The Research of Medical Science Review

Received: 15 October, 2024
Accepted: 15 November, 2024
Published: 30 November, 2024

ISSN: 3007-1208 | 3007-1216
Volume 2, Issue 3, 2024

PHYTOCHEMICALS, BIOACTIVITIES AND THERAPEUTIC APPLICATION OF THREE MEDICINAL PLANTS OF SINDH, PAKISTAN: IPOMOEA CARNEA, TINOSPORA MALABARICA, FAGONIA INDICA

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ABSTRACT

Research outcomes on the effectiveness and safety of existing and newly produced drugs recommend naturally produced therapeutic constituents due to safety and the least side effects. Hence, millions of researchers continuously explore medicinal plants to isolate known and new compounds and formulate safe therapeutics. Literature expands daily, covering such studies which need to be reviewed for summary, comparative survey and especially to present gapes suggested in previous studies which may be direction for future work and getting more out of species that still have potential for further scientific research work. In this context, three species of medicinal plants of Sindh origin, Pakistan, have been selected for this review.

***Ipomoea Carnea**: This plant has been chosen by folk medicine experts and is used as a raw material for Unani medicine. As per available literature, toxic effects were initially evaluated, followed by chemical exploration, and to some extent, its safety profile was also assessed.*

***Fagonia Indica**: Many varieties of Fagonia are commonly found throughout Pakistan, parts of India, and the Middle East. In the Sindh region of Pakistan, Indica is the most commonly found species of Fagonia, known as "Sachi Booti" that means true herb (Ali et al. 2021). Local medicinal uses are also reported which include treatment of cancer and issues caused due to any poisonous substances. Different compound have been isolated and bioactivity studies are also reported.*

***Tinospora Malabarica**: Species of the genus Tinospora are very close to each other; however, their difference from a botanical point of view and chemical profile is quite different and reported in literature. Various papers have reported the use of this plant as a source of therapeutics, and the isolation of known and new compounds has also been reported. Bioactivity studies of extracts and bioguided fractionation to isolate bioactive compounds have also been reported for this plant. Minimal work is reported on this species; however, due to its similarity with each other, data available on different species of genus Tinospora indicate that the species malabarica may also be very prominent as the choice of medicinal compounds and use as raw material in formulation to be used to cure ailments*

INTRODUCTION

Since ancient civilizations, people have relied on plants to get valuable material for making drugs to cure ailments. The choice of plants is due to their availability in large quantities, safety, and effective secondary metabolites (1). Thousands of research articles are published, and literature is enhanced for further directions to the scientific community. In this connection, published literature has also been reviewed to get data from one point and find the gaps in work and options for opportunities to work toward even better goals. Consequently, three species, i.e., *I. carnea*, *T. malabarica*, and *F. indica*, have been selected for review here, which grow worldwide and are the choice of natural product researchers. The selection of three species is due to their equal importance in the Umarkot region of Sindh province of Pakistan, where all these three plants are used by *Hakim* (local medical practitioners) to cure different ailments. The taxonomical details of three plant species (*I. carnea*, *T. malabarica*, and *F. indica*) are described in the Table. 1.

Table 1-Taxonomical details of three plant species [2]

	I. carnea	***T. malabarica***	***F. indica***
Class	Tracheobionta	Magnoliopsida	Magnoliopsida
Order	Solanales	Ranunculales	Zygophyllales
Family	Convolvulaceae	Menispermaceae	Zygophyllales
Genus	Ipomoea	Tinospora	Fagonia
Species	carnea	malabarica	indica

Ipomoea Carnea

Ipomoea Carnea plant is believed to be the origin of the American tropical region but is now sparely distributed in different areas and grows worldwide by 2 to 3 meters high as erect, leafed, and unbranched have cited sufficient literature in which claims of previous reports prove that *I. carnea* is grown worldwide for medicinal and ornamental purpose. Its ability to grow even in adverse climatic conditions has also been reported (3-5). Its different parts, including leaves and flowers, have been used as raw therapeutics to cure ailments such as venereal and skin diseases, immunodeficiency, dysentery, gout, rheumatism, and hypertension. Extracts of *I. carnea* have also demonstrated antimicrobial, anticancer, and antioxidant by reducing free radical, antidiabetic, immune-modulatory, wound healing, anticonvulsant, anxiolytic, anti-inflammatory, sedative, and hepatoprotective properties (6-15). As per earlier work done to determine the essential oil of *I. carnea*, they only did the same for Brazilian ecospecies for comparative analysis (16).

Its toxic effect has long been studied to know the primary constituent responsible for this activity, polyhydroxy alkaloids such as swainsonine and calystegines (5). The plant is also grown in line to make boundaries as a hurdle for animals, prevent the loss of valuable plants, and make the area beautiful by utilizing the ornamental property of this plant. Though some researchers have detected glycosides, chitinase phenolics, and alkaloids in leaves of *I. carnea*, comprehensive data has not yet been reported anywhere. Bioassay-guided isolation of antifungal unstable coumarates isomers has also been reported (17). Another study has also done similar work to see the effect of *I. carnea* alkaloids present in aqueous on the central nervous system of rats (18).

Fig 1. ***Ipomoea carnea***

Fagonia Indica

Various varieties of Fagonia are commonly found throughout Pakistan, parts of India, and the Middle East (19). In Sindh, Indica is the most found species of Fagonia, known as “Sachi Booti, " meaning actual herb. Local medicinal uses are also reported, including treating cancer and issues caused by poisonous substances. Tumor growth inhibition has also been observed as a prominent property of *Fagonia indica* aqueous extract from whole plants (20). This plant is found rich in saponin or triterpenoid glycoside as per isolation work carried out by different groups of scientists from time to time. Fagonia species are often used in traditional medicines, such as for the treatment of fever, jaundice (21), blood purification, cold, cough (22), asthma, skin infection, liver problems (23), carminative, emetic (24), and the extracts of these plants have been reported to exhibit antimicrobial, anti-inflammatory, analgesic and antipyretic activities (25-26).

Additionally, this plant is declared to be the remedy for cancer (27-29) and thalassemia (30). This plant's medicinal properties were attributed to its variety of active phytochemical constituents. Many saponins or triterpenoid glycosides have been isolated and characterized from various species of Fagonia (31). Additionally, sulfated triterpenoids and sulfated triterpenoid glycosides have been isolated from Fagonia species (32). Saponins are considered the major bioactive constituents of the drugs, mainly used for their hemolytic (33) anti-inflammatory activities (34). Some saponins from Fagonia species have been reported to exert anticancer, antidiabetic, molluscicidal, and antioxidant activities (35).

Fig 2. Fagonia indica

Tinspora Malabarica

Since the species of the genus *malabarica* are very closely related to each other in morphology hence, it becomes necessary to cite such a pair of species like it is done in India for *malabarica and Cordifolia*, and the same is applicable for *malabarica* and *crispa* is Sindh region of Pakistan. It is helpful for consumers and good healer practitioners to prevent adulteration and know the exact chemical and bioactivity profiles of each species. Both species are cited up to the best of our accessible literature.

Several studies have been conducted on the constituents of the genus Tinospora, and a variety of compounds have been isolated, including furanoditerpene lactones, steroids, flavonoids, lignans, alkaloids, and phenolics as per literature cited by (36) and (37). Malabarica is one of thirty species of the genus Tinospora. Due to their therapeutic importance, it is included in a few of those thirty species used as medicinal sources. Like other species, it also grows in tropical and subtropical regions, including South Asia (38). This plant is bitter due to a variety of alkaloids and is considered a tonic for treating jaundice, rheumatism, urinary disorders, gout, leprosy, and fever (39-41). Two more alkaloids, tinosporine and tinosporidine, have also been reported. Extracting the whole plant's dichloromethane revealed three compounds: N-cis-feruloyltyramine, N-transferuloyltyramine, and secoisolariciresinol. These compounds have shown antioxidant properties when tested per assay protocols of β-carotene and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (42). *T. malabarica* stem contains flavone O-glycosides (apigenin), picroretoside, berberine, palmatine, picroretine, resin & five flavonoids (42).

Literature reported on the isolation claims that a group of scientists has isolated the number of clerodane-type furanoditerpenoids that have shown activity against human oral epidermoid, medulla, and colon cancer cell lines; however, the relevant paper shows work done on *Casearia membranacea*. Cis-clerodane-type furanoditerpenoids from *T. malabarica* have also been isolated. Still, they were not found active when tested for cytotoxicity assays against human prostate cancer (PC-3) and the normal mouse fibroblast (3T3) cell lines (43, 44). Hot water extract *T. malabarica* showed protective effects on renal damage and hemolysis induced by malaria infection during experiments done on mice. Thus, this plant species was supposed to be a potential source in developing various herbal formulations for malarial treatment (45).

Tinspora malabarica

CHEMICAL EXPLORATION AND BIOACTIVITIES

Ipomoea Carnea

Phytochemical Analysis

Various extracts of *Ipomoea species* were prepared, such as chloroform benzene water, and an initial test to know the phytochemicals was performed for steroids, triterpenoids, carbohydrates, alkaloids, phenolic compounds, saponins, xanthoproteins, tannins and flavonoids (46). It is observed that chloroform was the best solvent for extracting most of the compounds screened except saponins and xantho proteins found in benzene and water, respectively. After the screening, GC-MS was used for the identification of compounds, and *I. carnea* was found to contain neophyadiene, 1-decanol, tetradecaine, pentadecane, 1-iodo-2-methylundecane, trans-caryophyllene, eicosane in benzene extract while 2-butenoic acid and cholestan-3-one in chloroform extract. The authors proposed considering this plant insecticidal due to the presence of cholestan-3-one.

Alkaloidal Components

An early report (47) about the toxicity of *I. carnea* is available in the literature. They conducted studies to know the toxic effect of this plant after receiving complaints from goatherd of the Sudan region about the death of goats that consumed *I. Carnea* in higher quantities, especially when other plants in the pasture were short. They allowed goats to eat leaves of *I. Carnea* started slowly, but goat consumption increased over time, and the effect was observed after 5 to 6 weeks. Initially, weakness was observed, but later on, animals could not be on land properly and finally faced death. However, it was an initial study to report the plant as toxic for goats, giving room for work to know the reason behind that through a systematic analysis.

Continuing the report about alkaloids in I. carnea, this isolated (through ion-exchange chromatography) and characterized (through NMR) alkaloidal components in Brazilian plant species' leaves, flowers, and seeds. They also studied the inhibitory activities of alkaloids toward rat lysosomal glycosidases fluorometrically. Identified alkaloids were seven in total which are swainsonine (1), 2-epi-lentiginosine (2), calystegines B1 (3), calystegines B2 (4), calystegines B3 (5), and Calystegine C1, (6), and N-methyl-trans-4-hydroxy-L-proline (7). Comparatively, seeds possessed higher quantities of alkaloids, while the most potent activity against rat

lysosomal â-glucosidase was shown by calystegines B1 (3), calystegines B2 (**4**) and Calystegine C1, (**6**) (48-50).

Further studies of *I. carnea* toxicosis were carried out by evaluating the role of alkaloids (51). The study observed rats' water/food consumption and weight-gaining behavior after administering aqueous fraction and pure alkaloids in the leaves of *I. carnea*. It was found that calystegines do not produce any toxic effect but are just promoters of swainsonine, which has been found to cause many complications, even cell death. The authors also suggest other animal models (such as goats) for proper studies of the effect of calystegines on the central nerve system, as rats are not a good choice for these studies. Their suggestion might be based on the scenario after the report (47), which was based on the toxic effect of this plant on goats.

Coumarates

Coumarates have isolated two coumarate isomers by activity-guided fractionation and HPLC separation (52). Antifungal activity monitoring was continued during fractionation by using *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* as test organisms. Mixture (E)-octadecyl p-coumarate and (Z)-octadecyl p-coumarate were identified in active fractions, structurally close isomers, but retention time in HPLC could identified as different compounds. However, their conversion from one to another isomer was observed after isolation through the column due to the effect of daylight. Identification was further confirmed through mass spectrometry and hydrogen NMR (H-NMR) and carbon NMR (C- NMR) techniques, and the difference of isomers was confirmed through 2 D NMR. The dose-dependent inhibition of the spore germination of Alternaria alternata and A. porri further confirmed the activity of the purified fraction.

Essential Oils

Considering the results of other species regarding essential oil reported by various scientists (particularly in the Brazilian region) for the genus *Ipomoea*, (53) focused on the least worked out species of the genus, i.e., *I. carnea* of the Egyptian region. Aerial parts, including leaves, stems, and flowers, each taken 250 g, and essential oil was obtained through hydro distillation. Extracts were analyzed on GC-MS, which covered a range of C-8 to C-22 compounds. Extracts were also tested for antioxidant activity using two assays, i.e., DPPH and ABTS. Antibacterial activity was also assessed by gram-positive and gram-negative bacteria following the agar diffusion method. The authors claimed the yield of essential oil from leaves was 0.034%, which is quite high compared to previous reports, which ranged from 0.01–0.13% (53). Obtained 31 compounds were divided into six groups: oxygenated monoterpenes (α-Fenchyl alcohol major component), sesquiterpene hydrocarbons (ar-Curcumene major component), oxygenated sesquiterpenes (tau-Cadinol significant components), diterpene hydrocarbons (Beyerene, only one component), carotenoid (E-β-Damascenone major component), and apocarotenoid-derived compound. Extracts demonstrated considerable antioxidant activity when compared to ascorbic acid as a model. Antimicrobial activity was also comparable with antibiotic drugs like cephalexin, tetracycline, ofloxacin and ampicillin against gram positive and negative (4 bacterial strains of each).

Fagonia Indica

Anticancer Activities

It was earlier reported in the literature that some species of fagonia have been used to treat cancer patients in local medication systems (54). Hence, a study was carried out on the rat model to check this property systematically (55). After continuous efforts and application of tumor-producing components, 26 out of 30 rats (in an equal division of male and female) got tumor nodules in the 40th week. When the nodule reached the size of 1 cm, the zero-day count started, and the experiment was initiated. All parts of *F. indica* except roots were soaked in water, and the extract prepared was fed to rats to treat tumors. It was observed that treated rats could survive longer than untreated. The female group treated with an aqueous extract of *F. indica* survived from 55 to 118 days with a mean value of 83, but the untreated lived from 21 to 57 days with a mean value of 40 days. The male group treated with aqueous extract of *F. indica* survived from 39 to 98 days with a mean value of 59.4 days, while the untreated male group could live only 10 to 27 days with a mean value of 17 days.

All these observations proved that the aqueous extract of F. indica possesses tumourostatic properties, which was earlier shown in studies done on patients with oral cavity carcinoma (unpublished double-blind trial). The author suggested further studies.

Surveying literature led to a recent report on bioactivity studies on *F. indica* towards breast cancer cells. Aerial parts of *F. indica,* including stems and leaves, were put in 70% methanol, and polyphenols were extracted by mixing and ultrasonic treatment. Dry residue was used for the analysis of LC-MS and bioactivity studies. Dry residue was dissolved in dimethylsulfoxide (DMSO) to study its effect on breast cancer MCF-7 (one of the most common cells out of 50 available cell lines, used to screen natural products for their potential anti-breast cancer activities). A part of the *F. indica* extract was hydrolyzed using HCl, and further experiments were performed on hydrolyzed and unhydrolyzed extracts. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on human breast adenocarcinoma MCF-7 cells was performed to check the bioactivity of hydrolyzed and unhydrolyzed extracts of *F indica*. Different concentrations of *F. indica* extracts were used from 1 to 1200 µg/mL to reduce the MCF-7 cells compared with reference xanthohumol (XN), a hop flavonoid known to be toxic to MCF-7 cells from 24 to 48 and 72 hours. No reduction was observed in 24 hours, but a considerable cell decrease in 48 hours was treated with unhydrolyzed extract. However, compared with previous reports, the activity of *F. indica* was not up to mark as *F. cretica* extract reduced 75% of cells at 250 µg/mL, which could not be done by F. indica even at 1200 µg/Ml (56).

Contrary to that, hydrolyzed extracts showed remarkable activity by reducing MCS-7 cells even > 90%, showing activity beyond xanthohumol, which could inhibit cells by fifty percent at 20 µM in 72 hours. 300 µg/mL of hydrolyzed extracts of *F. indica* can reduce MCF-7 cells in MTT assay, which is almost equal to xanthohumol, which can do it simultaneously at 20 µM. Besides that, LC-MS analysis revealed the presence of many saponins in the extract, which were identified through data library and NMR studies. It was apparent that glycosides in unhydrolyzed extracts and aglycones in hydrolyzed extracts. As per activities shown by extracts, it was concluded that aglycone of F. indica is more active than glycoside; hence, isolation, identification, or even modification of *F. indica* constituents is suggested (57).

Saponins

Encouraged by the medicinal importance of *F. indica*, saponins were investigated. Work led to the isolation and characterization of seven new compounds, which were triterpenoid glycosides (2), sulfated triterpenoid glycosides (5), and previously known triterpenoid glycosides (1). Author Nayab Kanwal named new compounds by her name, "Nayabin." All were assayed for cytotoxicity activities in MIN6 cell lines to evaluate their r insulin secretory activity. 7.5 Kg powdered *F. indica* was soaked in 80% methanol, and the extract was prepared by removing the solvent, followed by fractionation with hexane, ethyl acetate, and n-butanol. 500 g n-butanol was further separated on column chromatography repeatedly. A fraction was subjected to recycling HPLC, which separated all compounds claimed in the study. Each compound was identified through ^{1}H-NMR, ^{13}C-NMR, High-resolution electrospray ionization mass spectrometry, and infrared spectroscopy. Each saponin was hydrolyzed and analyzed on GC to confirm the glycoside structures. Islets isolation and insulin secretory biological assay were performed on mice following previously known methods. All compounds could not be tested due to limited quantity; however, none of the new compounds showed any promising activity; however, known compounds showed nominal activity (58).

Another attempt was carried out to isolate bioactive compounds of F. indica (59). A 4 kg aerial part was dried and soaked in ethanol, followed by fractionation in organic solvents, and the methanol part was selected for further work. Following the biological activity-guided fractionation approach, the most active component, i.e., novel saponin glycoside of a crude extract of F. indica, was isolated. The purified compound appeared as a light, greenish-white amorphous powder (yield 42 mg). The compound was identified based on studies that included hydrogen and carbon NMR 1 and 2D, IR, and LC-MS. The final IUPAC name was derived as 12-(4-methyl-pent-3-enoyloxy)-20-(4-methyl-pent-3-enoyloxy)-3β,12β,20β-trihydroxypregnane-3-yl*O*-β-$_{D}$-cymapyranosyl-(1→4)-3-methoxy-6-deoxy-bD-glucopyranoside. Assays pertinent to blood complication, apoptosis, and necrosis, was carried out on three cancer cell lines: MCF-7 oestrogen-dependent breast cancer, MDA-MB-468 estrogen independent breast cancer, and Caco-2 colon cancer cells. The isolated pregnane was

tested for growth inhibition of oestrogen-negative breast cancer cells (MDA-MB-468) and colorectal carcinoma cells (Caco-2), which was very prominent; however, it did not inhibit MCF-7 cells (only 42 to 50% at high concertation). Weaker attach on MCF-7 cells was seconded to earlier studies where a glycone was more active than saponin derivatives (59).

Atta-ur-Rahman and his co-workers have explored many species of the genus Fagonia. Likewise, they continued their interest in *F. indica* and isolated two saponins (60). Ethanol extract of *F. indica* prepared using an aerial part was fractionated, and one of the fractions tested positive for saponins' presence by showing precipitation in acetone. A saponin was isolated by repeated chromatography, while a second compound was isolated through Sephadex LH-20 and Biogel P-2. The structure of both saponins was derived through NMR spectroscopy, which gave clues about the presence of triterpenoic acid and sugar moieties. Hydrolyzing these saponins also witnessed the conversion of these compounds into earlier-known nahagenin. After detailed NMR studies and justification of every carbon and hydrogen through 1 and 2-dimensional NMR compounds, they were named saponin A (23,28-di-*O*-*β*-D-glucopyranosyltaraxer-en-28-oic acid) and saponin B (3*β*,28-di-*O*-*β*-D-glucopyranosyl-23-hydroxytaraxer-20-en-28-oic acid). Another interesting experiment was carried out to see the conversion mechanism of aglycone to nahagenin by hydrolyzing in D_2O and $H_2{}^{18}O$. Formed nahagenin was used by this approach as analyzed by mass spectrometry, which revealed that hydrogen isotope D was incorporated, but the isotope of oxygen 18O was not.

Glycosides

An attempt to isolate compounds from two species i.e., *Bassia muricata* and *F. indica*, has been reported (61). The air-dried *F. indica* aerial part (2.5 kg) was soaked in 17 liters of ethanol (85%), and after concentrating on reducing pressure, a 602-gram extract was obtained. After fractionation in organic solvents, the n-butanol fraction (53 out of 102 grams) was subjected to column chromatography. Semi-purified fractions were purified by passing through Sephadex LH-20 and polyamide material. In this way, three compounds were purified. Structure elucidation was done by using mass spectrometry and NMR spectroscopy techniques. Of three first already known compound was identified as flavonoid glycoside; 3-***O***-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]-kaempferol. Second was new triterpenoid saponin; 28-***O***-[β-D-glucopyranosylester-(1→3)-β-D-glucopyranosyl] oleanolic acid. The third was known from other sources: quinovic acid-3-***O***-(α-L-rahmnopyranosyl-)-28-***O***-β-D-glucopyranosly ester. All compounds isolated from Bassia muricata were tested for antioxidant activity following two assays, i.e., DPPH radical scavenging activity and Superoxide anion scavenging activity. DPPH assay was performed by preparing (25, 50, and 100 µg/ml) of each compound, and percent inhibition of DPPH was observed by reduction of its lambda max on the uv spectrophotometer at 520 nm. The first compound (kaempferol derivative) did not show activity at 25, 50 µg/ml, but it inhibited DPPH around 80 % at 100 µg/ml. The same was noted for a new compound that could inhibit DPPH only 30% at 100 µg/ml. The third compound (quinovic acid) was a relatively strong antioxidant that inhibited DPPH around 60% at all concentration levels. Contrary to the DPPH assay, the new compound was more active in superoxide anion scavenging, while both known compounds were moderate.

Terpenoids

F. indica collected in 1996 from Egypt was worked out to isolate terpenoid saponins in Germany, and work was reported in 1999 (Triterpenoid saponins from Fagonia indica). 4 kg whole plant soaked in 80 % methanol and the dried extract was fractionated in water and n-butanol. Later, fractions contained saponins separated on conventional column chromatography, and further purification was done through Sephadex LH-20, followed by running semi-pure fractions on preparative HPLC. In this way, four compounds with weight 7 to 18 mg were isolated. Upon structure elucidation on the excessive NMR technique tool, it was found that two were new compounds, and two were new from F. indica but were already known from other plants. New cmpounds identified as 3-O-{[b-D-glucopyranosyl-(1 4 2)]-[a-L-arabinopyranosyl-(1 4 3)]-a-L-arabinopyranosyl}- ursolic acid-28-O-[b-D-glucopyranosyl] ester (indicasaponin A), 3-O-{[b-D-glucopyranosyl-(1 42)]-[a-L-arabinopyranosyl-(1 43)]- a-L-arabinopyranosyl}-oleanolic acid-28-O-[b-D-glucopyranosyl] ester (indicasaponin B) and known were n triterpenoid saponins, 3- O-[b-D-glucopyranosyl-

(1 4 3)-a-L-arabinopyranosyl]-ursolic acid-28-O-[b-D-glucopyranosyl] ester, 3-O-[b-D-glucopyranosyl-(1 4 3)-a-L-arabinopyranosyl]-oleanolic acid-28-O-[b-D-glucopyranosyl] ester.

Another group of Pakistani scientists worked on *F. indica* to isolate triterpenoids and triterpenoid saponins (62). 5 kg aerial parts were collected from Hyderabad, Sindh, Pakistan, and soaked in methanol, followed by fractionation in an aqueous and n-butanol. 125 g obtained n-Butanol was further separated on conventional column chromatography to separate semi-pure fractions. Semi-pure fractions were purified using reverse phase (C-18) chromatography and kept viewing separation on TLC. A total of 7 compounds were isolated, and the author claimed two new, three already reported from *F. indica,* while the remaining two were reported from other sources but for the first time from *F. indica*. Structure elucidation as carefully carried out by using high resolution electron impact mass spectrometer HREIMS, 1D NMR (1 H, 13C NMR, and DEPT) and 2D NMR (COSY, NOESY, HSQC and HMBC), techniques and names of new compounds were derived as indicacin (3.5. 3-Oxo-12-en-23-O-β-$_D$-glucopyranosyl-27-hydroxyolean-28-oic acid) fagonicin (3 β, 20S-Dihydroxytaraxastane28-al). Both compounds (6.25 to 50 µM/mL) were assayed for bioactivity against colorectal cancer cells following MTT assay. % Inhibition was noted, which was 29.75 to 51.40 by *indicain* and 34.35181 to 48.37 by *fagonicin*, depending on concentration.

Tinospora Malabarica

Comparative studies of species

Species of the genus Tinospora are very close to each other in terms of appearance and physicochemical properties. However, their difference is well defined botanically, and studies are also available on this topic (63). A comparative study was reported comprising quantitative determination of four compounds, including 20β-hydroxyecysone, tinosporaside, cordioside, and columbin, using HPLC-DAD (64). Twenty-seven samples of each species (*T. cordifolia, T. malabrica, and T. crispa.* A) were collected from different locations in India. All four compounds (claiming the markers) were separated through chromatographic column fractionation and then identified on HPLC. The highest number of samples (27) were collected for *T. cordifolia* sps., while seven samples of *T. crispa.* were collected for analysis. Ethanol was used for initial extraction, and the percentage of extract yielded of *T. crispa.* for different locations ranged from 1.302 to 1.503. Individual compounds ranged from 20β-hydroxyecysone (0.02 to 0.117%), Tinosporaside (0.006 to 0.022%), Cordioside (0.002 to 0.016%), and Columbin (0.002 to 0.021%). The author also claimed to validate the developed method as an analytical tool for quantitative analysis of biomarkers of Tinospora species.

A research article reported four species of Tinospora from north to south; however, only two, i.e., *T.cordifolia* (Willd.) Miers. and *T. malabarica* Miers are found in the southern parts (65). Both are found to be active in curing different ailments either by themselves or mixing with other constituents/substances, such as *T. malabarica* Miers mixed with that of *Coleus amboinicus* Lour, and honey is used in the treatment of gonorrhea. However, the alarming situation is a matter of concern because Tinospora malabarica Miers is the major adulterant for Amrita, prepared by local herbal practitioners, who are adulterating without standardization. *T. malabarica* Miers can also be confused with *T. cordifolia (Willd.) Miers* for substitution and tampering purposes. The same is the case in Pakistan, where very close morphological features of *T. crispa* and *T. malabrica* can be deceptive; hence, reviewing both species over here is considered necessary. Since folk practitioners prepare both hot and cold extracts for healing, the difference in constituents is checked in both extracts. Carbohydrates, reducing sugar, monosaccharides, hexose sugars, non-reducing sugars, gums, mucilage, proteins, proteins containing sulfur, amino acids, steroids, glycosides, anthraquinone glycosides, saponins, coumarin glycosides, flavonoids, Alkaloids, tannins, and phenols, organic acids were tested by related standard reported methods in hot and cold extracts. All extracts, including petroleum ether chloroform acetone ethanol-water hot extract, were rich in positive compounds in both species. Another study is also reported, in which similarly the misuse of two identical species is discussed, quoting *Amrita* again (66).

It is claimed in the review that several species of the genus Tinospora grow in different parts of Asia and Africa and are well-known for their medicinal properties (67). Consequently, they have been investigated extensively over the last four decades. The related scientific work was briefly reviewed pertinent to the chemical

constituents found in these species and the biological activities reported from time to time till 1995, including the five species, i.e., *T. cordifolia, T .crispa, T.tuberculata, T .malabarica, T. capillipes* (68).

Antiulcer Activity

Petroleum ether, alcohol, and aqueous extract of *T. malabarica* were prepared, and flavonoids, carbohydrates, and amino acids in aqueous extract, flavonoids, alkaloids, carbohydrates, and steroids were known through initial phytochemical screening. Before doing an anti-ulcer activity that requires oral administration of extracts, an acute oral toxicity study was also carried out to understand the safe dose level, which was found to be up to 2000 to 5000 mg/kg for petroleum ether and aqueous alcohol extracts, respectively. Rats weighing 150 to 170 grams (mixed sex) were first starved, and then access water and libitum were given to cause ulcers. Then, all animals were kept individually to prevent attacking each other from being too hungry. After that, five groups, each containing six animals, were divided. Tween 80, standard drugs, i.e., ranitidine (20 mg/kg), aqueous, alcohol, and petroleum ether extract (500 mg/kg) were administered orally. The stomach was obtained by dissection in anesthesia and observed for ulcer spots. Besides that, another way to give ulcers was also tried, which was done by forces swimming and being hungry. Once the process was completed, the same dose was given, and again, swimming was forcefully done by animals. As per results, it was observed that in the first method of causing ulcers, the mean ulcer index of control (no treatment) was found to be 4.25±1.04, which was controlled by the drug ranitidine very well, and only 0.5±0.22 was observed. Aqueous extract could reduce ulcer index to 0.75±0.40 while alcohol and petroleum ether did 0.83±0.10 and 1.17±0.28, respectively. pH was also checked, which was very acidic in control 1.33±0.33. However, the aqueous extract was better in pH 5.16±1.04 followed by ranitidine 4.83±0.74, alcohol 4.66±0.47 and petroleum ether extract 3.66±0.95. In the second model (water immersion Stress-induced ulcer), the ulcer index was 4.67±0.494 which was cured by aqueous extract even better (0.67±0.210) than ranitidine (1.33±0.333) and no significant cure by alcohol and petroleum ether extracts. Overall protection was also very good in animal groups treated with aqueous extract, almost near the standard ranitidine drug. Researchers also believed that an aqueous extract of *T. malabrica* is best for anti-ulcer activity while suggesting that others isolate compounds responsible for this activity.

Phytochemical analysis and adaptogenic activity

Authors of this study have claimed that advanced studies, as compared to previous studies, which was based on the evaluation of the effect of *T. malabarica* and other compounds/elements (separately) on stress (induced on rats) (69, 70). A later study was carried out to examine the adaptogenic (stress-relieving) effects of different extracts (petroleum ether, alcohol, and water) from the stem of *T. malabarica* (Lamk.). The extracts were initially tested for their chemical components and oral toxicity. Then, their adaptogenic effects were tested using animal models, including mice and rats exposed to stress through three different conditions, i.e., lack of oxygen, forced swimming, and cold conditions. The chemical tests showed that the water extract contained flavonoids, carbohydrates, and amino acids, the alcohol extract had flavonoids, alkaloids, and carbohydrates, while the petroleum ether extract only contained steroids. The water and alcohol extracts were found to be non-toxic up to a dose of 5000 mg/kg, while the petroleum ether extract was safe up to 2000 mg/kg. Stress from forced swimming and cold temperatures changed various biological indicators in the animals, such as blood glucose, cholesterol, triglycerides, BUN, cortisol, and blood cell counts. The stress also affected the size of organs like the liver, spleen, and adrenal glands. Treatment with the water and alcohol extracts of *T. malabarica* helped reduce the elevated stress levels in the blood. It prevented changes in the size of the liver and adrenal glands while increasing the size of the spleen. In contrast to water and alcohol extracts, petroleum ether extract did not show any adaptogenic activity, proving that non-polar compounds of T. malabarica are not responsible for the activity; only polar compounds played the role. It was concluded that *T. malabarica* showed adaptogenic activity by reducing stress-related changes in blood chemistry and organ sizes.

ISOLATION OF AN ANTI-INFLAMMATORY COMPOUND.

Ethyl acetate extract of *T. malabrica* (50g) was prepared and processed for column chromatography (71). The column was run by elution of mobile phase Toulene and ethyl acetate starting with 8:2 to 4:6. The eighth fraction was treated with charcoal to get a pure compound, which was found to be steroidal saponin diosgenin through UV spectrophotometer, ^{1}H NMR, ^{13}C NMR and Mass spectrometry. The purified compound was tested for its anti-inflammatory activity on an animal model. 5 groups of animals (quantity in each is not provided) were given acute inflammation by up. In all groups, acute inflammation was produced by sub-plantar injection of 0.1ml of freshly prepared 1% carrageenan suspension in normal saline in the rats' right hind paws. Paw volume was measured plethysommetrically at 0 to 180 mins. The first group was controlled and only got normal saline, while groups II, III, IV, and IV were given diosgenin 100, 200, 400, and 100 µg/kg, respectively. Inhibition after 180 min was up to 82% at a 400 µg/kg dose. It was concluded that the isolated compound is an anti-inflammatory agent and a search for other compounds was also suggested.

ISOLATION OF DITERPENOIDAL CONSTITUENTS.

Isolation of two new furanoid diterpenes, malabarolide Br (1) and menispermacide, has been reported from the stems of *T. malabarica*. Stems of *T. malabarica* (120 kg) were extracted with Ethanol and obtained from crude gum (400 g) (72). The material was then subjected to treatment with HCl to remove basic materials. Forty-five grams of the neutral fraction was separated on column chromatography. The column was run by eluting mobile phase (Methanol and chloroform). A fraction obtained while the ratio of both solvents was 3:97 was evaporated and recrystallized from ether to get yellow needles of a pure compound, which was then confirmed by spectroscopic techniques.

The same group of researchers reported another paper, a new furanoid diterpene, ten g-hydroxycolumbin, was isolated from the fresh stems of *T. malaburica* (73). The procedure till column chromatography was the same. Still, the compound was isolated while the column was run with petrol and acetone (1:1), and the compound was purified from ***a*** fraction using preparative TLC. The structure of furanoid diterpene was established based on spectral studies.

DETECTION OF STEROIDS

Isolation of steroids has been reported from *T. malabarica*. However, this study is contrary to a later report (74, 75) in which the Salkowski test method was used to confirm the presence of steroids, and all extracts, including water and alcohol (cold and hot) and steroids, were not detected. Nevertheless, it is possible to accept both reports as accurate due to differences in the region from which plants were selected, and the extraction of steroids from this plant could only be made possible by hydro-alcoholic solvent instead of alcohol or water. Separation was carried out via TLC, and classical test methods were used to confirm the presence of steroids.

Alkaloids

To isolate the alkaloidal constituents, scientists from three countries collected *T. crispa* from a botanical garden in Malaysia (76). The plant was soaked in methanol, and the extract (284 g) was partitioned by fractionation in organic and aqueous solvents. N-butanol fraction was chosen for further work and ran on Diaion HP -20 resin column to elute using water-methanol as mobile phase. Monitoring of TLC helped to separate the different fractions while combining them, and chromatography was continued for better separation. Further purification was done through Sephadex LH-20 and recycling HPLC. A total of ten compounds were purified, and one was found to be new, which was characterized by spectroscopic techniques, and its name was N-formylasimilobine 2-O-β-$_{D}$-glucopyranoside (Alkaloid).

Furanoditerpenoids

Several cis-clerodane-type Furanoditerpenoids have been isolated from *T. crispa*, and bioactivity studies have been performed on them (77). To do so, *T. crispa* was collected from the Laboratory of Natural Products, University of Putra Malaysia (UPM) 's herbal garden in May 2003. Fractionation was the same as described (76). The same work led to the isolation and purification of 13 compounds, including nine new compounds.

As per the trend structure, elucidation was carried out using spectroscopic techniques. Compound 1 was a gummy solid that was identified as (2R,5R,6R,8S,9S,10S,12S)-15,16-epoxy-2-hydroxy-6-O-{-D-glucopyranosyl-(1f6)-R-D-xylopyranosyl}-cleroda3,13(16),14-trien-17,12-olid-18-oic acid methyl ester. Compound 2 was also a gummy solid and identified as (2R,5R,6R,8R,9S,10S,12S)- 15,16-epoxy-2-hydroxy-6-O-(-D-glucopyranosyl)-cleroda3,13(16),14-trien-17,12-olid-18-oic acid methyl ester. Compound 3 was identified as (5R,6R,8S,9R,10R,12S)- 15,16-epoxy-2-oxo-6-O-(-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oic acid methyl ester. Compound 4 (2R,7S,8S)-8-[(2S)-2-(3,4-dihydroxy2,5-dimethoxytetrahydro-3-furanyl)-2-hydroxyethyl] -2,8-dimethyl 10-oxo-11-oxatricyclo [7.2.1.02,7] dodec-3-ene-3-carboxylate and named, trivially, rumphiol E. Compound 5 (5R,6R,8S,9R,10S,12S) -15,16-epoxy-2-oxo-6-O- (-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oic acid methyl ester. Compound 6 was a colorless gummy material and identified as (2R,5R,6S,9S,10S,12S)-15,16-epoxy-2-hydroxy-6-O-(-D-glucopyranosyl)-cleroda-3,7,13(16),14-tetraen-17,12-olid-18-oic acid methyl ester. Compound 7 (5R,6S,9S,10S,12S)-15,16-epoxy-2-oxo-6-O-(-D-glucopyranosyl)-cleroda-3,7,13(16),14-tetraen-17,12-olid-18-oic acid methyl ester. Compound 8 (3R,4R,5R,6S,8R,9S,10S,12S)-15,16- epoxy-3,4-epoxy-6-O-(-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oic acid methyl ester. (1R,4S, 5R,8S,9R,10S,12S)-15,16-epoxy-4-O-(-D-glucopyranosyl)-cleroda2,13(16),14-triene-17(12), 18(1)-diolide. Known compounds were assayed for cytotoxicity activity on human prostate cancer cells, but all were active following previous reports (78-79).

Anti-proliferative and Antioxidant Effects

Water, methanol, and chloroform extracts of stems of *T. crispa* (collected from a village in Chenor, Pahang, Malaysia) were prepared for cytotoxicity testing and antioxidant determination (80). Before bioactivity studies, total phenols and total flavonoids were also calculated using previously known authentic methods. Total Phenolic content (mg Gallic Acid Equivalent/ g sample) was 79.00 ± 10.00, 255.33 ± 10.79, and 172.33 ± 22.30 in Water, methanol and chloroform extracts, respectively. Total flavonoids (mg Quercetin Equivalent/ g sample) were 2.67 ± 0.15, 9.53 ± 0.50, and 5.38 ± 0.06 in Water, methanol and chloroform extracts, respectively. Cell culture and cytotoxicity tests were performed using MTT assay. MCF-7, MDA-MB-231, HeLa, and 3T3 cell lines were used for the activity, and cell viability was checked after inducing *T. crispa* extracts. MCF-7 cell viability on various *T. crispa* extracts (water, methanol, and chloroform) and tamoxifen as control. A decrease of cell viability in dose dependent manner was observed and the IC_{50} were 42.75 ± 4.61 µg/ml, 33.75 ± 4.65 µg/ml and 38.90 ± 3.21 µg/ml for water, methanol and chloroform extracts, respectively. MDA-MB-231 cell viability decreased dose-dependent manner where the IC_{50} were 46.88 ± 1.75 µg/ml, 44.83 ± 1.21 µg/ml and 51.25 ± 3.62 µg/ml for water, methanol and chloroform extracts, respectively. There was linear correlation between total phenolics and cytotoxicity activity and methanol was on top among all three extract regarding both.

Activities Against Malerial Diseases

Plasmodium malaria caused hypoglycemia which is often observed as confirmation tool of severe malaria. A study on ability of *T. crispa* stem extract to prevent hypoglycemia induced by *P. berghei* infection is carried out (81). Stems of *T. crispa* were collected from Kanchanaburi province, Thailand. Ethanol extract from a 300-gram dry powdered plant was prepared, and doses were prepared by mixing tween-80. Mice were infected with 1×10^7 parasitized erythrocytes of *P. berghei* by intraperitoneal injection. Monitoring was continued until work started to administer extracts orally and observe the effect at concentrations 50, 100, and 200 mg/kg. It was observed that a prominent effect of *T. crispa* extracts was observed at 100 and 200 mg/kg at parasitemia count. Based on the results, the authors claimed T. crispa is a potent agent for an anti-hypoglycemic to be a potential medicinal remedy for malaria treatment with the least toxicity. However, the mechanism of action is still not precise and needs to be carefully studied.

Malaria causes renal damage and hemolysis, thereby leading to an increased chance of mortality in children; hence, the protective effects of *T. crispa* stem extract against these are evaluated (82). Plant material (stem parts) was collected from March to April and grown in Suphanburi province, Thailand. Plant material was

dried at 60 °C and soaked in water to prepare the aqueous extract, and further doses were also prepared in water. Pathogen-free female mice were used for experiments. Plasmodium berghei parasite strains were used to parasitize mice by intraperitoneal passage. Parasitemia was confirmed by monitoring two tools, i.e., examination of Giemsa stained thin blood smear and hematocrit %. Blood urea nitrogen and creatinine were used as indicators of renal function for assessment of renal function test. After two hours of inducing infection and monitoring, mice were orally administered T. crispa extract doses at 500, 1000, and 2000 mg/kg. Oral administration was continued continuously after 24 hours, two times, for four days. Blood urea nitrogen and creatinine increased after inducing infection and kept increasing administered with extract till 500 mg/kg dose compared to control. However, both values decreased in mice given doses of extract at 1000 and 2000 mg/kg. It was observed that extracts of *T. crispa* could considerably protect mice from renal damage and hemolysis. After other needful studies, the author suggested these extracts as possible sources of alternative antimalarial drug raw material.

HPLC METHOD DEVELOPMENT

Many medicinal plant species possess essential compounds that are present in varying quantities whenever the plant is grown and ready for use in the formulation of therapeutics. However, different factors affect their quantities; hence, using plants without carrying out quality control may either decrease the chance of efficacy if compound quantity is reduced for any reason or may be harmful if quantities abruptly increase due to external factors. Hence, analytical chemists continue to develop some protocols for the quality control of drugs.

In the above scenario, the study was carried out to develop an HPLC analytical method for the quantitative determination of four biomarkers, i.e., 20*β*-hydroxy ecdysone, Tinosporaside, Cordioside, and Columbin, which have long been detected in different species of Tinspora (83). Bulk quantities of four species of Tinospora (including crispa) were collected from various regions of India (including Jammu and Kashmir), and ethanol extract was prepared, which was further fractionated in organic solvents and water. N-butanol fraction was selected for further work, and 4 compound were purified through column chromatography and structure was elucidated by getting NMR spectra and comparing with literature. For HPLC analysis ethanol extract of 100 mg of each plant material was prepared in water–methanol 2 mL in equal ratio. Compounds earlier isolated in this study were used as reference standard and solution were prepared by mixing all four compounds ranging from 10 to 200 ng/µL to draw calibration curves. RP-18 column was used for separation and elution was monitored on HPLC detector set at 215 nm. The analysis was carried out using a gradient of water–acetonitrile initially at 90:10, changing to 0:100 in 40 min, followed by isocratic elution at 0:100 for 5 min, changing to 90:10 in 10 min, and, finally, it was an isocratic elution at 90:10 for 5 min. The total analysis time was 60 min and mobile was passed at the rate of 1 mL per minute. All compound were well separated and could easily be detected in sample and developed method was applicable to any plant species of this genus or else which could be possessing these biomarkers. A good quality control protocol for these four biomarkers was added in literature. Since *T. crispa* has been used as medicine to treat t diabetes mellitus in Malysia hence authors tried to detect active constituents of this species and evaluate their potential to inhibit α-Glucosidase and α-amylase which support digestion of dietary starch leading to increase blood sugar level (84). 5.0 Kg of *T. crispa* vines were collected in Bentong, Pahang, Malayisa and defatted by using hexane. Defatted plant was extracted in methanol water 4:1 and obtained extract was further treated in acidic and basic medium to separate and get alkaloidal part in methanol which was analyzed on UPLC-QToF/MS and many alkaloids were detected. 11 compounds were isolated in this study through column chromatography and structures were elucidated by mass spectrometry and NMR data. Identified compounds are Borapetoside C (1) 4-Hydroxybenzaldehyde (2) β-Sitosterol (3) Liriodenine (4) NA Lysicamine (5) Dihydrodiscretamine± 0.012 Columbamine (7) Magnoflorine (8) N-Formylannonaine (9) N-Formylnornuciferine (10) N-trans-Feruloyltyramine (11). All were checked for – α-Glucosidase and α-amylase inhibitory activity, which was considerably shown by all compounds except Liriodenine, which could only inhibit α-Glucosidase but not α-amylase. However, none of the compounds could exhibit enzymes equal to Acarbose, which was used as a control.

Rearranged Clerodane Diterpenoid

A new rearranged clerodane diterpenoid, tinocrispide, was isolated from the stems of Tinospora crispa along with thirteen known compounds, including eight clerodane diterpenoids. Among the known compounds, baenzigeride A, (6S, 9 R)-vomifoliol and steponine are being reported for the first time from *T. crispa*. Their structures were elucidated by 1 D and 2 D NMR and confirmed by HRESIMS. The 13C NMR data of borapetol A has been revised (85). 2kg *T. crispa* was collected from Thailand and soaked in 8-liter methanol, and a 110-gram residue was obtained. The extract was fractionated in different organic solvents. Hexane extract was loaded on column and chromatography was carried out till purification of compounds through Sephadex LH-20. New compound (Tinocrispide) white amorphous powder which was identified on the basis of IR, HRESIMS, ^{1}HNMR, ^{13}CNMR.

PREPARATION METHOD OF CUO NANOPARTICLES

Considering the importance of Copper oxide CuO nanoparticles in different sectors, an attempt for simple CuO NP preparation with focus on economical and environmentally friendly green method was reported for the first time using *T. crispa* leaves extract (86). *T. crispa* leaves were collected from Balitro, Bogor, West Java, Indonesia, dried and powdered. Water extract was prepared and used for synthesis of nanoparticles by adding into $Cu(NO_3)_2$ solution and stirred to obtain $Cu(OH)_2$. CuO was formed at 450 °C detected with presence of dark blue powders. Further confirmation as done using FT-IR and UV-Vis spectrophotometer and XRD pattern and found that nanoparticles were spherical in shape and particle size range of 10–40 nm.

Conclusion

All three species are very important and work done/reported so far is sufficient to know their importance and safe use. However, there is still a big room for work on different aspects such as isolation of new bioactive compounds, safety doses and systemic bioactivity work. It would lead to develop quality control protocol for getting maximum benefits of these species which can be choice in herbal medicine sector.

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