinetic parameters of lignans in rat plasma after oral administration are depicted in Table 3. Both lignans showed a rapid rise with a T_{max} of 1h followed by a gradual decline to 0 after 24h. Both compounds showed non-linear increase in $AUC_{0 \rightarrow 24h}$ values with increase in their oral doses. Being lipophilic (isolated from the hexane extract of *P.amarus*), dissolved lignans could penetrate the gastrointestinal tract more easily achieving their peak plasma concentration after 1h.

CONCLUSION

A simple, sensitive and validated HPLC–PDA detection method for the determination of phyllanthin and hypophyllanthin in solvent system and plasma has been developed. This method was accurate and precise for the quantitative analysis of lignans and it has additional advantages over known methods such as simple sample preparation, low detection and quantification limits. The developed method was successfully applied in the pharmacokinetic study of lignans in rats.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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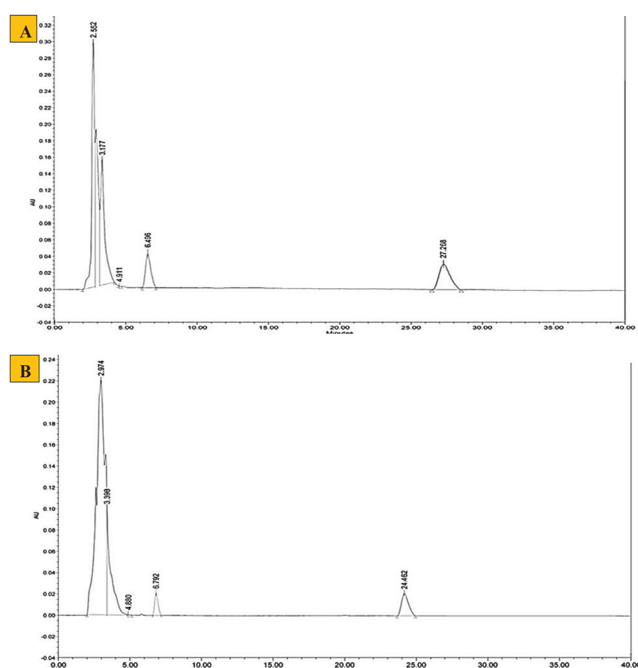

Figure 2A & B. HPLC chromatograms of rat plasma after administration of 5mg/kg of phyllanthin and hypophyllanthin.

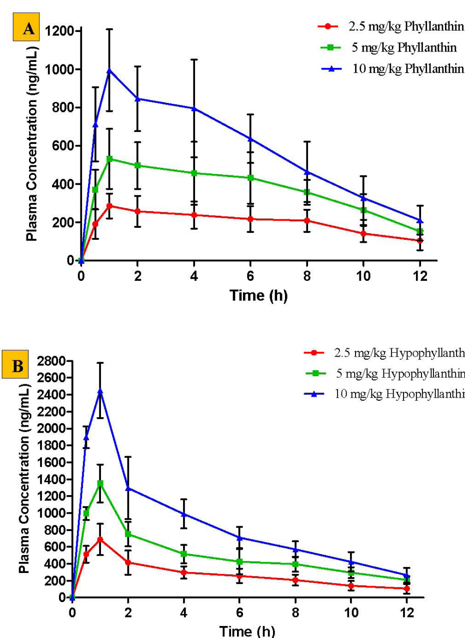

Figure 3A & B. Plasma concentration (mean ± SD, n=6) versus time profiles after oral administration of phyllanthin and hypophyllanthin at doses of 2.5, 5 and 10mg/kg to rats.

Table 2. Recovery, Intra-day and Inter-day precision values of phyllanthin and hypophyllanthin in rat plasma.

Drug concentration (µg/ml)	Recovery		Precision	
	Mean (± SD) amount (µg) recovered (n=6)	Mean (± SD) % of recovery (n=6)	Intra-day (% CV), n=6	Inter-day (%CV), n=6
Phyllanthin				
2	1.956 ± 0.25	97.12 ± 1.26	1.96 (0.458)	1.945 (0.97)
6	5.92 ± 0.14	98.56 ± 2.45	5.94 (1.79)	5.88 (3.31)
10	9.84 ± 0.15	98.42 ± 1.16	9.68 (3.8)	9.86 (0.81)
40	39.89 ± 0.27	99.74 ± 0.17	39.79 (0.51)	39.89 (0.18)
Hypophyllanthin				
2	1.94 ± 0.22	98.34 ± 1.04	1.96 (0.92)	1.962 (1.46)
6	5.91 ± 0.18	98.38 ± 0.69	5.91 (0.25)	5.86 (0.41)
10	9.84 ± 0.14	98.32 ± 1.31	9.72 (3.51)	9.73 (1.98)
40	39.89 ± 0.24	99.76 ± 0.15	39.62 (1.25)	39.84 (0.97)

Table 3. Pharmacokinetic parameters (mean ± SD, n=6) after oral administration of phyllanthin and hypophyllanthin to rats.

Parameters	Phyllanthin			Hypophyllanthin		
	2.5 mg/kg	5 mg/kg	10 mg/kg	2.5 mg/kg	5 mg/kg	10 mg/kg
C_{max}(µg/ml)	0.28 ± 0.06	0.53 ± 0.16	0.98 ± 0.22	0.68 ± 0.76	1.35 ± 0.23	2.45 ± 0.33
T_{max}(h)	1 ± 0.09[a]	1 ± 0.22	1 ± 0.25	1 ± 0.14[a]	1 ± 0.25	1 ± 0.18
AUC (µg h/ml)	2.81 ± 0.15	5.24 ± 0.26	8.09 ± 0.19	3.89 ± 0.11	7.37 ± 0.17	12.17 ± 0.27
k_{el}(h^{-1})	0.22 ± 0.03[a]	0.22 ± 0.07	0.19 ± 0.08	0.17 ± 0.02[a]	0.16 ± 0.05	0.14 ± 0.04
$t_{1/2}$ (h)	3.22 ± 0.28[a]	3.27 ± 0.14	3.53 ± 0.26	4.07 ± 0.35[a]	4.34 ± 0.17	4.54 ± 0.26
Cl (L/h)	0.87 ± 0.11[a]	0.96 ± 0.24	1.24 ± 0.21	0.64 ± 0.14[a]	0.68 ± 0.22	0.82 ± 0.25
Vz (L)	4.15 ± 0.94[a]	4.49 ± 1.15	6.28 ± 1.67	3.78 ± 1.05[a]	4.24 ± 0.86	5.37 ± 1.26

[a] Values were significantly different from 5 and 10mg/kg ($P<0.05$).

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