Comparative studies directed towards the bioactivity evaluation of *Iris* species for prospective resourcing as therapeutic agents

DISSERTATION

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BY

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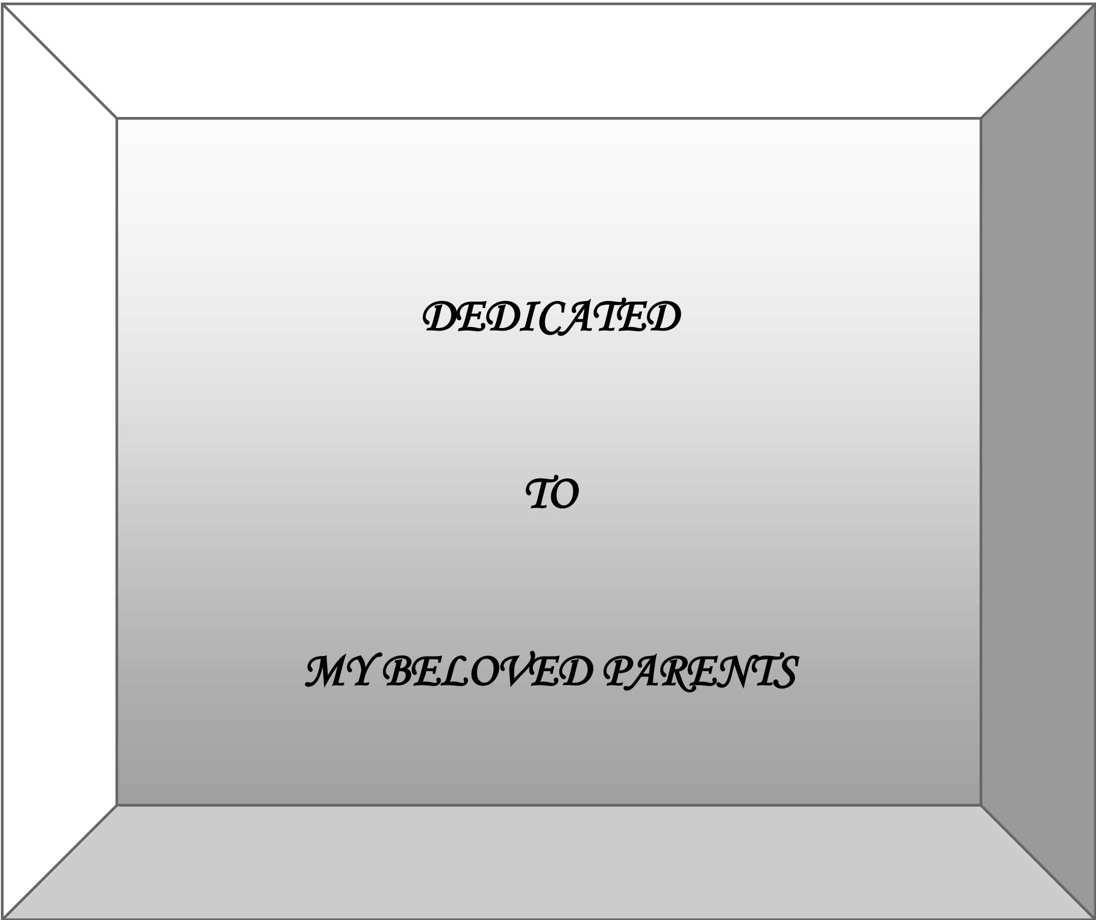
DEDICATED
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MY BELOVED PARENTS

Department of Chemistry,
University of Kashmir, Srinagar-190 006

Certificate

This is to certify that the work presented in this dissertation entitled **Comparative studies directed towards the bioactivity evaluation of *Iris* species for prospective resourcing as therapeutic agents** *is original and has been carried out by* ***Ms Soubiya Mukhtar Buchh*** *under my supervision. This work is suitable for submission for the award of M.Phil Degree in Chemistry. It is further certified that the work has not been submitted in part or full for award of any degree in this or any other University.*

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Declaration

I hereby declare that the work incorporated in this dissertation entitled Comparative studies directed towards the bioactivity evaluation of *Iris* species for prospective resourcing as therapeutic agents under the supervision of Prof. M. A. Qurishi submitted for Mphil degree was carried out by me in the Department of Chemistry, University of Kashmir, Srinagar-190006, J&K. The entire work or any part of it has never been submitted before for any prize or degree anywhere.

Dated:

Soubiya Mukhtar

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Soubiya Mukhtar

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Chapter 1

Introduction

1.1.NATURAL PRODUCTS AND DRUG DISCOVERY

Nature has always been a valuable source of drugs and continues to deliver lead compounds despite the unprecedented opportunities afforded by the medicinal chemistry. In the form of its vast repository (phyto-diversity), nature has provided a complete store house of remedies to cure most of the ailments of mankind. It is estimated that less than 10% of this world's biodiversity has been studied seriously as source of medicines[1]. Yet, from this small fraction, humanity has reaped enormous benefits in the form of bioactive natural products that span all the way from small molecules such as the toxin responsible for Dogger Bank itch[2] to complex, polycyclic compounds such as paclitaxel.

We are grateful to the plant kingdom for such useful drugs like vinblastine and vincristine from the African periwinkle, *Caratharanthus roseus* used for the treatment of pediatric leukemia and Hodgkin's disease[3]. Despite competition from other drug discovery methods, Natural Products (NPs) are still providing their fair share of new clinical candidates and drugs especially in the anticancer and antihypertensive therapeutic areas[4].

NP-derived drugs are well represented in the top 35 worldwide selling ethical drug sales of 2004, 2005 and 2006[5].

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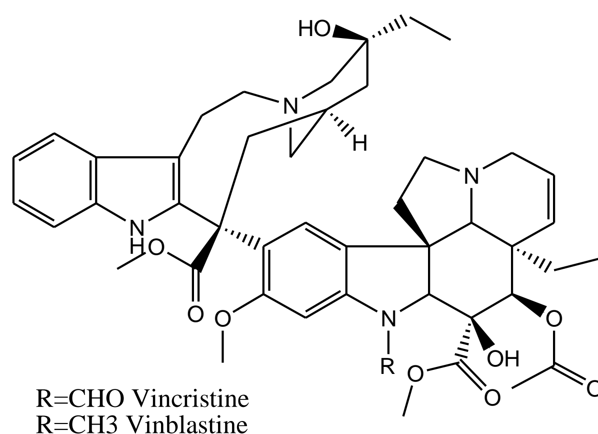
1 Verporte R. **1998** *Drug Discovery Today*, 3:232.

2 Charle JS Christophersen C. **1980** *J. Am. Chem. Soc.*, 102:5108.

3 Balick MJ, Cox PA. **1996** *In Plants, people and Culture: The Science of Botany*. Scientific American Library: New York, p-25.

4 Newman DJ, Cragg GM, Snader KM. **2003** *J. Nat. Prod.*, 66:1022.

5 Newman DJ, Cragg GM, Snader KM. **2004** *J. Nat. Prod.*, 67:1216.



In addition to the historical success in drug discovery, natural products are likely to continue to be source of new commercial viable drug leads. The chemical novelty associated with natural products is higher than that of any other source. Additionally, natural products that are biologically active in assays are generally small molecules with drug like properties. That is, they are capable of being absorbed and metabolized by the body. Finally it should not remain unmentioned that the 'Natural' aspect or origin of pharmaceuticals often leads to an enhanced consumer acceptance. A remarkable galaxy of pure compounds with different pharmacological activities has been isolated from natural sources.[6,7,8] This collection has also proved to be at once-teasing and mind expanding in its power to provoke the imagination of organic and medicinal chemist. A sampling of some of these natural products including compounds of historic interest and some of particularly novel structures is displayed in figure 1.1.

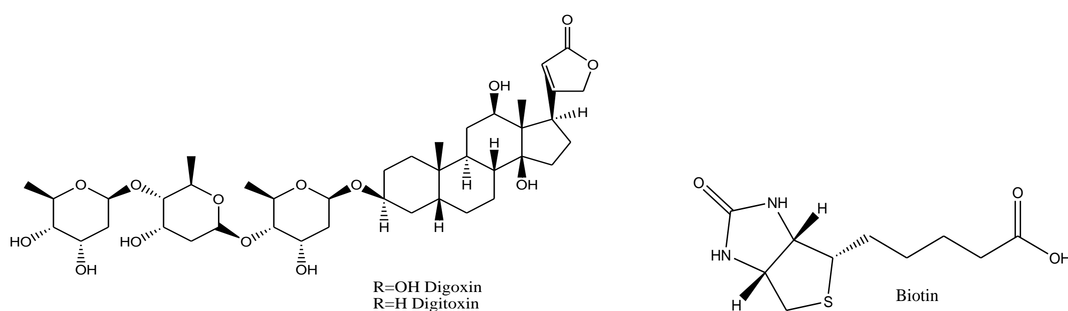


Fig.1.1. Structures of some representative natural product drugs.

[6] Butler MS. J. Nat. Prod. **2004** 67:2141. *J. Nat. Prod.*, 67:2141.

[7] Wilson RM, Danishefsky SJ. J. **2006** *Org. Chem.*, 71:8329.

[8] Newman DJ, Cragg GM, Snader KM. **1997** *J. Nat. Prod.*, 60:52.

1.2. FOLKLORE APPROACH TO PHARMACUETICAL DRUG DISCOVERY

Historically, ethno pharmacology was the origin of all medicines and plant products were the most important sources of drugs. Use of plants in the traditional systems of medicine dating back to empires of Mesopotamia, Egypt, Greece, Rome, China, India and many other cultures[9] has been extensively documented. This knowledge of drugs has accumulated over thousands of years as a result of mans inquisitive nature, so that today we possess many effective means of ensuring health care. In pre-industrialized society and in Agrarian society, plant-derived natural products were used by indigenous population as therapies for many diseases ranging from infections to emphysema. On numerous occasions, the folklore records of many different cultures have provided leads to plants with useful medicinal properties.[10, 11] Clinical, pharmacological and chemical studies of these traditional medicines, which were derived pre dominantly from plants were the basis of most early medicine such as aspirin, morphine, quinine, pilocarpine and digitoxin.

Aspirin is a powerful synthetic drug that is used to treat a wide variety of ailments mostly as an anti-inflammatory drug and pain reliever. The natural product that provides the basis for aspirin is salicylic acid, which is isolated from the bark of the willow tree.[12] Use of the willow tree for medicinal purposes dates back nearly 2500 years to the time of the ancient Mediterranean empires.[13]

One of the side effects of salicylic acid is gastric discomfort and irritation, so the acetyl derivative of salicylic acid (acetyl salicylic acid or aspirin) is used clinically to partially reduce the side effects.[13] Aspirin also functions as an important preventative treatment against heart disease because of its inhibition of prostaglandins, which affect the clotting of blood.[14]

Codeine and morphine are two other well known and often prescribed analgesics. Both of these similarly structured alcholoides come from unripened seed pods of the opium poppy plant. Use of morphine as a drug dates back many centuries to a time

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[9] Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. **1985** *In Medicinal Plants in Therapy. Bull*. WHO, 63:965

[10] Swain T. **1972** In *Plantsin the Development of Modern Medicine*. Havard University Press: Cambridge, Ma.

[11] Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH. **1985** *Science*, 228:1154.

[12] Swerdlow JL. **2000** *Nature's Medicine: Plants that Heal*. National Geographic Society: Washington, D.C.

[13] Grabley S, Thiericke R. **1999.** The impact of natural products on drug discovery. In *Drug Discovery from Nature*;Grabley S, Thiericke R, Eds. Springer: Berlin/Heidelberg.

[14] Dewick PM. **1997** *Medicinal Natural Products: A Biosynthetic Approach*. John Wiley & Sons Ltd.: Chichester.

when monks saw an anesthetic and pain relieving properties of *Papaver somniferum*. While codeine is not nearly as affective in its pain relieving abilities as morphine, it also can be used as cough suppressant and it is a considerably less addictive drug, producing fewer affects of euphoria as compared to narcotic cousin.[14]Inspite of side effects and the possibility of addiction, morphine remains one of the most powerful and effective medicines for intense pain in clinical situations, an advantage that cannot be matched by any human made compound.[15]

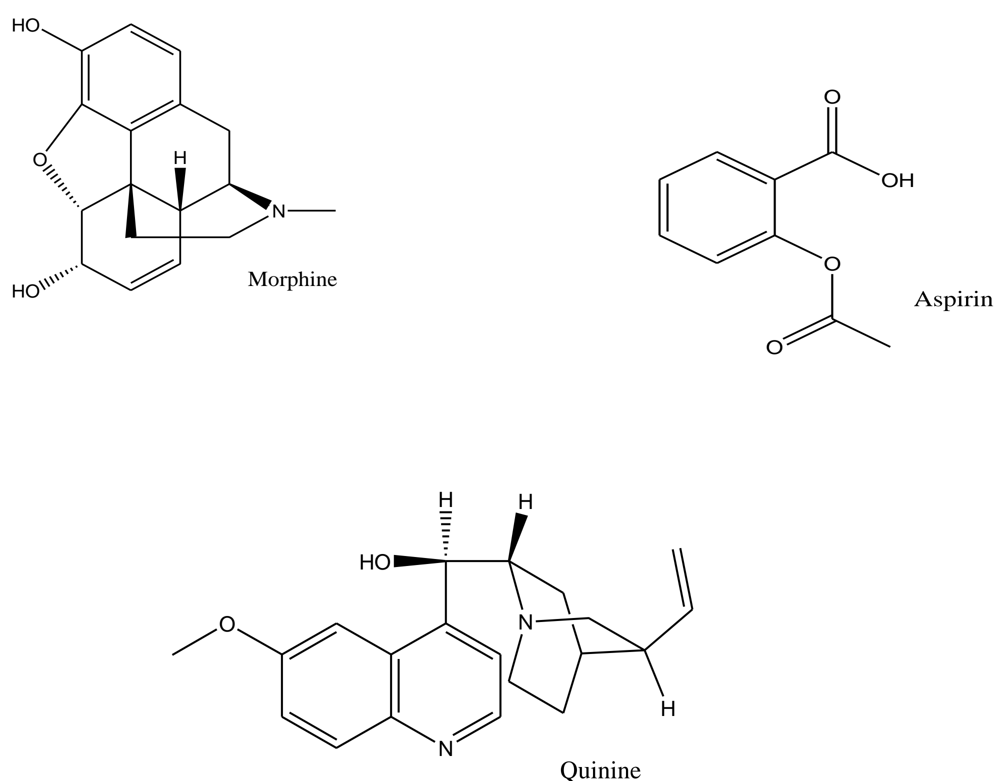
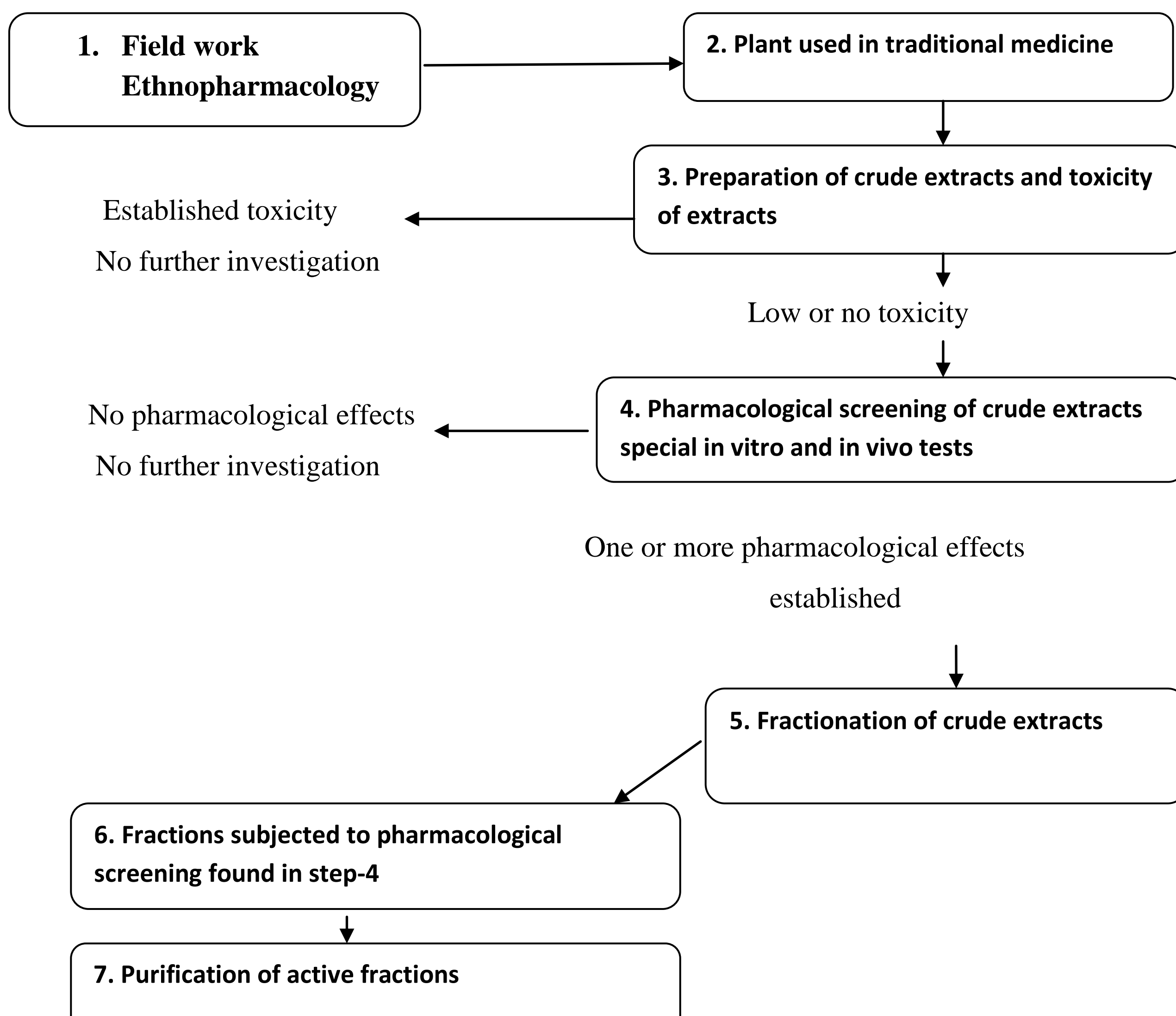


Fig. 1.2. Structures of some representative traditional drugs derived from plants.

[15] Harry L, Brielmann **1999** *J. Phytochemicals: The Chemical Components of Plants*. CRC Press: Boca Raton, FL.

Ethnopharmacological approach for obtaining active principles from medicinal plants

It is often claimed that using similar ethno pharmacological information will greatly increase the chance of discovering novel chemical entities having biological activities that may be useful in the quest for finding drugs with greater efficacy and specificity for the treatment of many human diseases. The approach adopted for such a purpose is shown in scheme 1.1.



1.3. CURRENT STATUS OF NATURAL PRODUCT RESEARCH

Given that NPs have historically provided many novel drug leads, one would assume that NPs would still play a pivotal role in the drug discovery strategy of Big Pharma. However, most Big Pharma companies have terminated or significantly scaled down screening natural resource collections because they are not thought to fit into modern High Throughput Screening (HTS) strategies and with the expectation of reaping rich dividends in terms of a multiplicity of novel drugs by screening compounds produced Combinatorial Libraries. The impending structure of the human genome and the promise of a plethora of new targets added to the excitement of the time. The basic premise was the combinatorial chemistry would generate libraries consisting of millions of compounds, which would be screened by HTS and produce drug leads by sheer weight of numbers. The leads would be delivered in quicker time and in greater numbers for all therapeutic areas compared to traditional drug discovery methods, and as a consequence, it was not surprising tha NP research was often assigned a lower priority. The expected surge in productivity, however, has not materialized. Despite this puzzling (and seemingly disastrous) decision to significantly downplay the role of NPs in medicinal research in favour of far less validated discovery platform, a disproportionate number of new chemical entities approved even over past 10 years have in fact been natural products or natural product-based.[16] The impact of natural products on drug development can be felt across virtually every major therapeutic area. Moreover, NP derived drugs are well presented in the top 35 world wide selling ethical drug scales of 2000 to 2007. A total of 3116 new drugs having natural origin were launched from January 2000 to March 2007. These include new drug types such as antimalarials, antibiotics, anticancer drugs and other therapeutic areas where lead compounds from synthetic libraries are lacking while as natural products have proven their worth.

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[16] Newman DJ, Cragg GM. 2007 J. Nat. Prod., 70:461.

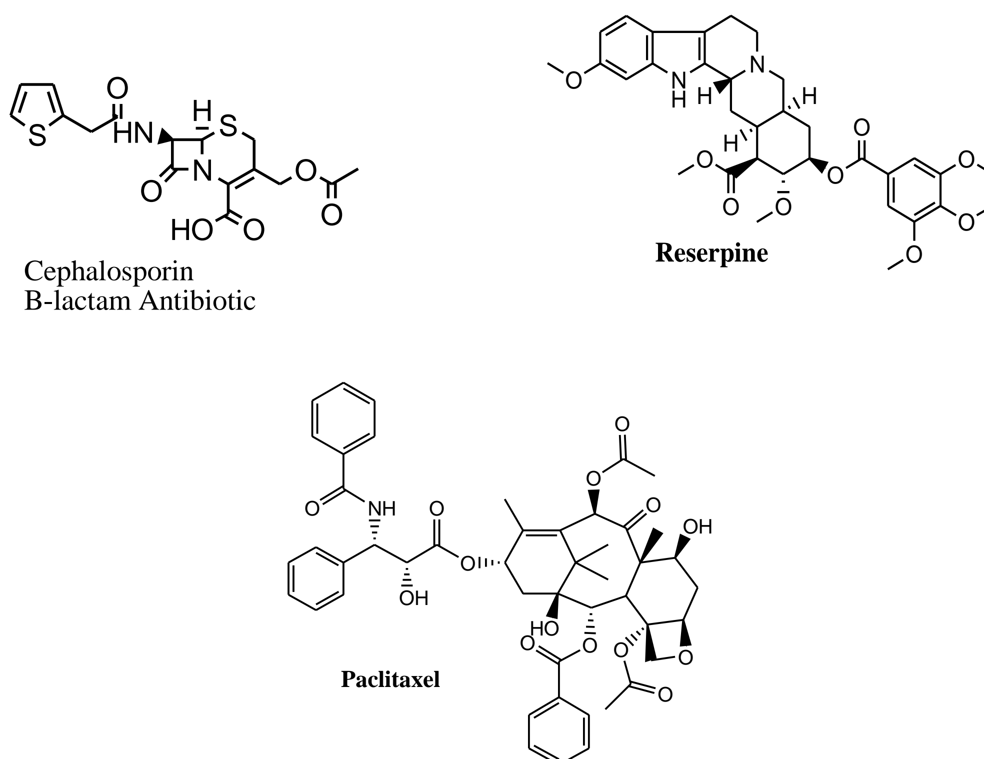

Fig.1.3. Some representative drugs derived from natural products.

1.4. FLAVONOIDS- AN OVERVIEW

Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine[17]. These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves[18]. Research on flavonoids received an added impulse with the discovery of the French paradox, i.e. the low cardiovascular mortality rate observed in Mediterranean populations in association with red wine consumption and a high saturated fat intake.

[17] Middleton EJ **1998** Effect of plant flavonoids on immune and inflammatory cell function. *AdvExp Med Biology* 439:175-182.

[18] de Groot, H., Reun U **1998** Tissue injury by reactive oxygen species and the protective effects of flavonoids. *FundamClin Pharmacology* 12: 249-255.

The flavonoids in red wine are responsible, at least in part, for this effect[19]. Furthermore, epidemiologic studies suggest a protective role of dietary flavonoids against coronary heart disease[18]. The association between flavonoid intake and the long term effects on mortality was studied subsequently[20] and it was suggested that flavonoid intake is inversely correlated with mortality due to coronary heart disease[21]. Until 50 years ago, information on the working mechanisms of flavonoids was scarce. However, it has been widely known for centuries that derivatives of plant origin possess a broad spectrum of biological activity[22]. In 1930 a new substance was isolated from oranges, which is believed to be a member of a new class of vitamins, and was designated as vitamin P. When it became clear that this substance was a flavonoid (rutin), a flurry of research began in an attempt to isolate the various individual flavonoids and to study the mechanism by which flavonoids act.

The flavones characterized by a planar structure because of a double bond in the central aromatic ring of flavonoids. One of the best described flavonoids, quercetin, is a member of this group. Quercetin is found in abundance in onions, apples, broccoli, and berries. The second group is the flavanones, which are mainly found in citrus fruit. An example of a flavonoid of this group is narigin.

Flavonoids belonging to the catechins are mainly found in green and black tea and in red wine[23], whereas anthocyanins are found in strawberries and other berries, grapes, wine, and tea. An important effect of flavonoids is the scavenging of oxygen-derived

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[19] Formica J V Regelson W **1995**Review of the biology of Quercitin and related biflavonoids. *Food Chem Toxicology*. 33:1061-1080.

[20] Hertog M G Kromhout D Aravanis C **1995** Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch Intern Medicine*. 155:381-386.

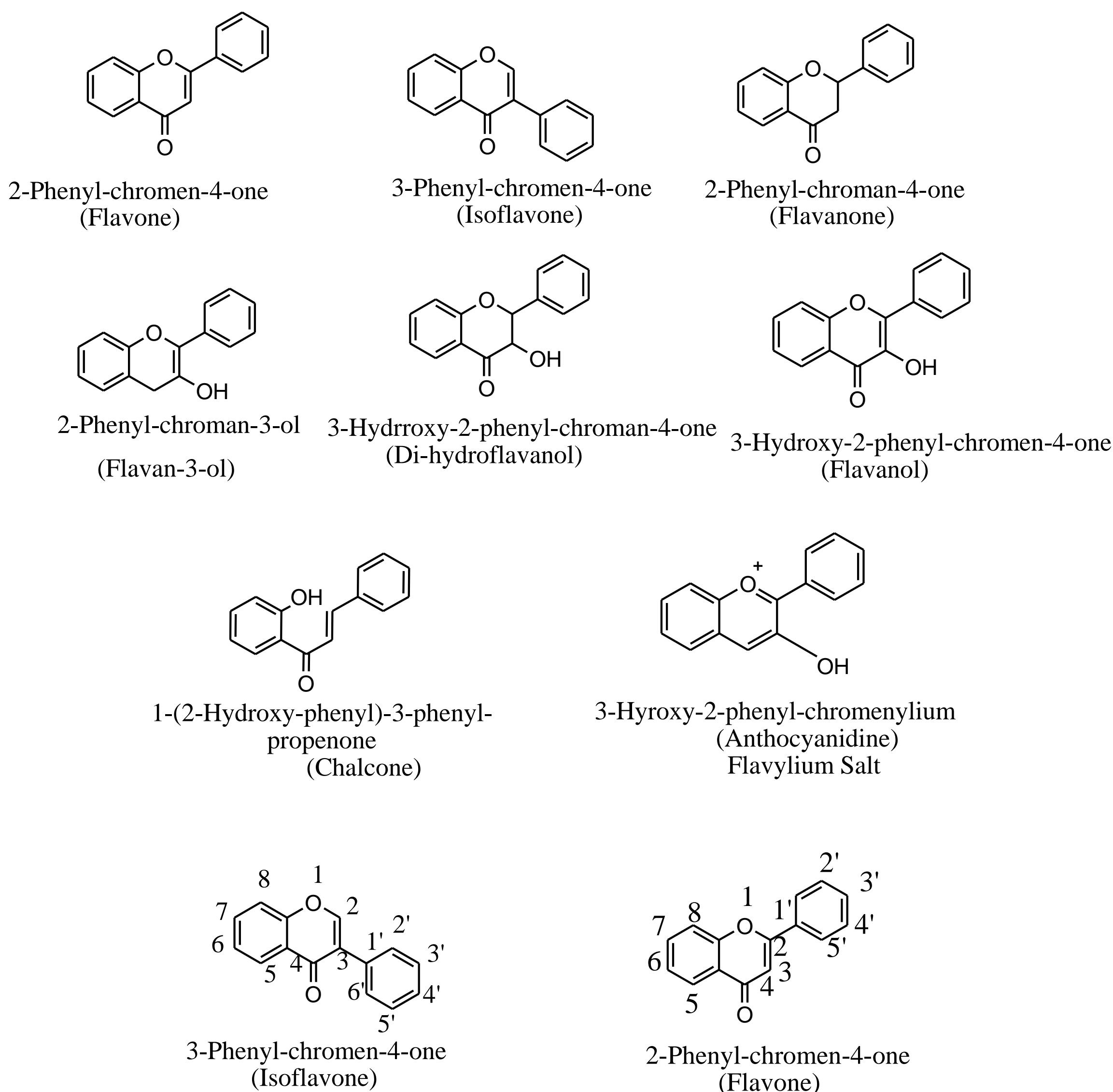
[21] Knekt P JarveninRuenanen A Mateela J **1996** Flavonoid intake and mortality rate in finland:A cohort study. *BMJ* 312: 478-481.

[22] Robak J Gryglewisky R J **1996** Bioactivity of flavonoids*Pol J Pharmacol*. 48: 555-54.

[23] Hollman, P.C., Katan, M.B**. 1999**. Dietary flavonoids: intake, health effects and bioavailability. *Food ChemToxicol*. **37**: 937–942.

free radicals. In vitro experimental systems also showed that flavonoids possess anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties[24].

Flavonoids can be divided into various classes on the basis of their molecular structure[25]. The molecular structure of each group of flavonoids is given under:



[24] Hollman, P.C., van Trijp, J.M., Mengelers, M.J., de Vries, J.H., Katan, M.B**. 1997**. Bioavailability of the dietary antioxidant flavonol quercetin in man. *Cancer Lett*. **114**: 139–140.

[25]Piskula, M.K., Terao, J. **1998**. Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr*. **128**: 1172–1178.

Intake: The average daily flavonoid intake is estimated to be 23 mg/d[26]. Intake of flavonoids exceed those of vitamin E and β-carotene, whereas the average intake of vitamin C is 3 times higher than the intake of flavonoids. Flavonoid intake seems to vary greatly between countries; the lowest intakes (2.6 mg/d) are in Finland and the highest intakes (68.2 mg/d) are in Japan (Friesenecker, 1994). Quercetin is the most important contributor to the estimated intake of flavonoids, mainly from the consumption of apples and onions[27]. A major problem in cohort studies of flavonoid intakes is that only a limited number of flavonoids can be measured in biological samples, and more importantly, only a relatively small number of fruit and vegetables are used to make an accurate estimation.

Absorption: Data on the absorption, metabolism and excretion of flavonoids in humans are contradictory and scarce[28,29,30,31,32]. Naturally occurring flavones exist predominantly in a glycosylated form rather than in their aglycone form. The form of the flavonoid seems to influence the rate of absorption. Hollman and Katan[32] suggested that the glycosylated forms of quercetin are absorbed more readily than are the aglycone forms; however, this has been questioned by other researchers[33]. Some studies showed that the most intensely studied dietary flavonoid, quercetin, is absorbed in significant amounts[34]. The role of flavonoid glycosylation in facilitating

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[26] Haenen, G.R., Bast, A. **1999**. Nitric oxide radical scavenging of flavonoids. *Methods Enzymol*. **301**: 490–503.

[27] Knekt, P., Jarvinen, R., Seppanen, R. **1997**. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am J Epidemiol*. **146**: 223–230.

[28] Hollman, P.C., van Trijp, J.M., Buysman, M.N. **1997**. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett*. **418**: 152–156.

[29] Hollman, P.C., Gaag, M., Mengelers, M.J., van Trijp, J.M., de Vries, J.H., Katan, M.B. **1996**. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free RadicBiol Med*. **21**: 703–707.

[30] Hollman, P.C., Katan, M.B. **1997**. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed Pharmacother*. **51**: 305–310.

[31] Hollman, P.C., van Trijp, J.M., Mengelers, M.J., de Vries, J.H., Katan, M.B. **1997**. Bioavailability of the dietary antioxidant flavonolquercetin in man. *Cancer Lett*. **114**: 139–140.

[32] Hollman, P.C., Katan, M.B. **1999**. Dietary flavonoids: intake, health effects and bioavailability. *Food ChemToxicol*. **37**: 937–942.

[33] Manach, C., Morand, C., Demigne, C., Texier, O., Regerat, F., Remesy, C. **1997**. Bioavailability of rutin and quercetin in rats. *FEBS Lett*. **409**: 12–16.

[34] Young, J.F., Nielsen, S.E., Haraldsdottir, J. **1999**. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *Am J ClinNutr*. **69**: 87–94.

absorption is questioned by the fact that catechin, which is not glycosylated in nature, is absorbed relatively efficiently[35].

Conjugation: It is generally accepted that the conjugation pathway for flavonoids (catechins) begins with the conjugation of a glucuronide moiety in intestinal cells. The flavonoid is then bound to albumin and transported to the liver[36,37]. The liver can extend the conjugation of the flavonoid by adding a sulfate group, a methyl group, or both. The addition of these groups increases the circulatory elimination time and probably also decreases toxicity. There are several possible locations for the conjugates on the flavonoid skeleton. The type of conjugate and its location on the flavonoid skeleton probably determine the enzyme-inhibiting capacity, the antioxidant activity, or both of the flavonoid. Recent data suggest that the regular intake of flavonoids results in a more predominant formation of several conjugates, which probably results in greater activity. A detailed example is given in the study by Manach *et al*[35], in which a high dose of quercetin was administered to a group of rats adjusted to flavonoid intake and to a non-adjusted group. Results of this study indicated that the conjugated compound isorhamnetin was formed in higher quantities in the adjusted group, which is important because it is known to be even more active than is the aglycone form of quercetin on xanthine oxidase inhibition[35].

Concentrations of individual flavonoids and their biologically active conjugates may not be high enough after occasional intake to explain the low mortality rates from cardiovascular disease in Mediterranean countries. However, because the half-lives of conjugated flavonoids are rather long (23–28 h)[34], accumulation may occur with regular intakes, which may in turn result in sufficiently active flavonoid concentrations.

Toxicity: Formica and Regelson[38] gave an interesting overview of the in vitro and in vivo studies on quercetin. The early data on toxic side effects are mainly derived from

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[35] Okushio, K., Matsumoto, N., Kohri, T., Suzuki, M., Nanjo, F., Hara, Y. **1996**. Absorption of tea catechins into rat portal vein. *Biol Pharm Bull*.**19**: 326–329.

[36] Manach, C., Morand, C., Texier, O. **1995**. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J Nutr*. **125**: 1911–1922.

[37] Piskula, M.K., Terao, J. **1998**. Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr*. **128**: 1172–1178.

[38] Formica, J.V., Regelson, W. **1995**. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicology*. **33**: 1061–1080.

in vitro studies. At a conference of the Federation of American Societies for Experimental Biology in 1984 on mutagenic food flavonoids, carcinogenicity was reported in just 1 of 17 feeding studies conducted in laboratory animals[39,40]. Dunnick and Hailey[41] reported that high doses of quercetin over several years might result in the formation of tumors in mice. However, in other long-term studies, no carcinogenicity was found[42]. In contrast with the potential mutagenic effects of flavonoids in earlier studies, several more recent reports indicate that flavonoids, including quercetin, seem to be antimutagenic in vivo [43,44]. A large clinical study by Knekt*et al*[45], in which 9959 men and women were followed for 24 years, showed an inverse relation between the intake of flavonoids (eg, quercetin) and lung cancer. One possible explanation for these conflicting data is that flavonoids are toxic to cancer cells or to immortalized cells, but are not toxic or are less toxic to normal cells. If this is true, flavonoids might play a role in the prevention of cancer that is worthy for further investigation.

Clinical Effects: An overview of the hypothetical links between the working mechanisms and clinical effects of flavonoids is given in Figure 2.

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[39] Ertrurk, E., Hatcher, J.F., Pamukeu, A.M. **1984.** Bracken fern carcinogenesis and quercetin. *Fed Proc*. **43**: 2344.

[40] Starvic, B. (1984). Mutagenic food flavonoids. *Fed Proc*. **43**: 2344. Dunnick, J.K., Hailey, J.R. (1992). Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Fundam Appl Toxicol*.**19**: 423–431.

[41] Dunnick, J.K., Hailey, J.R. **1992**. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Fundam Appl Toxicol*.**19**: 423–431.

[42] Zhu, B.T., Ezell, E.T., Liehr, J.G. **2001**. Catechol-*o*-methyl transferase catalysis rapid *O*-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. *J Biol Chem*. **269**: 292–299.

[43] Kato, K., Mori, H., Fujii, M. **1984**. Lack of promotive effect of quercetin on methylazoxymethanol acetate carcinogenesis in rats. *J Toxicol Sci*. **9**: 319–325.

[44] Plakas, S.M., Lee, T.C., Wolke, R.E. **1985**. Absence of overt toxicity from feeding the flavonol, quercetin, to rainbow trout (*Salmogairdneri*). *Food ChemToxicol*. **23**: 1077–1080.

[45] Knekt, P., Jarvinen, R., Seppanen, R. **1997**. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am J Epidemiol*. **146**: 223–230.

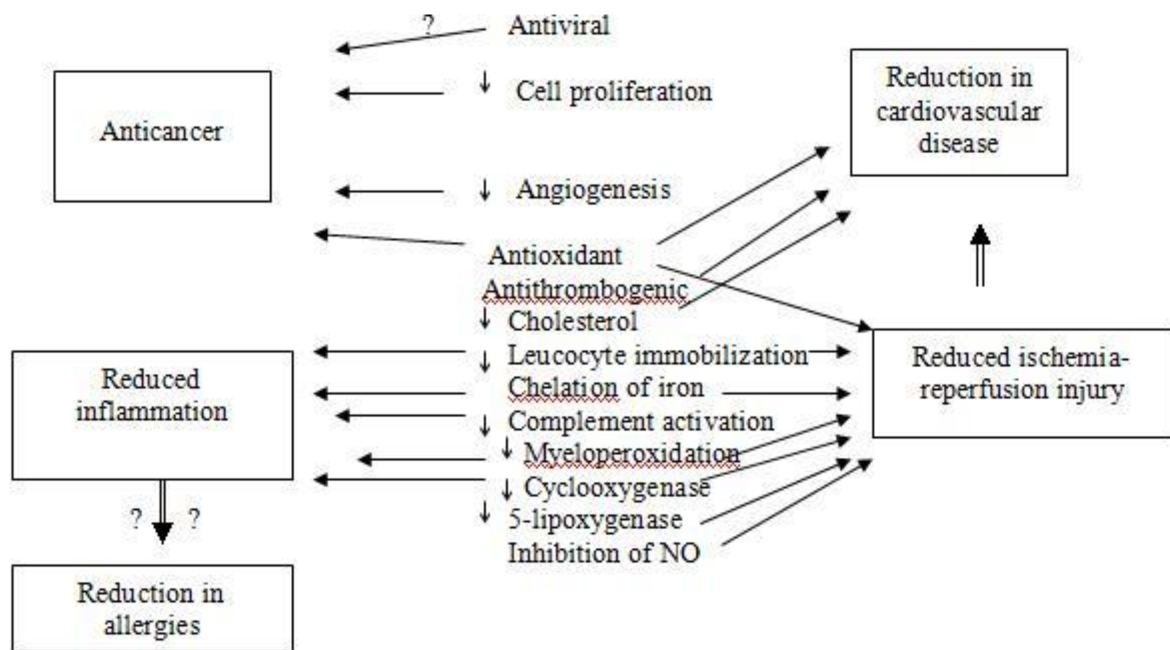

Fig1.4.Hypothesis of the links between the working mechanisms of flavonoids and their effects on disease.

Antioxidative effects: The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species. Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage[46,47]. The increased production of reactive oxygen species during injury results in consumption and depletion of the endogenous scavenging compounds. Flavonoids may have an additive effect to the endogenous scavenging compounds.

Radical scavenging activity: Flavonoids can prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen derived radical

[46] Chu, Y.H.; Chang, C.L.; Hsu, H.F. Flavonoid content of several vegetables and their antioxidant activity. **2000** *J. Sci. Food Agric.*, *80*, 561-566.

[47] Grace, P.A. **1994**. Ischaemia-reperfusion injury. *Br J Surg*. **81**: 637–647.

called peroxynitrite. Epicatechin and rutin are also powerful radical scavengers[48]. The scavenging ability of rutin may be due to its inhibitory activity on the enzyme xanthine oxidase. By scavenging radicals, flavonoids can inhibit LDL oxidation in vitro[49]. This action protects the LDL particles and, theoretically, flavonoids may have preventive action against atherosclerosis.

Anti-inflammatory effects: Cyclooxygenase and lipoxygenase play an important role as inflammatory mediators. They are involved in the release of arachidonic acid, which is a starting point for a general inflammatory response. The exact mechanism by which flavonoids inhibit these enzymes is not clear. Quercetin, in particular, inhibits both cyclooxygenase and lipoxygenase activities, thus diminishing the formation of these inflammatory metabolites[50].

Antitumour effects: Antioxidant systems are frequently inadequate, and damage from reactive oxygen species is proposed to be involved in carcinogenesis[51,52]. Reactive oxygen species can damage DNA, and division of cells with unrepaired or misrepaired damage leads to mutations.

It has been stated that flavonoids, as antioxidants, can inhibit carcinogenesis[53]. Some flavonoids- such as fisetin, apigenin, and luteolin are stated to be potent inhibitors of cell proliferation[54]. Furthermore, it has been speculated that flavonoids can inhibit angiogenesis[55]. Angiogenesis is normally a strictly controlled process in the human

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[48] Hanasaki, Y., Ogawa, S., Fukui, S. **1994**. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free RadicBiol Med*. **16**: 845–850.

[49] Kerry, N.L., Abbey, M. **1997**. Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation in vitro. *Atherosclerosis*. **135**: 93–102.

[50] Robak, J., Gryglewski, R.J. **1996**. Bioactivity of flavonoids. *Pol J Pharmacol*. **48**: 555–564.

[51] Zhao M.; Yang B.; Wang J.; Liu Y.; Yu L.; Jiang Y.M. **2007.** Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis*Sonn)pericarp. *Int.Immunopharmacol. 7*, 162-166.

[52] Pryor, W.A. **1997**. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect*. **105** (suppl): 875–882.

[53] Stefani, E.D., Boffetta, P., Deneo-Pellegrini, H. **1999**.Dietary antioxidants and lung cancer risk: a case-control study in Uruguay .*Nutr Cancer*. **34**: 100–110.

[54] Fotsis, T., Pepper, M.S., Aktas, E. **1997**. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res*. **57**: 2916–2921.

[55] Caltagirone, S., Rossi, C., Poggi, A. **2000**. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int J Cancer*. **87**: 595–600.

body. The process of angiogenesis is regulated by a variety of endogenous angiogenic and angiostatic factors. It is switched on, for example, during wound healing. Pathologic, unregulated angiogenesis occurs in cancer[56]. Angiogenesis inhibitors can interfere with various steps in angiogenesis, such as the proliferation and migration of endothelial cells and lumen formation. Among the known angiogenesis inhibitors, flavonoids seem to play an important role[57].

Antithrombogenic effects: Platelet aggregation contributes to both the development of atherosclerosis and acute platelet thrombus formation, followed by embolization of stenosed arteries. Selected flavonoids, such as quercetin, kaempferol, and myricetin were shown to be effective inhibitors of platelet aggregation in dogs and monkeys[58].

Antiviral effects: The antiviral activity of flavonoids was shown in a study by Wang *et al*[59]. Some of the viruses reported to be affected by flavonoids are herpes simplex virus, respiratory syncytial virus, parainfluenza virus, and adenovirus. Quercetin was reported to exhibit both antiinfective and antireplicative abilities. The interaction of flavonoids with the different stages in the replication cycle of viruses was previously

described[60]. For example, some flavonoids work on the intracellular replication of viruses, whereas others inhibit the infectious properties of the viruses.

[56] Fan, T.P., Jaggar, R., Bicknell, R. **1995**. Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol Sci*. **16**: 57–66.

[57] Paper, D.H. **1998**. Natural products as angiogenesis inhibitors. *Planta Med*. **64**: 686–695.

[58] Osman, H.E., Maalej, N., Shanmuganayagam, D., Folts, J.D. **1998**. Grape juice but not orange or grapefruit juice inhibits platelet activity in dogs and monkeys. *J Nutr*. **128**: 2307–2312.

[59] Wang, H.K., Xia, Y., Yang, Z.Y., Natschke, S.L., Lee, K.H. **1998**. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. *AdvExp Med Biol*. **439**: 191–225.

[60] Kaul, T.N., Middleton, E. Jr., Ogra, P.L. **1985**. Antiviral effect of flavonoids on human viruses. *J Med Virol*. **15**: 71–79.

Table1. Enzyme modulator activity of flavonoids

S.No.	ENZYME	FLAVONOID	MECHANISM
1.	Protein kinase	Quercitin	Inhibited the phosphorylated activity of the rous sarcoma virus-transforming gene.
2.	Phospholipase A 2 (pla2) Phospholipase C	Kaempferol	Inhibition of phossphorylase kinase and also of protein tyrosine kinase.
3.	ATPases	Kaempferol	Catalyzes the Myosin light chain kinase (MLICK)
4.	Lipoxygenases and Cyclooxygenases	Quercetin.Kaempferol-3-O -glycoside	Inhibition of PLA3 from human & rabbit leukocytes.
		scutellarein	Blocking the PLC activation & formation of inositol trisphosphate (IP3) & diacylglycerol (DGA).Mg21–ectolATPase of human leukocytes.
		Genistein Quercetin	Increase in ATPase activity by conformational changes in the structure of myosin.
		Quercetin	Effect on arachidonic acid metabolism via the LO and CO (TxB2, PGE2, 6-keto-PGFIA) pathways.

1.5. GENUS *IRIS*

NATURALLY OCCURRING FLAVONOIDS OF *IRIS*

There have been a variety of flavonoid compounds isolatedfrom *Iris* during 1999-2008. These compounds can be classified in the following six sections (isoflavones & their glycosides, flavones & their glycosides, flavonols & their glycosides, flavanones & flavanonols, peltogynoids and coumaronochromones & rotenoids), which follow a

standard pattern so far as is practicable. The normal flavonoid constituents of *Iris* reported during 1999-2008 can be classified, according to their skeletons, into five groups: isoflavones & their glycosides, flavones & their glycosides, flavonols & their glycosides, flavanones and dihydroflavonols. They constitute a relatively homogeneous group of compounds, almost all being derivative of the basic tricyclic structure of isoflavone or flavones with simple patterns of *O*-substitution (*e.g.* hydroxy, methoxy and methylenedioxy) and glycosylation. They differ only in the nature of the substituent position of rings A and B. There are relatively common 5,7-di- or 5,6,7-tri-*O* substitution pattern in the A-ring. Importantly, the most pronounced feature in this ring is 6,7-methylenedioxy substitution (32 out of 94 compounds possess the substitution pattern), which is less common in flavonoids obtained from other genus of Iridaceae. Furthermore, in the B-ring they often possess 2'- or 4'-O-substitution, 2',3'-, 3',4'- or 4',5'-di-O-substitution and 3',4',5'-tri-O-substitution, but it is noteworthy that 2'-O-substitution & 2',3'-di-O-substitution are rare in the aglycones of their glycosides. Finally, the majority of flavonoid glycosides characterized to date are either 6-,7-, 3- or 4'- glycosides. There are also classes of modified flavonoids found in *Iris* species, which in formal terms can be regarded as cyclized derivatives of the normal flavonoids mentioned above, namely peltogynoids, coumaronochromones and rotenoids. Similarly with the regular flavonoids, they possess simple patterns of O-substitution. The common feature of the compounds is that the substituent at C-2 or C-3 position is alwaysfound in the form of an oxygen-bearing ring fused with rings B and C.

As demonstrated previously by Iwashina*et al.*[61],isoflavones (mainly contained in the rhizomes) and glycosyl flavones (mainly found from the leaves and flowers of almost *Iris* species), either C-glycosyl flavones or O-glycosyl flavones, are major components of the genus *Iris*. We further find that O-glycosyl isoflavones (mainly contained in the rhizomes) are also major components of *Iris* species. The residue flavones, flavonols & their glycosides, flavanones, dihydroflavonols, peltogynoids, coumaronochromones and rotenoids are all minor components. Importantly,

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[61] Iwashina, T.; Otani, S. **1998** Flavonoids of the Genus Iris; Structures, Distribution and Function (Review). *Ann.Tsukuba Bot. Gard.*, 17,147-183.

Choudhary*et al.* first isolated five new peltogynoids from *Iris bungei* in 2001, and that was the first report of peltogynoid-type compounds found in the family of Iridaceae.[62]

1.5.1. Isoflavones and their Glycosides

Isoflavones and their glycosides are the most important flavonoid compounds isolated from the genus *Iris*. In most reports they were found in the rhizomes, rare in the flowers and leaves. The number of the isoflavones and their glycosides described in the literature during the reporting period exceeds that of any other subclass of flavonoids.

Isoflavones

As many of the likely permutations of hydroxy, methoxy, and methylenedioxy substitutions have already been described, not surprisingly, the number of new isoflavones reported between 1999 and 2008 is relatively small. There are only five records (1-5) characterized. 5,7,3',4'-tetrahydroxy-6-methoxyisoflavone (irilin D, 1) was isolated from the underground parts of *Iris bungei*, a species cited as a medicinal plant in Mongolian, along with four new flavonols, irisflavone A-D (26-29), and three known isoflavones, irilins A-B and tlatancuayin[62] irilin D (1) had been earlier reported as a synthetic product[63], and that was the first time it was characterized as a new natural product. The whole plant of *Iris tenuifolia* yielded 5,2',3'- trihydroxy-6,7-methylenedioxyisoflavone (tenuifone, 2)[64], which possesses the uncommon 6,7-methylenedioxy substitution in the A-ring.

Another example with this substitution is 5,3'-dihydroxy-4'-methoxy-6,7-methylenedioxyisoflavone (Soforanarin A, 3) from methanolic extracts of the rhizomes of *Iris soforana* of Turkish origin, which also yielded 6,3',4'- trihydroxy-5,7,5'-trimethoxyisoflavone (Soforanarin B, 4) and other six known isoflavones[65]. To

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[62] Choudhary,M. I.; Nur-e-Alam M.; Baig, I. **2001** Four new flavones and a new isoflavone from Iris bungei. *J. Nat. Prod.*, , 64, 857- 860.

[63] Horie, T.; Shibata, K.; Yamashita, K.; Fujii, K.; Tsukayama, M.; Ohtsuru, Y. **1998** Studies of the Selective O-Alkylation and Dealkylation of Flavonoids. XXIV. A Convenient Method for Synthesizing 6- and 8-Methoxylated 5,7-Dihydroxyisoflavones. *Chem. Pharm.Bull.*, ,46,222-230.

[64] Choudhary, M. I.; Hareem, S.; Siddiqui, H.; Anjum, S.; Ali, S.; Rahman, A.-U.; Zaidi, M. I. **2008**, A benzil and isoflavone from Iris tenuifolia.*Phytochemistry*, 69,1880-1885.

[65] Rahman,A.-U.;Nasim,S.;Baig, I.;Sener,B.;Orhan, I.;Ayanoglu, F.; Choudhary,M.I. **2004**.Two new isoflavanoidsfromthe rhizomes of Iris soforana.*Nat.Prod.Res.*, ,18,465-471.

the best of our understanding, it was the first phytochemical work conducted on this species. Compounds (1-4) are examples exhibiting relatively common 5,6,7-tri-O-substitution pattern of the A- ring, but 7, 8, 5'-trihydroxy-6, 3', 4'-trimethoxyisoflavone (5) obtained from the underground parts of *Iris potaninii*[66] is unusual to some extent, which possesses 8-hydroxy substitution in the A-ring. This substitution is relatively uncommon in isoflavones[67].

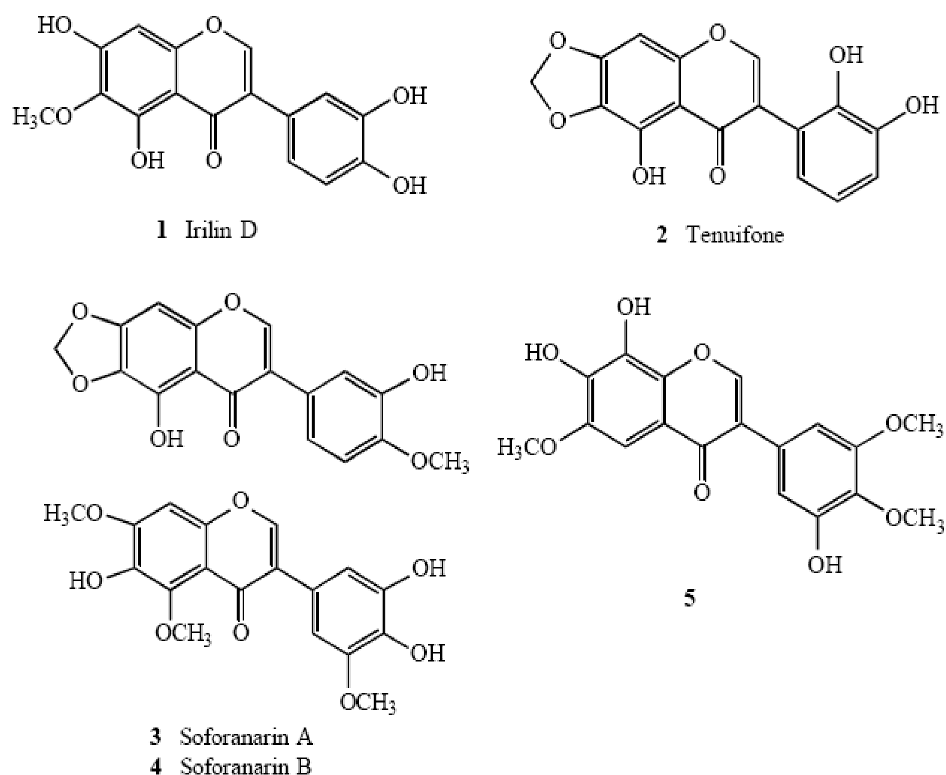

Fig.1.5.Structures of new isoflavones (1-5) isolated from *Iris*

[66] Purev, O.; Purevsuren, C.; Narantuya, S.; Lkhagvasuren, S.; Mizu- kami, H.; Nagatsu, A. **2002** New isoflavones and flavanol from Iris potaninii.*Chem.Pharm.Bull.*, ,50,1367-1369.

[67] Veitch, N. C. Isoflavonoids of the Leguminosae. Nat. Prod. Rep., 2007,24,417-464.

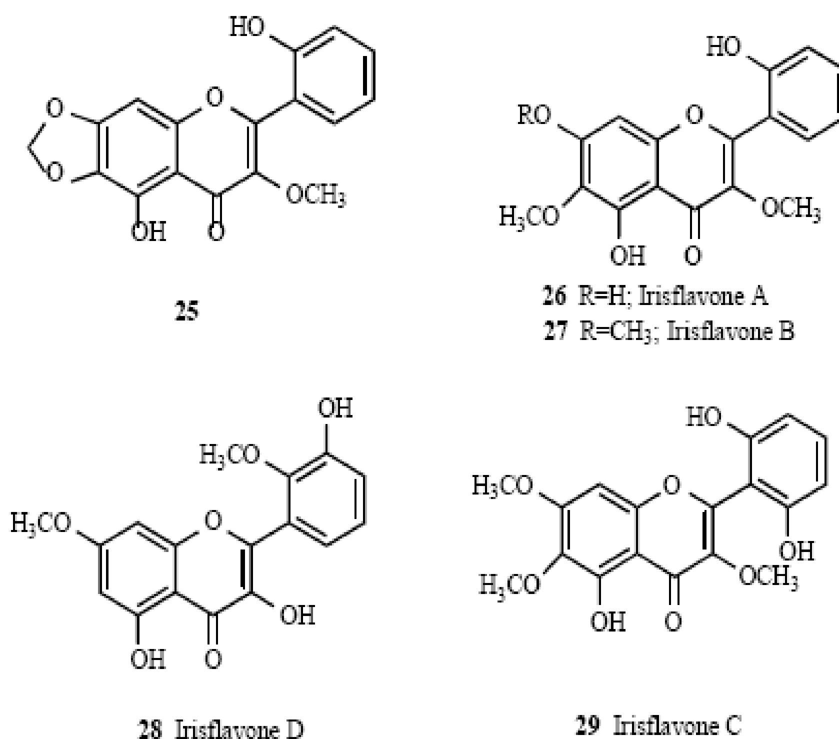

Fig.1.6. Structures of new flavones (25-29) isolated from *Iris*.

Isoflavone Glycosides

Most of the saccharides found in isoflavone glycosides reported between 1999 and 2008 are monosaccharides or disaccharides. The monosaccharides are normally D-glucopyranosides

(D-Glc*p*), and similarly, the disaccharides are mainly D-Glc*p*-(1,6)- D-Glc*p*. They usually form O-glycosides, with the sugar moiety often linked to C-7or C-4' position of the known aglycones. Among the new isoflavone glycosides isolated from the genus *Iris* during the reporting period, there are five containing monosaccharides, nine containing disaccharides and one containing trisaccharide. In the following part they are elucidated in detail. Compounds (**6**) (*Iris Carthaliniae*)[68], (**7**) and (**8**) (*Iris germanica*) [69] are relatively common 7-O- , 4'-O- D-glucopyranosyl isoflavones obtained from *Iris* species.

Two unusual isoflavone monosaccharides are iris leptophyllidin(**9**) from rhizomes of *Iris leptophylla* and 8,3',5'-trihydroxy-7,4'-dimethoxy-6-O-D-glucopyranosyl

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[68]Farag, S. F.; Backheet, E. Y.; El-Emary, N. A.; Niwa, M **,1999** Isoflavonoids and flavone glycosides from rhizomes of Iris carthaliniae.*Phytochemistry*,50,1407-1410.

[69]Rahman, A.-U.; Nasim, S.; Baig, I.; AraJahan, I.; Sener, B.; Orhan, I.; Choudhary, M. I. **2002** Isoflavonoid glycosides from the rhi- zomes of Iris germanica. *Chem. Pharm. Bull.*, 50, 1100- 1102.

isoflavone (**10**) from underground parts of *Iris potaninii*[66], which are 3'-O- and 6-O-D-glucopyranosyl isoflavone, respectively.

Similarly with 7, 8, 5'-trihydroxy-6, 3', 4'-trimethoxyisoflavone (**5**), the aglycone of compound (**10**) also possesses the uncommon 8-hydroxy substitution in the A-ring. Compounds (**11**), (**15**), (**17**) (*Iris germanica*)[71], (**13**) (*Iris pseudopumila*)[72], (**12**), (**16**) (*Iris carthaliniae*)[68]contain relatively common 7-O-, 4'-O -D-Glc*p*-(1,6)-D -Glc*p*.The uncommon disaccharide L-Rha*p*-(1,2)-D-Glc*p* was reported in a 4'-O-glycoside of irilone, irilone-bioside (**14**) obtained from rhizomes of *Iris leptophylla* together with iris leptophyllidin (**9**)[70].

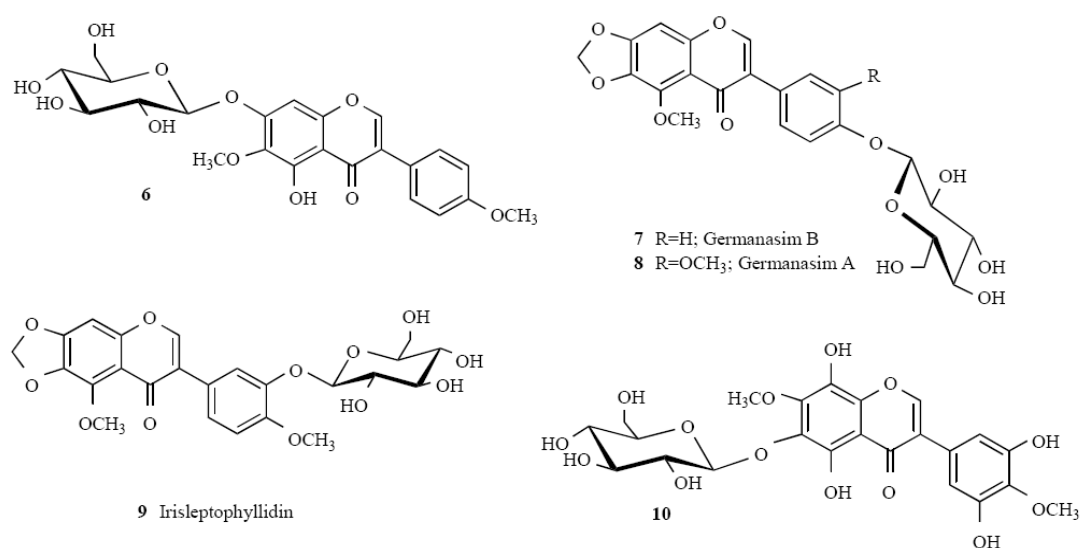

Fig.1.7. Structures of new monosaccharides (6-10) isolated from *Iris*

Compounds (**11-17**) are all linear disaccharides, but (**18**) (*Iris spuria*)[73] and (**19**) (*Iris germanica*)[71] are uncommon examples of two monosaccharides bound to different positions of the aglycones, respectively. (**13**) (*Iris pseudopumila*)[72], contain relatively common 7-*O*-, 4'-O-D-Glc*p*-(1,6)-D -Glc*p*. (**18**) (*Iris spuria*), a monosaccharide is bound to different positions of the aglycones.[73]

[70] Qin, M. J.; Li, R.; Wang, X.; Ye, W. C. **2007.** New isoflavonoidglycosides from the rhizomes of Iris leptophylla Lingelsh. *J. Integr.PlantBiol*., ,49,213-217.

[71] Rahman,A.-U.;Nasim,S.;Baig,I.; Orhan,I.;Sener,B.;Ayanoglu, F.; Choudhary, M. I. **2003** Isoflavonoid Glycosides from the Rhizomes of Iris germanica. *Helv. Chim. Acta*,, 86, 3354- 3362.

[72] Rigano, D.; Formisano, C.; Grassia, A.; Grassia, G.; Perrone, A.; Piacente, S.; Vuotto, M. L.; Senatore, F. **2007** Antioxidant flavonoids and isoflavonoids from rhizomes of Iris pseudopumila. *PlantaMed*. ,73,1-4.

[73] Singab, A. N. B. **2004** Flavonoids from Iris spuria (Zeal) cultivated in Egypt. *Arch.Pharm.Res* ,27,1023-1028.

The more noteworthy one is germanasim G (**19**), which is a 3',4'-di-*O*- D-glucopyranosyl isoflavone with 2-methoxy-4-acetylphenyl group bound to the C-2 position ofthe 4'-glucopyranoside. The acylated phenyl derivative of a glucose moiety is very rare among the isoflavone glycosidesobtained from the genus *Iris*. The unique example of new isoflavone glycoside containing a linear trisaccharide is germanasim F(**20**) isolated from rhizomes of *Iris germanica*, together with germanasim C-E and G (**11**, **15**, **17**, **19**).[71] The linear trisaccharide of germanasimF(**20**), -D-Glc*p*-(1 2)- L-Rha*p*-(1,6)-DGlc*p*, is characterized in a 4'-O-glycoside of nigricin (irisolone).

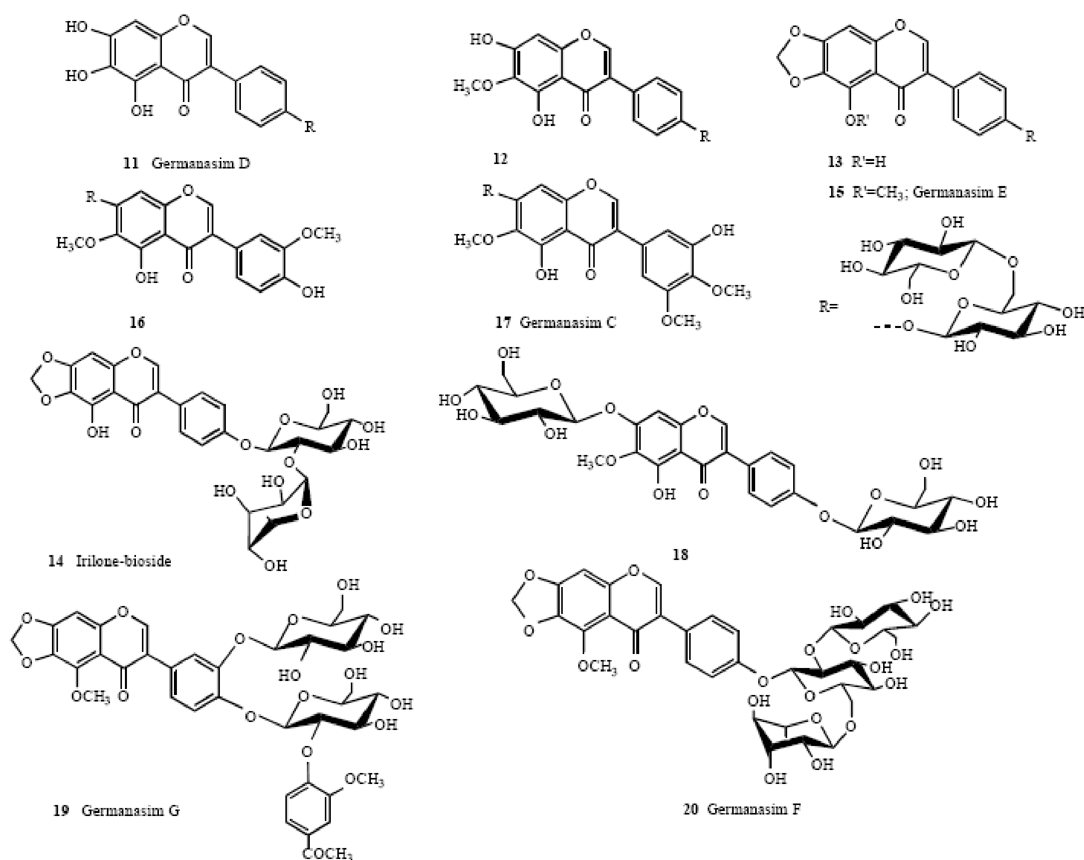

Fig.1.8.Structures of isoflavone disaccharides and trisachharides isolated from *Iris*

1.5.2. CHEMICAL AND SPECTRAL METHODS OF CHARACTERIZATION:

Chemical methods for the structure determination of isoflavones are well documented (ollis,1962; Dean,1963). Flavonoids are generally soluble in water and alcohol, but insoluble in organic solvents; the genins are only sparingly soluble in water but

are soluble in ether. Flavonoids dissolve in alkalis, giving yellow solutions which on the addition of acid become colorless. Isoflavones are much more susceptible to alkali hydrolysis than flavones. The production of a deoxybenzoin under mild conditions, together with formic acid, constitutes important evidence for an isoflavone structure. Confirmation may further be obtained by resynthesis of the isoflavone from the deoxybenzoin with the appropriate reagent.

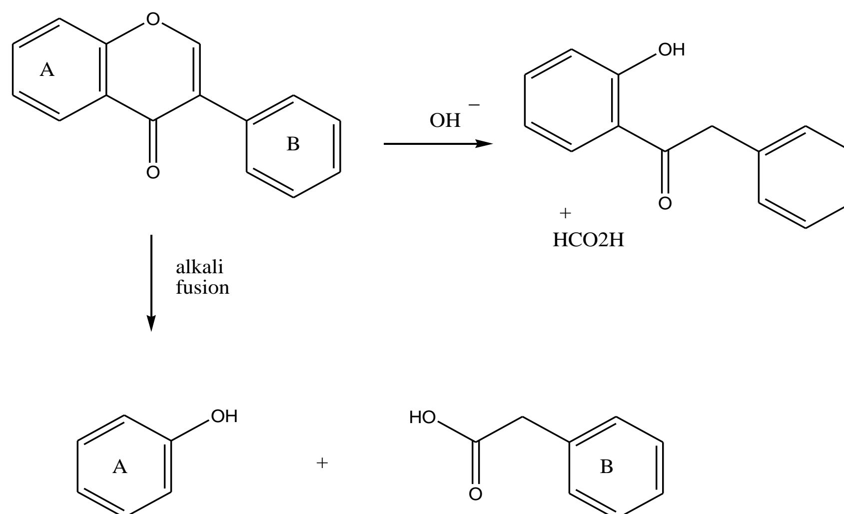

Fig.1.9. Alkali degradation of isoflavones.

CHEMICAL METHODS:

1) FLUORESCENCE QUENCHING TEST

A novel fluorescence quenching test for the detection of flavonoid degradation by microorganisms was developed. The test is based on the ability of the flavonoids to quench the fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH). Several members of the anthocyanidins, flavones, isoflavones, flavonols, flavanones, dihydroflavanones, chalcones, dihydrochalcones and catechins were tested with regard to their quenching properties. The anthocyanidins were the most potent quenchers of DPH fluorescence, while the flavanones, dihydroflavanones and dihydrochalcones, quenched the fluorescence only weakly. The catechins had no visible impact on DPH fluorescence. The developed test allows a quick and easy differentiation between flavonoid-degrading and flavonoid-non-degrading bacteria. The investigation of individual

reactions of flavonoid trans- formation with the developed test system is also possible.[74]

2) SHINODA TEST

To dry powder or extract, add 5ml. 95% ethanol. Few drops conc. HCL and 0.5 g magnesium turnings. Pink color observed. To small quantity of residue, add lead acetate solution. Yellow colored precipitate is formed. Addition of increasing amount of sodium hydroxide to the residue shows yellow coloration which decolorises after addition of acid.[75]

SPECTRAL METHODS:

The combination of HPLC with mass spectrometric detection, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) has proved to be a useful tool in analysing natural products. The combination of HPLC–APCI allows efficient separation and identification of isoflavones with greater efficiency than HPLC (with UV detection) alone. Negative-ion APCI provides quality mass spectra and by variation of conditions, the collision-induced dissociations which are generated permits structure elucidation. Further information on isoflavone structure can be obtained by addition of deuterium oxide to the sample which induces peak shifts in the mass spectra and helps to identify the number of exchangeable hydrogen atoms in each molecule. The use of capillary electrophoresis (CE) in the determination of iso-flavones has also been described. In contrast to HPLC, the CE method is rapid, it does not require solvent gradient or elution and it does not consume organic solvents for elution. Capillary electrophoresis in combination with electrospray ionization–mass spectrometry (ESI–MS) has been shown to be a suitable technique for the determination of isoflavones.

The use of CE–ESI–MS which permits the efficient separation and identification of isoflavones with higher specificity than CE (with UV detection) alone, allows both the determination of the molecular mass of isoflavones and the recognition of various functional groups through analysis of several diagnostic fragment ions. Two dimensional NMR techniques including ^{1}H–^{1}H (COSY), ^{13}C–^{1}H (HETCOR), ^{13}C–^{1}H (COLOC) and NOESY are now routine methods for the structure elucidation of isoflavonoids. Application of 2D NMR techniques has led to the unambiguous ^{1}H and

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[74] L Schoefer, A Braune, M Blaut, **2001**. A fluorescence quenching test for the detection of flavonoid transformation, *FEMS microbiology letters,* 204. 2, 277-280.

[75] Khandelwal K. R., **2007**.Practical pharmacognosy, Techniques &Experiments, 18.153. 12

^{13}C NMR assignments of the isoflavonoids 5,7-dihydroxy-4'-methoxyisoflavone (biochanin A), 2',5,7-trihydroxy-4'-methoxyisoflavone and 5,7-dihydroxy- 2',4'-dimethoxyisoflavone, isolated from Virola caducifolia.[76]

1.5.3. Spectral properties of Isoflavones:

The isoflavones remain by far the most common of the isoflavonoids. Most of the isoflavones known are simple isoflavones which can be grouped into the 18 oxygenation patterns. The remainder may be conveniently called complex isoflavones, since each compound contains one or more isoprenoid substituents usually further cyclised with a hydroxyl group. Isoflavones can be distinguished from flavones and isoflavanones by UV and NMR spectroscopy. The simple isoflavones have intense absorption at 255-275 nm and generally a less intense band or inflection at 310-330 nm. The low intensity of absorption of the second band of isoflavones is a valuable diagnostic feature. The NMR signal of the olefinic proton at C-2 in isoflavones appears as a characteristic down field singlet at 7.8δ (8.3δ in DMSO) as compared to 6.7δ for the C-3 proton in flavones. The impact of NMR on structure determination is most evident in the complex isoflavones; the presence and nature of the isoprenoid substituents in these compounds is readily revealed.

Mass spectrometry cannot distinguish between flavones and isoflavones since fragmentation via the retro Diels-Alder process results in identical fragments for both classes of compounds. The presence of 2′-methoxyl group in isoflavones, however, has been found to influence the fragmentation pattern profoundly. These compounds show a strong M-31 peak due to loss of methoxyl; this probably occurs via ring closure.

[76] S.A. dos Santos, M.G. de Carvalho and R.Braz-Filho, **1995** *J. Braz. Chem. Soc.*, , 6,349.

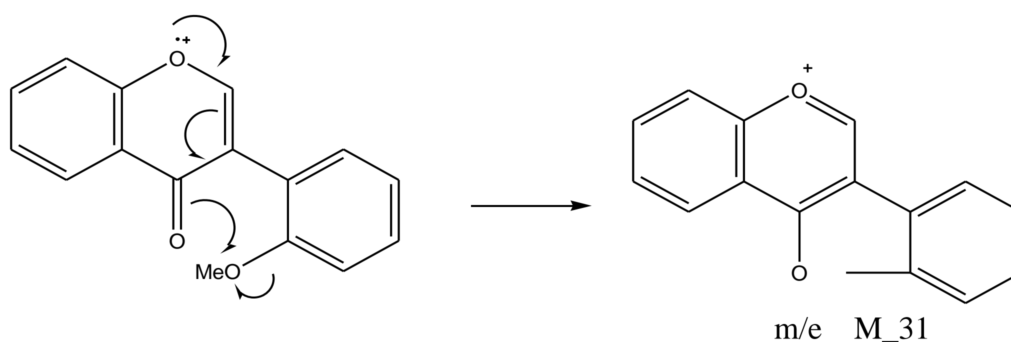

Fig.1.10. MS fragmentation of 2′- methoxyisoflavones.

1.5.4. BIOLOGICAL PROPERTIES OF ISOFLAVONES:

A distinctive feature of the isoflavonoids is the possession of biological activity within the group. In contrast to other flavonoids which on the whole are innocuous substances (whalley, 1959), isoflavonoids have oestrogenic, insecticidal, piscicidal and anti-fungal properties.

Isoflavones have a strikingly similar chemical structure to mammalian estrogens. Therefore, it is not surprising that isoflavones bind to estrogen receptors and affect estrogen-regulated gene products.[77] Traditionally, isoflavones have been considered to be very weak estrogens, possessing the activity between 1 x 10^{-4} and 1 x 10^{-2} of 17ß-estradiol on a molar basis.[78] Even these estimates of estrogenic activity suggest isoflavones likely exert physiological activity in vivo, since people who consume soyfoods may have serum isoflavone levels up to 10,000 fold higher than endogenous estrogen levels.[79] The high serum and tissue isoflavone concentrations compensate for their relative weakness. However, for several reasons, these older views of the relative estrogenicity of isoflavones may need to be revised. First, genistein binds with almost the same affinity to the recently discovered second estrogen receptor, estrogen receptor beta (ERb), as 17ß -estradiol.[80] Until recently, estimates of the estrogenic activity of isoflavones were based largely on estrogen receptor alpha (ERa), for which genistein has much less affinity. Second, isoflavones may bind less tightly than

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[77] Markiewicz L, Garey J, Adlercreutz H, Gurpide E. **1993** In vitro bioassays of non-steroidal phytoestrogens. *J Steroid Biochem Mol Biol*; 45:399-405.

[78] Mayr U, Butsch A, Schneider S. **1992** Validation of two in vitro test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. *Toxicology*; 74:135-49.

[79] King RA, Bursill DB. **1998** Plasma and urinary kinetics of the isoflavonesdaidzein and genistein after a single soy meal in humans. *Am J ClinNutr*; 67:867-72.

[80] Kuiper GG, Lemmen JG, Carlsson B, et al. **1998** Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology; 139:4252-63.

estrogen to serum proteins making them more available to the tissues.[81] Third, isoflavones may be tissue-selective, exerting quite pronounced estrogenic activity in some tissues,[82] but not in others.[83] The ability to exert tissue-selective effects likely stems in part from the different tissue distribution of ERb and ERa.[84] This observation has prompted speculation that isoflavones are natural selective estrogen receptor modulators (SERMs), like the drugs tamoxifen and raloxifene,[85] and therefore might provide the benefits of estrogen without the disadvantages – but this is still speculative. Although isoflavones possess estrogenic and possibly antiestrogenic activity,[86] the physiological effects of isoflavones, especially genistein, are likely only partially related to direct interaction with or binding to estrogen receptors.

This is evident by the finding that genistein inhibits the growth of a wide range of both hormone- dependent and independent cancer cells in vitro[87] – a result thought to be due to the ability of genistein to influence signal transduction.[88] In vitro, genistein inhibits the activity of many enzymes and cellular factors that control the growth of cells.[89] Isoflavones also possess antioxidant activity.[90]

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[81] Nagel SC, vomSaal FS, Welshons WV. **1998** The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. *Proc Soc Exp Biol Med*; 217:300-9.

[82] Walker HA, Dean TS, Sanders TA, Jackson G, Ritter JM, Chowienczyk PJ. **2001**.The phytoestrogen genistein produces acute nitric oxide-dependent dilation of human forearm vasculature with similar potency to 17b-estradiol. *Circulation*; 103:258- 262.

[83] Upmalis DH, Lobo R, Bradley L, Warren M, Cone FL, Lamia CA. **2000**. Vasomotor symptom relief by soy isoflavone extract tablets in postmenopausal women: a multicenter, double-blind, randomized, placebo-controlled study. *Menopause*; 7:236-42.

[84] Cassidy A, Faughnan M. **2000**. Phyto-oestrogens through the life cycle. *Proc Nutr Soc;* 59:489-96.

[85] Gustafsson JA. **1998**. Therapeutic potential of selective estrogen receptor modulators. *Curr Opin Chem Biol*; 2:508-11.

[86] Folman Y, Pope GS. **1966** The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero- vaginotrophic compounds of low potency. *J Endocrinol*; 34:215-25.

[87] Adlercreutz H, Mazur W. **1997**. Phyto-oestrogens and Western diseases. *Ann Med*; 29:95-120.

[88] Weber G, Shen F, Yang H, Prajda N, Li W. **1999.** Regulation of signal transduction activity in normal and cancer cells. *Anticancer Res*; 19:3703-9.

[89] Kim H, Peterson TG, Barnes S. **1998**. Mechanisms of action of the soy isoflavonegenistein: emerging role for its effects via transforming growth factor beta signaling pathways. *Am J ClinNutr*; 68:1418S-1425S.

[90] Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. **1997** Antioxidant activity of phytoestrogenic isoflavones. *Free Radic Res*; 26:63-70.

The myriad biological effects of isoflavones accounts for their possible beneficial roles in diseases as diverse as osteoporosis,[91] coronary heart disease,[82] malaria,[92] cystic fibrosis,[93] and alcoholism.[94] In clinical studies demonstrating the health benefits of soyfood consumption, subjects typically ingest between 40 and 150 mg of isoflavones per day. Some studies report average Japanese consumption may be as high as 50 mg/day. Obviously, a sizeable portion of the population consumes more than this amount. In fact, several studies reported that isoflavone intake by approximately five percent of the Japanese population is 100 mg/day. Therefore, a reasonable upper adult daily limit for isoflavone intake is considered to be about 100 mg (aglycone units), an amount found in approximately three servings of traditional soyfoods. Consuming three servings of soyfoods per day is much more than most Americans currently consume, but still consistent with recommendations to choose a variety of foods. Furthermore, clinical studies conducted for as long as one year in which subjects have consumed more than 100 mg of isoflavones per day have not reported any adverse effects.

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[91] Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JW, Jr. 1998. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am J Clin Nutr; 68:1375S-1379S.

[92] Kraft C, Jenett-Siems K, Siems K, Gupta MP, Bienzle U, Eich E. **2000**. Antiplasmodial activity of isoflavones from andirainermis. *J Ethnopharmacol*; 73:131-5.

[93] Lansdell KA, Cai Z, Kidd JF, Sheppard DN. **2000.** Two mechanisms of genistein inhibition of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in murine cell line. *J Physiol*; 524 Pt 2:317-30.

[94] Keung WM, Vallee BL.. **1998.** Kudzu root: an ancient Chinese source of modern antidipsotropic agents. *Phytochemistry*; 47:499-506.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE:

Genus Iris belonging to family Iridaceae represents one such genera which has a worldwide distribution comprising 1630-1750 species classified into 77[95] genera. India is represented by 34 species and 5 varieties under 14 genera of Iridaceae[96]. Kashmir is represented by 12 species[97]. Out of these species, two less explored species have been selected for the studies.

Iris species have an immense medicinal importance. Iris species are used in treatment of biliousness with liver dysfunction[98], cancer, inflammations in addition to bacterial and viral infections[99].They are used as antispasmodic[100], emetic, laxative[101], antidote and haemostatic agents[102]. Furthermore, they exhibit molluscicidal activity[103] and significant anthehminitic activity[104]. The compounds isolated from these species are reported to have anti tumor[105], piscicidal, antioxidant, antituberculousis, anti-inflammatory[106], antiplasmodial and antifungal[107] properties.

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[95] Goldblatt, P. **1990**. Phylogeny and classification of Iridaceae. *Annuals of Missouri Botanical Garden* 77(4):607-627.

[96] Bahali, D.D., Sanjappa, M. and Rata, S.P**.2004**. Geographical distribution of Iridaceae in India. *Indian Journal of Forestry* 27(3):251-256.

[97] Karihaloo V, Kachroo P, Koul AK. **. 1984.** *Cytomorphology of Iris*.

[98] Evans, W.C., **2002**. Trease and Evans *Pharmacognosy,* 15:37, 417.

[99] Atta-ur-Rahmann, Nasim,S., Baig, I., Jalil, S., Orhan, I., Sener, B.Choudhary, M.I., **2003.** *J. Ethonopharmacol*.86, 177-180.

[100] Atta-ur-Rahmann, Nasim, S.,Baig, I., Jalil, S., Orhan, I., Sener, B., Choudhary, M.I., **2003***.J. Ethonopharmacol*.86, 177-180.

[101] Seki, K., Tomihari, T., Haga, K., Kaneko, R., **1994***. Phytochemistry* 36, 433-438.

[102] Seki, K., Haga, K., Kaneko, R., **1995**. *Phytochemistry* 38, 965-973.

[103] Atta-ur-Rahmann, Nasim, S., Baig, I., Jalil, S.Orhan, I., Sener, B., Choudhary, M.I., **2003**. *J. Ethonopharmacol*.86, 177-180.

[104] Tariq,K.A.,Chishti,M.Z.,Ahmad,F.,Shawl,A.S.,Tantary,M.A.**,2008***.J.Helminthol*.82,135-141.Singab,A.n., Ahmed, A.H, Sinkkonen, J., Ovcharenko, V., Philaja,K., 2006. Z. Naturforsch. C 61, 57-63.

[105] Atta-ur-Rahmann, Nasim, S., Baig, I., Jalil, S., Orhan, I., Sener, B., Choudhary, M.I.,**2003** *J. Ethonopharmacol*.86, 177-180.

[106] Benoit-Vical, F., Imbert, C., Bonfils, J.P., Sauvaire, Y., **2003**. *Phytochemistry* 62, 747-751.

[107] Chopra R.N., Nayer, S. L. and Chopra**. 1956**. *Glossary of Indian medicinal plants*.

In addition to these properties, Iris have the property of skin lightening and showing estrogenic activities. Also roots and leaves of Iris are given in fever[108]. They find their use as aperients[109], diuretic[110]and in the treatment of bruise, back pain and diarrhea[111] as well. Iris nepalensis rhizomes are used in Indian Indigenous System of Medicines under the name of “Sosan” for a variety of heart diseases[112].

The major groups of compounds that are present in the Iris are flavonoids and titerpenoids. Other components present are anthocyanins, flavonols, flavones, isoflavones, flavonones, xanthones and quinones.

Table 2. Phytoconstituents of various species of *Iris*.

S. No.	**Species**	**Compounds reported**	**References**
1	*I. lactea* (LEAVES)	C-glycosylflavones IrislactinA and IrislactinB	W. J. Shen, M. J. Qin, P. Shu and C. F. Zhang (2008). ***Chinese chemical letters,*** 19 (821-824).
2	*I. ensata*	5-hydroxy-4′-methoxyflavone, 5-hydroxy-3′-methoxyflavone, 5-hydroxy-2′-methoxyflavone. new type ofmajor anthocyanins medium chain fatty acids	E.V. Boltenkov, V.G. Rybin, and E.V. Zarembo (2005). ***Chemistry of Natural Compounds,*** 41 (5). J. B. Kim, J. B. Kim, K. H. Kim, S. K. Hwang, Y. H. Kim, K. J. Cho, Y. S.Hwyang, R.D. Park. (2001). ***Hanguk Nonghwa Hakhocchi.***44(1), 20-23.

[108] Chopra R.N., Nayer, S. L. and Chopra. **1956**. *Glossary of Indian medicinal plants*.

[109] Chopra R.N., Nayer, S. L. and Chopra.**1956**. *Glossary of Indian medicinal plants.*

[110] Chopra R.N., Nayer, S. L. and Chopra. **1956**. *Glossary of Indian medicinal plants.*

[111] Yabaya, T., Imayama, T., Shimomura, T., Urushihara, R., Yamaguchi, M. **2001**. *Euphytica.* 118(3), 253-256.

[112] Jacob Vaya and SnaitTamir. (2004). Current medicinal chemistry, 11, 1333-1343..

3	*I. germanica*	Triterpenoids	G.T. Verlag .(2001). **Plant Med**. 67(1),79-81.

4	*I. petrana*	xanthose and isoflavones	T . Aburjai., B. Amro, S. Al-Khalil, D.Al-eisawi. (2000). ***Acta Technol. Legis Med***. 11(3), 137-145.
5	*I. germanica L.* var. alba (RHIZOMES)	isoflavonoid glycoside, 8,4′-dimethoxyisoflavone-7-O-glucoside together with irisflorentin, irisolone, irisolone-4′-diglucoside, iristectorin A and irisxanthone	A.A. Ali, N.A. El-emary, F.M. Darwish. (1993) ***Bull. Pharm. Sci***., 16(2), 159-62.
6	*I. germanica* L.var.alba (LEAVES)	flavone-C-glycoside, swertisin, xanthone glycoside mangiferin and isomangiferin	A.A. Ali, N.A. El-emary, F.M. Darwish. (1993) ***Bull. Pharm. Sci***., 16(2), 159-62.
7	*I. pseudacorus* L.	two isoflavones- irisolidone and irigenin with an isoflavone glycoside iridin	A.A. Ali, N.A. El-emary, F.M. Darwish(1993) ***Bull. Pharm. Sci.,*** 16(2), 159-62.
8	*I. tectorum*	iristectorenes A and C-G, monocyclic triterpene esters	S. Katsura, T. Toshiya, H. Kazuo, K.Ryohei. (1994), ***Phytochemistry.*** 36 (2), 425-431.
9	*I. ensata* (FLOWERS)	p-coumaroyl glycosides of cyanidin and peonidin	T. Yabuya, M. Nakamura, A. Yamasaki. (1993). ***Euphytica.*** 74(1-2), 47-50.

10	*I. pseudacorus* (LEAVES)	5,7,3′-trihydroxy-4′-methoxycoumaronochromone (ayameninE), two flavones (apigenin and hispidulin), two flavanonols (alpinone and 7-O-methyldihydrokaempferol) and 5,7,2′-trihydroxyflavanone.	H. Fujinori, T. Satoshi, M. Junya. (1991). ***Phytochemistry.*** 30(7), 2197-2198.
11	*I. ensata*	C-glycosides of luteolin	N.I . Pryakhina, K.F. Blinova. (1984). ***Khim. Prir. Soedin***. 1, 109-110
12	*I. germanica*	three isoflavonoids irisolidone, irigenin, iridin	A.A. Ali, N.A. El-Emary, M.A. El-Moghazi,F.M. Darwish, A.W. Frahm. (1983). ***Phytochemistry.*** 22(9), 2061-2063.
13	*I. versicolor* L (RHIZOMES)	monocyclic C 31-triterpenoid	K. Wolfgang, M. F. Josef, J.Lothar, Z. C. Naturforsch. (1983). ***Biosci***. 38c (9-10), 689-92.
14	*I. kumaonensis*	isoflavone iriskumaonin	A.K. Kalla, M.K. Bhan,K.L. Dhar. (1978). ***Phytochemistry***, 17(8), 1441-2.
15	*I. ensata* (AERIAL PARTS)	4′-7-dimethoxyapigenin-6-C-β-D-glucopyranosyl-O-L-rhamnose.	K.F. Blinova, V.I. Glyzin, N.I. Pryakhina. (1977). ***Khim. Prir. Soedin***., (1), 116.
16	*I. hollandica* (BULBS)	3-demethylplastoquinone 9 and 3-demethylplastoquinone 8	E.G. Claudine, T. Claude, P. Judith. (1977). ***Nouv. J. Chim*** 1(4), 323-325.
17	*I. kashmiriana*	Irisolidone	K.L. Dhar, A.K. Kalla. (1975). ***J. Indian Chem. Soc***. 52(8), 784.
18	*I. pseudacorus*	Irisquinone	S. katsura, K. Ryohei.(1975) ***Chem, Ind***., (8), 349-50.
19	*I. germanica*	Carotenoids	B. Richard, L.J. Synnove (1975) ***Phytochemistry***, 14 (3), 851-2.

20	*I. hollandica*	Terpenes	L.S. George, A.A. De hertogh, A. Amedo. (1972) ***J. Amer. Soc. Hort. Sci***., 97(2), 189-91.
21	*I. tectorum*	isoflavone glycoside	M. Naokata, S. Masami, S. Mineo, A. Munehisa. (1972) ***Chem. Pharm. Bull.***, 20(4), 730-3.
22	*I. hollandica*	Steroids	L.S. George, A.A. De hertogh, A. Amedo.(1972) ***J. Amer. Soc. Hort. Sci.***,7(4), 411-12.
23	*I. kumaonensis*	Isoflavones	K.L. Dhar, A.K. Kalla. (1972) ***Phytochemistry***, 111(10), 3097-8
24	*I. tingitana*	delphanin and C-glycosyl flavones	A. Sam, N.R. Stewart, K.H. Norris, D.R. Massie. (1970) ***Phytochemistry***, 9(3), 619-27.
25	*I. germanica*	flavone C-glycosides	A. Kawase, K. Yagishita. (1968). ***Agr. Biol. Chem***. 32(4), 537-538.
26	*I.ensata (garden iris)*	mangiferin and glycophenolics	E.C. Bate-Smitha, J.B. Harborne. (1963) ***Nature 198***, 1307-1308.

2.1. OBJECTIVES FOR UNDERTAKING STUDIES OF *IRIS ENSATA* AND *IRIS HOOKERIANA*:

Flavonoids are ubiquitous in plant foods and drinks and therefore, a significant quantity is consumed in our daily diet. These are variously associated with the sensory and nutritional quality of our plant foods. A number of biological activities for flavonoids have been described extensively in literature[113]. An isoflavone genistein has been shown to block the action of a transcription factor, known as CCAAT binding factor neutralizing it before the switch is tripped, so that the cancer cell starves, withers and dies. Thus genistein commonly consumed as a component of soya bean, is a flavonoid capable of stopping cancer growth and angiogeesis. Crucially, it

[113] Mirheidar M, Moaref G. 1996 Tehran: Daftar Nashar Farhang-e-Islami,6:326.

has no harmful effect on normal healthy cells.[114] The estrogenic activity of genistein, daidzen and equol are currently being extensively investigated at the molecular, pre-clinical and clinical levels to determine their potential for treatment of chronic diseases such as hormone dependent cancer, cardiovascular diseases and osteoporosis.[115]

Inspired by a broad spectrum of biological activities possessed by flavonoids and a rich folklore record of Iris species, we decided for I.ensata and I.hookeriana from pharmacological point of view. Detailed literature available on phytochemical investigation of the genus[116] established the fact that it is a good source of biologically active compounds particularly isoflavones. These facts prompted us to undertake Iris ensata and Iris hookeriana,the two less explored species of kashmir for the biological activity evaluation.

[114] Coghlan A. 1998 New Scientist, 14 March, 14.

[115] Wiseman H. 2000 Exp. Opin. Investig. Drugs, 9:1829.

[116] Wollenweber E, Stevens JF, Kilmo K, Knauft J, Frank N, Gerhauser. 2003. Planta Medica, 69:15.

Chapter 3

Bioactivity evaluation of extracts of Iris ensata

Kingdom	**:**	**Plantae**
Subkingdom	**:**	**Tracheobionta**
Superdivision	**:**	**Spermatophyta**
Division	**:**	**Magnoliophyta**
Class	**:**	**Liliopsida**
Subclass	**:**	**Liliadae**
Order	**:**	**Liliales**
Family	**:**	**Iridaceae**
Genus	**:**	***Iris***
Species	**:**	***ensata***

IRIS ENSATA

Japanese iris or Japanese water iris is a rhizomatous beardless perennnial iris that grows in slowly expanding clumps to 2-4′ tall. Sword shaped, linear green leaves (to 24″ long) have prominent midribs. Flowers (typically 3-6″ across) have a distinctively flatened appearance. Cultivars come in single, double and peony flowered forms in a wide range of colours including shades of blue, lavender, violet-red, pink and white.

3.1. ANTITUMOR ACTIVITY OF RHIZOMES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris ensata* were subjected to screening for antitumor activities against various human cell lines. The reference compound used was 5-florouracil. Results are displayed in table-3.

Table 3. Antitumor activity of rhizomes of *Iris ensata*

Tissue Type			Leukemia	Prostate	Colon	Lung
Cell Line Type			THP-1	PC-3	HCT-15	A549
S.No.	Code	Conc. (µg/ml)	% GROWTH INHIBITION			
1.	DCM	100	**99±0.02**	**82±0.07**	**93±0.04**	**79±0.02**
2.	DCM	50	**91±0.04**	**59±0.05**	**85±0.05**	**62±0.03**
3.	PE	100	37±0.05	**67±0.03**	**79±0.02**	**62±0.04**
4.	PE	50	18±0.03	32±0.05	**54±0.07**	34±0.06
5.	MeW	100	**73±0.06**	47±0.04	34±0.04	64±0.07
6.	MeW	50	56±0.05	26±0.03	15±0.06	36±0.05
7.	MeOH	100	**76±0.06**	35±0.05	**76±0.07**	**82±0.07**
8.	MeOH	50	49±0.07	15±0.03	52±0.01	**76±0.02**
9.	EtOAc	100	**93±0.04**	47±0.05	**63±0.03**	**78±0.01**
10.	EtOAc	50	**73±0.03**	26±0.03	35±0.05	**62±0.07**
11.	5-FU	$1x10^{-5M}$	**92±0.03**	43±0.01	**90±0.02**	**62±0.04**

Results and Discussion

The dichloromethane extract showed the highest %growth inhibition of the cancer cell lines followed by the methanol water extract and then petroleum ether extract. The dichloromethane extract showed the potency for leukemia, prostate, colon and lung cancer whereas methanol water showed potency for leukemia and petroleum ether for colon cancer respectively.

Dichloromethane extract showed the presence of steroids and flavonoids whereas methanol water extract contained glycosides. So we could infer that the antitumor activity against the cell lines THP-1, PC-3, HCT-15, A549 is possibly due to the presence of these compounds either at individual level or through a synergistic mode within the class of compounds or between inter class of compounds. The antitumor activity of the extracts of *Iris ensata* is being reported for the first time.

3.2. ANTIOXIDANT ACTIVITY OF RHIZOMES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris ensata* were subjected to screening for antioxidant activities against DPPH. The standard used was quercetin. Results are displayed below:

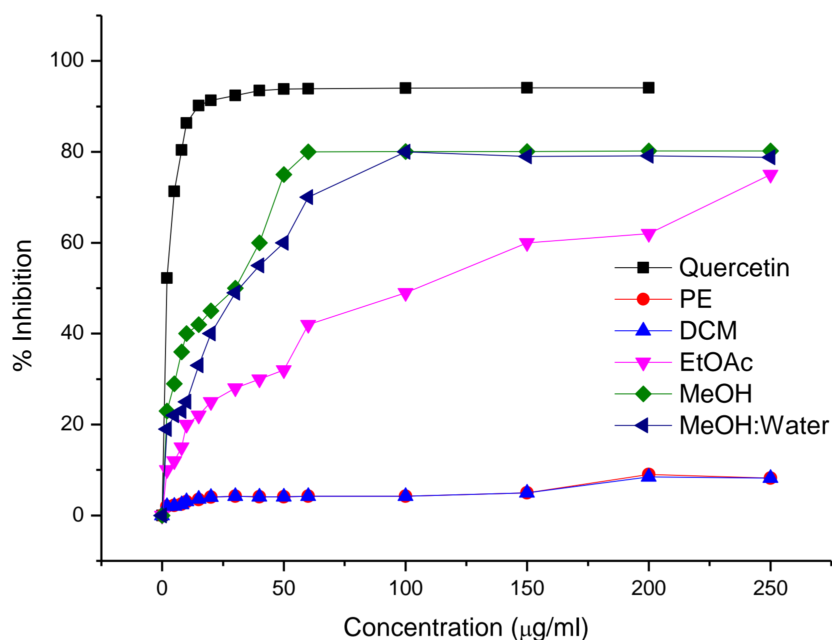


Table 4. IC_{50} (μg/ml) values of the extracts of *Iris ensata*.

Extract	IC_{50} (μg/ml)
Standard	2.09
PE	NA
DCM	NA
EtOAc	49.93
MeOH	29.18
MeOH:H_2O	31.43

NA: Not Active

Results and Discussion

The antioxidant activity performed on the extracts of the rhizomes showed that the methanol extract exhibited the highest activity with IC_{50} equal to 29.18 whereas methanol water showed less activity compared to the methanol extract.

Ethyl acetate extract also showed some good results. Methanol extract possessed flav onoids due to which the possible antioxidant activity is reported. The presence of glyc osides in the methanolwater extract can be attributed to some degree of antioxidant ac tivity. At higher concentration of the extract the ethylacetate showed the good amount of antioxidant activity which could be attributed due to the presence of principal class of compound present in it such as flavonoids, terpenoids and glycosides.

3.3. ANTIMICROBIAL ACTIVITY OF RHIZOMES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris ensata* were subjected to screening for antimicrobial activities against various microbial strains. Results are displayed in table-4

Primary Screening: The samples were tested at 256µg/ml concentration for antimicrobial activity.

Table 5. Antimicrobial activity of rhizomes of *Iris ensata*

S.No:	Tested Sample	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	*C. albicans* ATCC 90028	*A. fumigatus*
1.	DCM	Inactive	Inactive	Inactive	Inactive
2.	MeOH	Inactive	Inactive	Inactive	Inactive
3.	EtOAc	Inactive	Inactive	Inactive	Inactive
4.	PE	Inactive	Inactive	Inactive	Inactive
5.	MeW	Inactive	Inactive	Inactive	Inactive

Results and Discussion

Preliminary screening of the extracts showed no significant antimicrobial activity. None of the extracts were potent for the said activity.

3.4 ANTITUMOR ACTIVITY OF LEAVES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of leaves of *Iris ensata* were subjected to screening for antitumor activities against various human cell lines. The reference compound used was 5-florouracil. Results are displayed in table-5.

Table 6. Antitumor activity of leaves of *Iris ensata*

Tissue Type			**Breast**	**Lung**	**Pancreatic**	**Prostate**
Cell Line Type			**MCF-7**	**A-549**	**MiaPaca**	**PC-3**
S.No.	**Code**	**Conc. (μg/ml)**	**% Growth Inhibition**			
1.	PE	**10**	**57±0.04**	28±0.14	33±0.25	8±0.09
2.		**50**	**67±0.01**	44±0.06	41±0.34	22±0.13
3.	DCM	**10**	**68±0.01**	**67±0.23**	31±0.42	9±0.04
4.		**50**	**76±0.03**	**76±0.08**	**59±0.18**	33±0.04
5.	EtOAc	**10**	**53±0.05**	26±0.02	21±0.23	4±0.11
6.		**50**	**83±0.03**	29±0.08	36±0.04	13±0.09
7.	MeOH	**10**	8±0.09	21±0.08	18±0.11	7±0.11
8.		**50**	11±0.89	**62±0.05**	**54±0.03**	17±0.02
9.	MeW	**10**	6±0.02	15±0.09	40±0.20	**60±0.03**
10.		**50**	**59±0.11**	26±0.12	**68±0.24**	**98±0.05**
11.	5-FU	**20μM**	-	**77±0.09**	-	**64±0.06**

Results and Discussion

The ethyl acetate and dichloromethane extract showed the highest %growth inhibition of the cancer cell lines followed by the methanol water, methanol and petroleum ether extract. The dichloromethane extract showed the potency for breast, lung and pancreatic cancer whereas ethyl acetate extract showed highest % growth inhibition for breast cancer respectively. Methanol extract showed potency for lung and

pancreatic cancer whereas methanol water showed potency for breast, pancreatic and prostate cancer respectively. Petroleum ether showed promising % growth inhibition for breast cancer.

Dichloromethane extract showed the presence of steroids, flavonoids and glycoside whereas ethyl acetate extract contained terpenoids in addition to the above mentioned principal class of compounds. Methanol and methanol water extract showed the presence of terpenoids, flavonoids and glycosides respectively. Petroleum ether extract showed the presence of steroids. So we could infer that the antitumor activity against the cell lines MCF-7, A-549, MiaPaca, PC-3 is possibly due the presence of these compounds giving the results either individually or synergistically.

The antitumor activity of the extracts of *Iris ensata* is being reported for the first time. The results shown can be utilized for obtaining the corresponding leads so that the work done can prove beneficial in the herbal medicine.

3.5. ANTIOXIDANT ACTIVITY OF LEAVES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of leaves of *Iris ensata* were subjected to screening for antioxidant activities against DPPH. The standard used was quercetin. Results are displayed below:

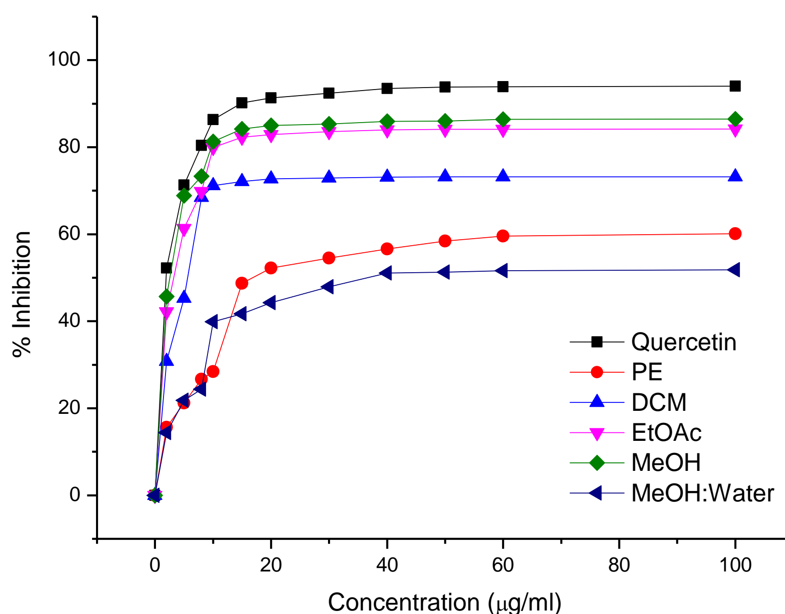


Table 7. IC_{50} (μg/ml) values of the extracts of *Iris ensata*.

Extract	IC_{50} (μg/ml)

Standard	2.09
PE	16.18
DCM	5.65
EtOAc	3.24
MeOH	2.34
MeOH: Water	36.35

Results and Discussions

The antioxidant activity performed on the extracts showed that methanol extract exhibited the highest activity with IC_{50} equal to 2.34 almost comparable to that of the standard followed by ethyl acetate extract with IC_{50} equal to 3.24. Dichloromethane extract also showed some good results. Methanol extract possessed flavonoids and glycosides. Ethyl acetate extract possessed flavanoids besides glycosid es due to which the possible antioxidant activity is reported. The presence of flavonoi ds and glycosides as principal class ofcompounds in the dichloromethane extract can be attributed to antioxidant activity. At higher concentration of the extract even the p etroleum ether showed some antioxidant activity due to expected presence of trace amount of these compounds in it.

3.6. ANTIMICROBIAL ACTIVITY OF LEAVES OF *IRIS ENSATA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of leaves of *Iris ensata* were subjected to screening for antimicrobial activities against various microbial strains. Results are displayed in table-6.

Primary Screening: The samples were tested at 256µg/ml concentration for antimicrobial activity

Results and Discussions

No significant results could be observed for the extracts subjected for the antimicrobial activity.

Table 8. Antimicrobial activity of leaves of *Iris ensata*

S.No:	**Tested Sample**	***S. aureus* ATCC 29213**	***E. coli* ATCC 25922**	***C. albicans* ATCC 90028**	***A. fumigatus***
1.	EN/L-Z	Inactive	Inactive	Inactive	Inactive
2.	EN/L-Y	Inactive	Inactive	Inactive	Inactive
3.	EN/L-X	Inactive	Inactive	Inactive	Inactive
4.	EN/L-W	Inactive	Inactive	Inactive	Inactive
5.	EN/L-V	Inactive	Inactive	Inactive	Inactive

Chapter 4

Bioactivity evaluation of extracts of Iris hookeriana

Kingdom	**:**	**Plantae**
Subkingdom	**:**	**Tracheobionta**
Superdivision	**:**	**Spermatophyta**
Division	**:**	**Magnoliophyta**
Class	**:**	**Liliopsida**
Subclass	**:**	**Liliadae**
Order	**:**	**Liliales**
Family	**:**	**Iridaceae**
Genus	**:**	***Iris***
Species	**:**	***hookeriana***

IRIS HOOKERIANA

Roots slender, fleshy. Rhizome slender, knobbly. Leaves up to 40.0 (2 cm. Peduncle (stem) 5-15 (-30) cm. Bracts 4.5-7 cm long. 2-flowered; pedicel very short. Flowers blue-purple, with blotches; tube 1.2-3.0 cm long; falls 5-6.5 cm long, c. 2.0 cm broad; haft cuneate, blade oblong, beard white, tips coloured. Standard 5.0(2.0 cm, haft canaliculate, blade oblong. Filaments blue, as long as creamy anthers. Ovary c. 1.2-1.3 cm long, trigonal, style sharply keeled, crest triangular; stigma with serrated edge. Capsule 5-6 cm long, broadly elliptic, terminating into a conspicuous beak with dried flower parts; stipe (1.5-) 2.0-2.5 cm; dehiscence longitudinal. Seeds pyriform, red, aril yellowish.

4.1. ANTITUMOR ACTIVITY OF RHIZOMES OF *IRIS HOOKERIANA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris hookeriana* were subjected to screening for antitumor activities against various human cell lines.The reference compound used was 5-florouracil. Results are displayed in table-7.

Table-9: Antitumor activity of rhizomes of *Iris hookeriana*

Tissue Type			Leukemia	Prostate	Colon	Lung
Cell Line Type			THP-1	PC-3	HCT-15	A549
S.No.	Code	Conc. (µg/ml)	% GROWTH INHIBITION			
1.	DCM	100	26±0.04	**63±0.07**	**71±0.008**	**58±004**
2.	DCM	50	13±0.03	40±0.04	55±0.04	32±0.03
3.	MeOH	100	**67±0.03**	52±0.05	26±0.03	32±0.04
4.	MeOH	50	39±0.05	26±0.01	14±0.02	13±0.06
5.	MeW	100	**67±0.02**	**79±0.05**	51±0.05	45±0.02
6.	MeW	50	36±0.01	**58±0.03**	22±0.06	21±0.01
7.	PE	100	**82±0.06**	25±0.06	**92±0.03**	**69±0.04**
8.	PE	50	**59±0.03**	11±0.01	**87±0.02**	45±0.06
9.	EtOAc	100	**78±0.06**	**97±0.05**	**98±0.01**	**89±0.08**
10.	EtOAc	50	54±0.01	**93±0.02**	**92±0.07**	60±0.05
11.	5-FU	$1x10^{-5M}$	**92±0.05**	43±0.04	**90±0.05**	62±0.01

Results and Discussions

The ethyl acetate extract showed the highest %growth inhibition of the cancer cell lines followed by the dichloromethane extract and then petroleum ether extract. The ethyl acetate extract showed the potency for leukemia, prostate, colon and lung cancer whereas dichloromethane showed potency for prostate, lung and colon and petroleum ether for leukemia, colon and lung cancer respectively. Ethyl acetate extract showed the presence of terpenoids, flavonoids and glycosides whereas dichloromethane extract contained terpenoids and flavonoids. Petroleum ether extract showed the presence of steroids and terpenoids. So we could infer from the results of the antitumor activity against the cell lines THP-1, PC-3, HCT-15, A549 is possibly due the presence of these compounds giving the results either individually or synergistically. The antitumor activity of the extracts of *Iris hookeriana* is being reported for the first time.

4.2. ANTIOXIDANT ACTIVITY OF RHIZOMES OF IRIS HOOKERIANA

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris hookeriana* were subjected to screening for antioxidant activities against DPPH. The standard used was quercetin. Results are displayed below:

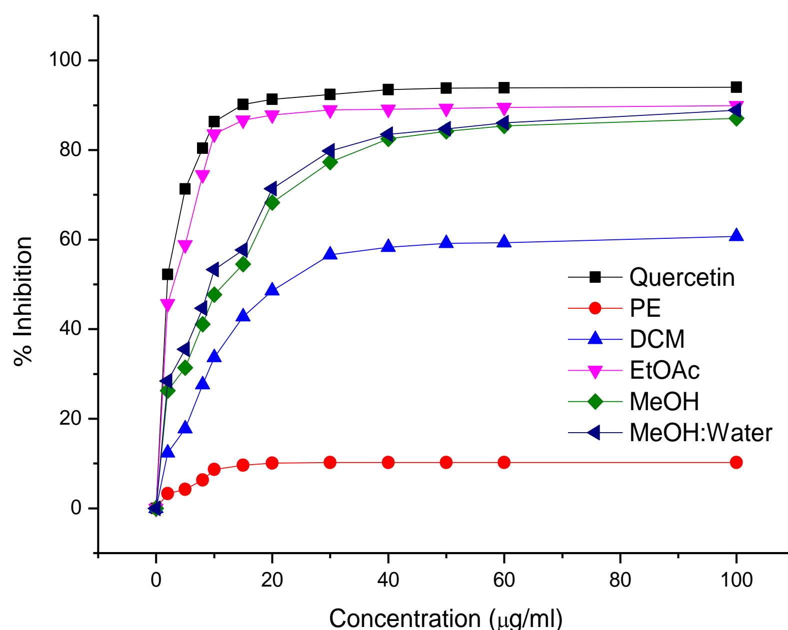


Table 10. IC_{50} (μg/ml) values of the extracts of *Iris hookeriana* rhizomes

Extract	IC_{50} (μg/ml)
Standard	2.09
PE	NA
DCM	21.90
EtOAc	2.94
MeOH	11.67
MeOH: Water	9.26

Results and Discussion: The antioxidant activity of the extracts showed that ethyl acetate extract exhibited the highest activity with to 2.94 which is comparable to that

of the quercetin followed by methanol water extract. Methanol extract also showed so me significant results.

The potential antioxidant activity of ethyl acetate extract could mainly be attributed to the presence of flavonoids and to some extent to glycosides and terpenoids present in it. Same holds true about methanol and methanol water extract.

4.3. ANIMICROBIAL ACTIVITY OF OF IRIS HOOKERIANA

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris hookeriana* were subjected to screening for antimicrobial activities against various microbial strains. Results are displayed in table-8.

Primary Screening: The samples were tested at 256μg/ml concentration for antimicrobial activity

Table 11. Animicrobial activity of of *Iris hookeriana*

S.No:	Tested Sample	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	*C. albicans* ATCC 90028	*A. fumigatus*
1.	MeW	Inactive	Inactive	Inactive	Inactive
2.	MeOH	Inactive	Inactive	Inactive	Inactive
3.	DCM	Inactive	Inactive	Inactive	Inactive
4.	EtOAc	Inactive	Inactive	Inactive	Inactive
5.	PE	Inactive	Inactive	Inactive	Inactive

Results and Discussion

All the extracts were inactive towards antimicrobial activity.

4.5. ANTITUMOR ACTIVITY OF LEAVES OF *IRIS HOOKERIANA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of leaves of *Iris hookeriana* were subjected to screening for antitumor activities against various human cell lines. The reference compound used was 5-florouracil. Results are displayed in table-9.

Table 12. Antitumor activity of leaves of *Iris hookeriana*

Tissue Type			**Breast**	**Lung**	**Pancreatic**	**Prostate**
Cell Line Type			**MCF-7**	**A-549**	**MiaPaca**	**PC-3**
S.No.	**Code**	**Conc.(µg/ml)**	**% growth inhibition**			
1.	PE	**10**	**89±0.05**	**60±0.14**	**63±0.01**	**87±0.02**
2.		**50**	**99±0.13**	**81±0.02**	**79±0.03**	**98±0.01**
3.	DCM	**10**	**53±0.08**	**77±0.04**	**65±0.06**	**54±0.05**
4.		**50**	**96±0.03**	**97±0.08**	**95±0.08**	**77±0.04**
5.	EtOAc	**10**	10±0.08	6±0.01	**52±0.01**	29±0.01
6.		**50**	**52±0.89**	35±1	**80±1**	**56±1**
7.	MeOH	**10**	4±0.08	3±0.09	46±0.24	10±0.09
8.		**50**	10±0.15	13±0.03	**55±0.09**	**68±0.05**
9.	MeW	**10**	4±0.04	32±0.08	27±0.01	19±0.02
10.		**50**	10±0.03	**65±0.18**	40±0.36	**52±0.04**
11.	5-FU	**20µM**	-	**77±0.09**	-	**64±0.06**

Results and Discussion

The petroleum ether and dichloromethane extract showed the highest %growth inhibition of the cancer cell lines followed by the ethyl acetate, methanol and methanol water extracts. The dichloromethane extract showed the potency for breast,

lung, pancreatic and prostate cancer whereas ethyl acetate extract showed high % growth inhibition for breast, pancreatic and prostate cancer at higher concentration. Methanol extract showed potency for pancreatic and prostate cancer whereas methanol water showed potency for lung and prostate cancer respectively at higher concentration.

The presence of the principal class of compounds viz. flavonoids, terpenoids and glycosides present in these extracts, could be responsible for the reported activities either at individual level or through synergistic mode. The dichloromethane extract showed the highest possible activity for the cancer cell lines. Petroleum extract is equally potent. Therefore further studies on those extracts if undertaken based on the isolation and characterization of the compounds can fetch us a good lead so that a better pharmacopeia can be added to the literature of the herbal medicine.

Dichloromethane extract showed the presence of terpenoids and flavonoids whereas petroleum ether extract contained steroids and terpenoids as the principal class of compounds. Ethyl acetate extract showed the presence of terpenoids, flavonoids and glycosides. Methanol and methanol water extracts contained flavonoids and glycosides respectively. So we could infer that the antitumor activity against the cell lines MCF-7, A-549, MiaPaca, PC-3 is possibly due the presence of these compounds giving the results either individually or synergistically.

The antitumor activity of the extracts of *Iris hookeriana* is being reported for the first time.

4.4. ANTIOXIDANT ACTIVITY OF LEAVES OF *IRIS HOOKERIANA*

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris hookeriana* were subjected to screening for antioxidant activities against DPPH. The standard used was quercetin. Results are displayed below:

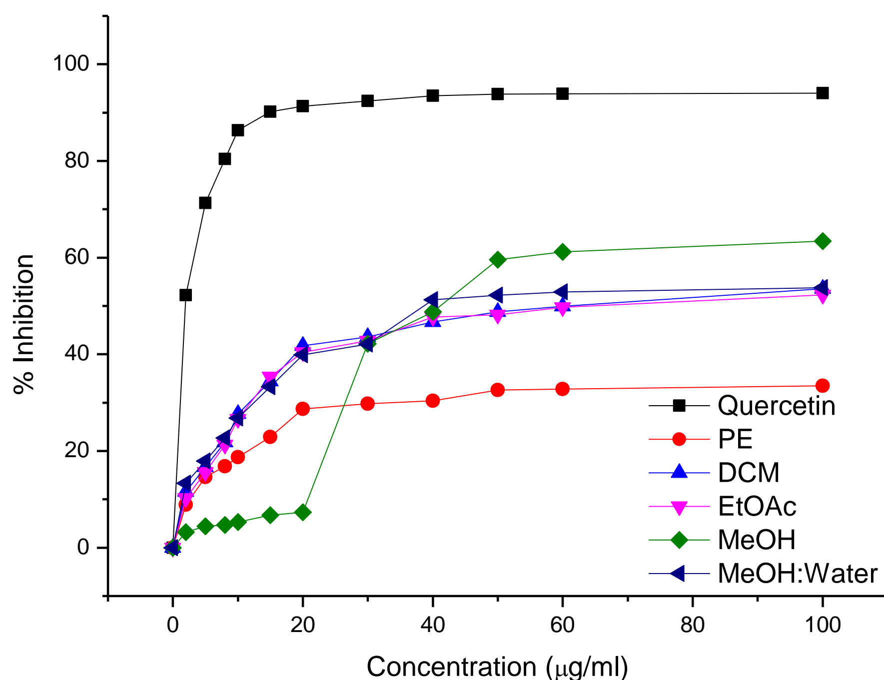


Table 13. IC_{50} (μg/ml) values of the extracts of *Iris hookeriana* leaves

Extract	IC_{50} (μg/ml)
Standard	2.09
PE	NA
DCM	62.64
EtOAc	67.45
MeOH	41.27
MeOH: Water	38.26

Results and Discussion

The antioxidant activity performed on the extracts showed that methanol water extract exhibited the highest activity followed by methanol extract. Dichloromethane and ethyl actetate extract also showed some amount of activity.

Methanol water extract possessed flavonoids and their glycosides due to which the po ssible antioxidant activity is reported. The presence of flavonoids and glycosides as pr incipal class of compounds in the methanol extract as well can be attributed to antioxi dant activity. At higher concentration of the extract, the dichloromethane and ethyl

acetate extract showed some amount of antioxidant activity presumably due to the lower concentration of flavonoids and their glycosides respectively.

4.5. ANTIMICROBIAL ACTIVITY OF LEAVES OF *IRIS HOOKERIANA*.

The petroleum ether, dichloromethane, ethyl acetate, methanol and methanol water extract of rhizomes of *Iris hookeriana* were subjected to screening for antimicrobial activities against various microbial strains. Results are displayed in table-10.

Primary Screening: The samples were tested at 256µg/ml concentration for antimicrobial activity.

Table 14. Antimicrobial activity of leaves of *Iris hookeriana*.

S.No:	Tested Sample	S.aureus ATCC 29213	E.coli ATCC 25922	C.albicans ATCC 90028	A.fumigatus
1.	PE	Inactive	Inactive	Inactive	Inactive
2.	DCM	Inactive	Inactive	Inactive	Inactive
3.	EtOAc	Inactive	Inactive	Inactive	Inactive
4.	MeOH	Inactive	Inactive	Inactive	Inactive
5.	MeW	Inactive	Inactive	Inactive	Inactive

Results and Discussion

No antimicrobial activity could be observed for the extracts.

Chapter 5

Experimental

5. EXPERIMENTAL WORK:

Collection of the plant material

The plant material was collected during their blooming seasons respectively. *Iris ensata* was collected from Pantha Chowk area of the Valley during the month of May and was identified by the Institute of Plant taxanomy, university of kashmir and was registered under vocher number 35606(KASH). *Iris hookeriana* was collected from Doodpathrie area of the Valley during the month of June and was identified by the Istitute of Plant taxanomy, university of kashmir and was registered under vocher number 1210(KASH).

Drying of plant material

The plant material obtained was subsequently separated into its different parts viz, rhizomes, leaves and flowers respectively. The plant parts were shade dried.

Pulverization of different parts of the plants

The dried plant material was then pulverized partwise and extracted separately using soxhlet apparatus with solvents of increasing polarity starting from pet ether, dichloromethane, ethyl acetate, methanol to methanol water for different time periods.

PLANT PART: RHIZOMES

The rhizome part of *Iris ensata* and *Iris hookeriana* was subjected to soxhlet extraction with following solvent system for the given period of time. The respective extracts were dried with rotavapour. The time period and the yields obtained are tabulated as under.

Table 15. Yield of extracts of rhizomes.

Extracts	*Iris ensata* Time/weight of the residue	*Iris hookeriana* Time/weight of the residue
Pet ether	8 hours/8.7 gms	12 hours/5.34 gms
DCM	11 hours/5.57 gms	28 hours/64.6 gms
Ethyl acetate	13 hours/5.4 gms	40 hours/31.98 gms
Methanol	17 hours/27.36 gms	48 hours/169.74 gms

Methanol water	13 hours/13 gms	32 hours/110.02 gms

Table 16. Qualitative Chemical analysis.

Extracts	**Steroids**	**Terpenoids**	**Flavanoids**	**Glycosides**	**Alkaloids**
Pet ether Ie/Ih	positive/ positive	negative/ positive	negative/ negative	negative/ negative	negative negative/
DCM Ie/Ih	positive/ negative	negative/ positive	positive/ positive	negative/ negative	negative/ negative
Ethyl acetate Ie/Ih	positive/ negative	positive/ positive	positive/ positive	positive/ positive	negative/ negative
Methanol Ie/Ih	negative/ negative	negative/ positive	positive/ positive	negative/ positive	negative/ negative
Methanol:water Ie/Ih	negative/ negative	negative/ negative	negative/ positive	positive/ positive	negative/ negative

Observation based on chemical analysis:

Qualitative chemical tests performed on the extracts of the rhizomes of *Iris ensata* and *Iris hookeriana* showed the presence of different principal class of compounds present in them.

The petroleum ether extract of iris ensata showed the presence of steroids. The dichloromethane extract showed the presence of steroids and flavonoids. The ethyl acetate extract showed the presence of steroids, terpenoids, flavonoids and their glycosides. The methanol extract contained flavonoids and methanol water extract contained glycosides respectively.

Similarly the petroleum ether extract of rhizomes of iris hookeriana showed the presence steroids and terpenoids. The dichloromethane extract showed the presence of terpenoids and flavonoids. The ethyl acetate extract showed the presence of terpenoids, flavonoids and their glycosides. The methanol extract contained terpenoids, flavonoids and their glycosides. The methanol water extract contained flavonoids and glycosides respectively.

PLANT PART : LEAVES

The leaves part of *Iris ensata* and *Iris hookeriana* was subjected to soxhlet extraction with following solvent system for the given period of time. The respective extracts were dried with rotavapour.The time period and the yields obtained are tabulated as under:

Table 17. Yield of extracts of leaves.

Extracts	***Iris ensata*** **Time/weight of the residue**	***Iris hookeriana*** **Time/weight of the residue**
Pet ether	4 hours/12.67 gms	10 hours/16.34 gms
DCM	16 hours/7 gms	5 hours/8.6 gms
Ethyl acetate	5 hours/10 gms	10 hours/11.98 gms
Methanol	13 hours/27.36 gms	11 hours/60.74 gms
Methanol:water	5 hours/13 gms	5 hours/11.02 gms

Table 18. Qualitative chemical analysis

Extracts	**Steroids**	**Terpenoids**	**Flavanoids**	**Glycosides**	**Alkaloids**
Pet ether Ie/Ih	Positive/ Positive	Negative/ Positive	Negative/ Negative	Negative/ Negative	Negative/ Negative
DCM Ie/Ih	Positive/ Negative	Negative/ Positive	Positive/ Positive	Positive/ Negative	Negative/ Negative
Ethyl acetate Ie/Ih	Positive/ Negative	Positive/ Positive	Positive/ Positive	Positive/ Positive	Negative/ Negative
Methanol Ie/Ih	Negative/ Negative	Positive/ Negative	Positive/ Positive	Positive/ Positive	Negative/ Negative
Methanol water Ie/Ih	Negative/ Negative	Positive/ Negative	Positive/ Positive	Positive/ Positive	Negative/ Negative

Observation based on chemical analysis:

Qualitative chemical tests performed on the extracts of the rhizomes of *Iris ensata* and *Iris hookeriana* showed the presence of different principal class of compounds present in them.

The petroleum ether extract of leaves of *Iris ensata* showed the presence of steroids. The dichloromethane extract showed the presence of steroids, flavonoids and glycosides. The ethyl acetate extract showed the presence of steroids, terpenoids, flavonoids and glycosides. The methanol and methanol water extracts contained terpenoids, flavonoids and glcosides respectively.

Similarly the petroleum ether extract of leaves of *Iris hookeriana* showed the presence steroids and terpenoids. The dichloromethane extract showed the presence of terpenoids and flavonoids. The ethyl acetate extract showed the presence of terpenoids, flavonoids and glycosides. The methanol extract contained flavonoids and glycosides. The methanol water extract also contained flavonoids and glycosides respectively.

ANTI-TUMOR ACTIVITY:

Cytotoxicity assay by Sulphorhodamine B Dye (SRB):

SRB assay is a rapid, sensitive and inexpensive method for measuring the cytotoxic potential of test substances, based on the cellular protein content of adhered suspension cultures in 96 well plates.

This method is suitable for ordinary laboratory purposes and for large-scale applications like high through put *in vitro* screening in anticancer drug discovery. The anticancer activity was determined by the cytotoxic potential of the test material using human cancer cell line, which was allowed to grow on tissue culture plate in the presence of test material. The cell growth was measured on ELISA reader after staining with Sulphorhodamine B (SRB) dye which binds to basic amino acid residues in trichloroacetic acid (TCA) fixed cells

Principle of assay:

1. SRB is an anionic bright pink amino xanthene protein dye with two sulfonic groups. Its molecular formula and molecular weight are $C_{27}H_{30}N_2O_7S_2$ and 558.66 respectively.
2. The SRB assay is based on the ability of the SRB dye to bind electrostatically and pH dependent on protein basic amino acid residues. Under mild acidic conditions, SRB binds to protein basic amino acid residues of trichloroacetic acid (TCA)–fixed cells. It can be quantitatively extracted from cells and solubilized for optical densitiy (OD) measurement by weak bases such as tris base.

3. Color development in SRB assay is rapid, stable and visible. The end point of the SRB assay is colorimetric, nondestructive, and indefinitely stable. The SRB staining method is nondestructive in the sense that it is not necessary to digest samples.

   The method appears to offer several advantages over the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays- other cytotoxicity assays. It is simpler, faster and more sensitive than the MTT assay, provides better linearity with cell number, permits the use of saturating dye concentrations and is less sensitive to environmental fluctuations. It is independent of intermediatory metabolism and provides a fixed end point that does not require a time sensitive measurement of initial reaction velocity.

Procedure:

1. The plates were taken out from the incubator after 48 h of adding test samples.
2. To stop the reaction, 50µl of chilled 50% TCA (trichloroacetic acid) to each well of the plate was added, making final concentration to 10%.
3. Incubated the plates at 4°C for 1h to fix the cells attached to bottom of the wells.
4. Washed the plates 5-6 times with distilled water.
5. Plates were air-dried.
6. Added 100 µl of SRB dye (0.4% in 1%acetic acid) to each well of the plate and left the plates at room temperature for 30 min.
7. Washed the plates with 1% acetic acid after 30 min.
8. Plates were again air-dried.
9. Added 100µl of tris buffer (10.5 M) to each well.
10. Shaked the plates gently for 10-15 minutes on a mechanical shaker.
11. Recorded the optical density with ELISA reader at λ= 540nm.

Calculations:

The viability and growth in the presence of test material is calculated as

OD change = Mean of OD of test sample – mean of OD of blank

% Growth in presence of the control = 100/OD change in presence of control

% Growth in the presence of test sample = (% growth in presence of control) X
(OD change in presence of test sample)

% Inhibition by the test sample = 100 - % growth in the presence of test sample

$$\text{\% Inhibition by the Test sample} = \frac{\text{growth in presence of test material}}{\text{growth in absence of test material}} \times 100$$

T / C value for 50% growth inhibition for each test material is calculated from its three concentrations. The plant extract showing >70% growth inhibition at 100ug/ml and compound (small molecule) showing >50% growth inhibition at 1X10-5 M is considered to be active.

ANTIOXIDANT ACTIVITY:

Scavenging free radical potentials were tested in a methanolic solution of DPPH. The degree of decoloration of the solution indicates the scavenging efficiency of the added extract. To a 100 micro molar solution of DPPH, 0.01-0.15ml of 5mg/ml extract was added. Sixty minutes later, the absorbance was measured at 517 nm. The antiradical activity was calculated as a percentage of DPPH decoloration using the following equation:

Antiradical activity =100 × (1 - absorbance of sample/absorbance of reference)

ANTI-MICROBIAL ACTIVITY:

Antibacterial activity assay :

The extracts prepared were tested against the following bacterial strains: Gram positive Staphylococcus aureus ATCC 29213, Gram negative Escherichia coli ATCC 25922 . The bacterial cultures were maintained on Tryptone soya agar and stored at 70°C containing 50% glycerol. Ciprofloxacin obtained from Sigma-Aldrich was used as standard antibacterial agent for this study. Stock solution was prepared at 1mg/ml.. Bacterial suspensions were prepared by suspending 18-24 hrs grown bacterial cultures in sterile normal saline. The turbidity of the bacterial suspension was adjusted to 0.5

McFarland standards (equivalent to 1.5 × 108 CFU/ml) at wavelength 625nm. The 2-fold serial of compounds and extracts (stock solution prepared in DMSO) were prepared in MHB (Mueller Hinton Broth; DIFCO laboratories) in 100µl volume in 96-well U bottom microtitre plates (Tarson, Mumbai, India). The above mentioned bacterial suspensionwas further diluted in the MHB and 100µl volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of 5 × 105 CFU/ml in the well and the final concentrations of the samples ranged from 2000 to 3.90µg/ml till the 10th column. Column 11 and column 12 containing 100µl and 200µl of medium without drug served as growth and media control respectively. The plates were incubated at 37°C for 18 hrs and were read visually and the minimum concentration of the compound showing no turbidity was recorded as MIC.

Antifungal activity assay :

The disc diffusion method was used to determine the antifungal activity of the extracts using Sabouraud Dextrose Agar (SDA) (Hi-Media). The SDA plates were prepared by pouring 15ml of SDA into sterile Petri plates. The plates were allowed to solidify for 15 minutes and 0.1 ml (0.5 McFarland) inoculum suspension was swabbed uniformly and the plates were allowed to dry for 5 -10 minutes. 50 µg of the different extracts was loaded on 6 mm sterile discs (Hi Media). The loaded discs were placed on the surface of the medium and the samples were allowed to diffuse for 5 minutes and the plates were kept for incubation. Inhibition zones formed around the discs were measured with transparent ruler (in mm). Antibiotic disc (Nystatin) and solvent (methanol) were taken as positive and negative controls respectively.

Chapter 6

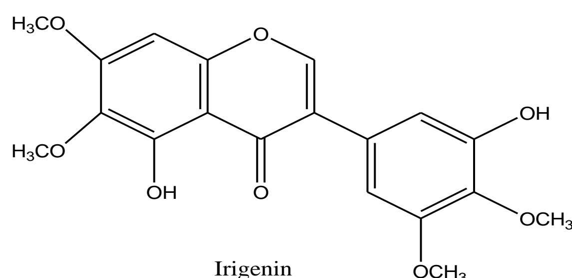
Isolation & characterization of Ih/DC and Ih/DC-2

6. ISOLATION OF Ih/DC AND Ih/DC-2

During soxhlet extraction of rhizomes of *Iris hookeriana* with dichloromethane for 18 hrs a residue was left out (8gm). On checking its purity on TLC it showed a multispot behaviour. On treating it with different solvents of varying polarity to carry out its crystallisation, two compounds were obtained. One of them was i dentified as 3′,5-dihydroxy 4′,5′,6,7-tetramethoxy isoflavone commonly known a s irigenin and another one was identified as 5′-hydroxy,3′,4′-dimethoxy-6,7-methylenedioxy isoflavone.

CHARACTERISATION OF Ih/DC

The compound Ih/DC was isolated as yellow needles (MeOH), m.p. 186-187 °C. The mass spectrum showed molecular ion peak at M^+ 374 analysed for $C_{19}H_{18}O_8$ that showed a molecular ion peak at m/z 374[M+1]. The UV spectrum showed absorption bands at λmax 201, 265, and 323 nm, suggesting its isoflavone nature, further confirmed by the singlet signal at δH 8.42 for H-2 in its ^{1}HNMR spectra. The IR spectrum showed absorption bands at 3437, 2956, 1651, and 1065 cm^{-1}, indicating the presence of O-H, aromatic C-H, α ,β-unsaturated carbonyl, and C-O functions in the molecule. The ^{1}H-NMR spectrum showed resonances for 18 protons; three aromatic protons signals at δH 8.42 (s, H-2), 6.61 (s, H-8), 6.75 (d, J = 1.5 Hz, H-2', 6'), four methoxy group signals at δH 3.70 (4'-OCH_3), 3.76 (6-OCH_3), 3.79 (5'-OCH_3), and 3.88 (7-OCH_3), 3' and 5 chelated hydroxy groups at δH 9.31 and 12.97.The ^{13}CNMR showed nineteen carbon signals, The placement of the methoxy groups at C-4', C-6, C-5', and C-7 was supported by the spectrum that displayed correlations between 4'-OCH_3 and C-4' (δC 136.1), 6-OCH_3 and C-6 (δC 132.1), 5'-OCH_3 and C-5' (δC 152.2), as well as 7-OCH_3 and C-7 (δC 154.5). On the basis of the above evidences, the structure of Ih/DC was elucidated as 3',5-dihydroxy-4',5',6,7-tetramethoxyisoflavone (irigenin).

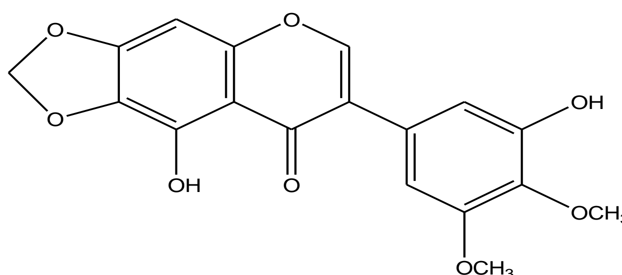


Irigenin

CHARACTERISATION OF Ih/DC-2

The compound Ih/DC-2 derived as yellow amorphous solid from the dichloromethane extract of *Iris hookeriana*, m.p. 174°C. The mass spectrum showed ion peak M^+ at

358 analysed for $C_{18}H_{14}O_8$. The compound exhibited UV absorption at 270 and 340 nm characteristic of an isoflavonoid. IR absorption bands were found at 3240 (OH), 1660 (C=O), 1610 (C=C) 940 cm^{-1} (methylenedioxy) and sharp two proton singlet at δ 6.2 in the ^{1}HNMR spectrum, which showed additionally two low field singlets at δ 9.1 and 12.9 characteristic for the hydroxyl proton at position 3′ and 5 respectively. Chemical shifts of two singlet signals for the H-2 and H-8 protons fell in the normal shift region for the isoflavonoid nucleus. The 3′4′5′ oxygenation pattern of ring C could be derived from the two meta coupled doublets at δ 6.58 and 6.83. Methylation of the free hydroxyl groups gave a product with mp and ^{1}HNMR data identical with those reported for irisflorentin (5,3′,4′,5′-tetramethoxy-6,7methylenedioxyisoflavone). The ^{13}CNMR showed 18 carbon signals. Having the same basic skeleton as that of the irigenin the differentiating signal is at 102.32 corresponding to O_CH2_O. Therefore the structure of Ih/DC-2 got confirmed as 5,3′-dihydroxy-4′,5′-dimethoxy-6,7-methylenedioxyisoflavone.



5'-hydroxy,3',4',-dimethoxy-6,7-
methylenedioxy isoflavone.

6.1. SAR STUDIES OF Ih/DC VIS-À-VIS ANTITUMOR ACTIVITY

In order to carry out some SAR studies, the irigenin was subjected to acetylation and methylation and two compounds obtained were subjected for antitumor activity vis a vis the irigenin and the result obtained is given below:

GENARAL PROCEDURE FOR ACETYLATION

20-25 mg of the compound dissolved in C_5H_5N (5ml) was treated with Ac_2O (2ml) and left for 24-48 hrs. The reaction was monitored by TLC. The acetates were treated with acidulated water; the ppt. was filtered, dried and dissolved in $CHCl_3$. The mixture was separated by column chromatography using petrol: benzene (3:7) and the acetates were crystallized from $CHCl_3$-MeOH.

GENERAL PROCEDURE FOR METHYLATION

20-30 mg of the compound was dissolved in dry MeOH (25-40 ml) and anhydrous K_2CO_3 (0.5-1gm) was added to the solution. The MeI (5ml) was now added dropwise with constant shaking to the solution. The mixture was refluxed on water bath for 2 hrs, filtered and freed from solvent and crystallized from C_6H_6-MeOH.

RESULTS OF SAR STUDIES:

The parent compound, irigenin possessed the significant antitumor activity against leukemia, lung and colon cancer respectively. On methylation the %growth inhibition got increased for leukemia, lung and liver cancer cell lines whereas the activity got decreased for colon cancer at higher concentration but at lower concentration it showed an increase in the activity. The acetylated irigenin showed an overall decrease in the % growth inhibition of all the cancer cell lines respectively. It could be inferred that length of carbon chain has a pivotal role in the antitumor activity of the compound. These results reveal that for better activity the optimum length of the carbon chain of the substituents should not exceed beyond two carbon atoms.

The standards used in carrying out SAR studies vis a vis antitumor activity were 5-FU, Paclitaxel and Mito-C respectively.

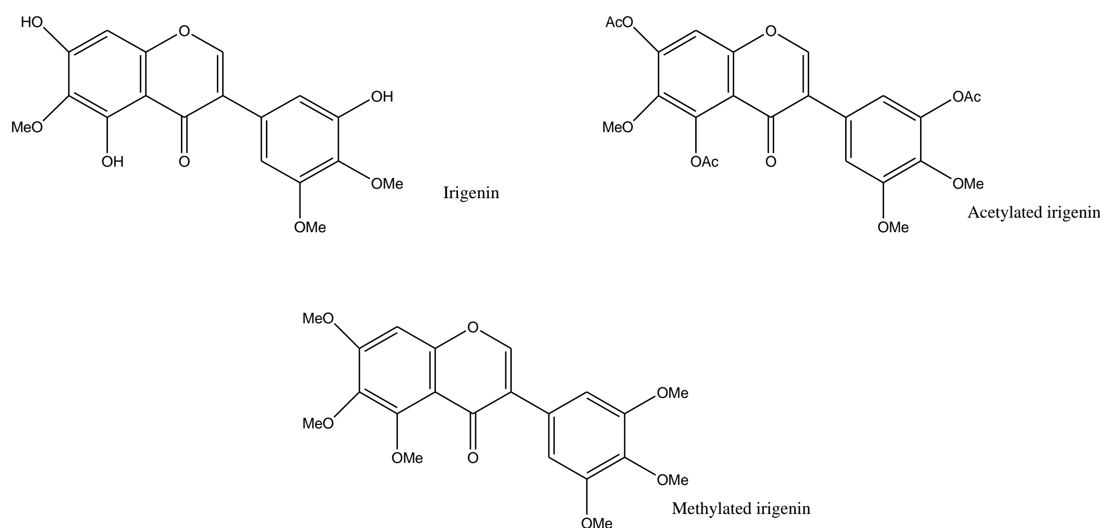


Table 19: Anticancer activity of Irigenin and its acetylated and methylated derivative

	LIVER	LUKEUMIA	LUNG	COLON
	HEP-2	THP-1	A-549	COLO-205

Code	Conc.(ug/ml)	% GROWTH INHIBITION			
Ih/DC	100	57±0.05	**81±0.03**	**78±0.02**	**86±0.06**
	50	28±0.04	65±0.04	51±0.03	49±0.03
Ih-I	100	**69±0.01**	**91±0.05**	**84±0.05**	**78±0.05**
	50	38±0.03	56±0.01	45±0.04	51±0.03
Ih-II	100	36±0.04	50±0.02	35±0.06	43±0.05
	50	05±1	26±0.03	12±0.03	13±0.02
5-FU	1x10-5	23±0.08	38±0.05	20±0.03	**78±0.01**
Paclitaxel	1x10-5	28±0.06	45±0.06	**79±0.08**	15±0.03
Mito-C	1x10-5	**89±0.05**	43±0.05	37±0.05	12±0.04

Ih/DC is irigenin

Ih-I is methylated irigenin

Ih-II is acetylated irigenin

Summary

PLANT PART: RHIZOMES

Comparative antitumor activity of extracts of rhizomes of *Iris ensata* and *Iris hookeriana*.

SulphaRhodamine B assay (SRB) was performed which revealed increase in growth inhibition during 48h incubation against a panel of human cancer cell lines. The increase in growth inhibition depicted that the incubation of the cells for 48h with the test material (DCM) at 100 and 50µM concentration resulted in growth inhibition of leukemia (THP-1), prostate (PC-3), colon (HCT-15) and lung (A549) cancer cell lines. Interestingly, leukemia was showing promising inhibition compared to rest of the cell lines tested. Ethyl acetate extract of *Iris hookeriana* exhibited promising inhibition among all the cell lines tested for anti-cancer activity. Taken together, the results depicted that the incubation of different human cancer cell lines of varied tissue origin even with 50µM of dichloromethane extract of *Iris ensata* and ethyl acetate extract of *Iris hookeriana* for 48h imparted cytotoxic effects on cellular cytotoxicity to a great extent.

Comparative antioxidant activity of extracts of rhizomes of *Iris ensata* and *Iris hookeriana*

DPPH radical scavenging activity showed *Iris hookeriana* to be more potent than *Iris ensata*. The IC_{50} of ethyl acetate extract of *Iris hookeriana* equal to 2.94 which was almost comparable with the IC_{50} of the quercetin used as a standard. Methanol and methanol water extracts of rhizomes of *Iris hookeriana* also showed some amount of antioxidant activity.

Comparative antimicrobial activity of rhizomes of *Iris ensata* and *Iris hookeriana*.

All the extracts of the two species showed no significant activity towards inhibition of microbial growth. Preliminary screening of the extracts of the two species for the microbial strains *S. aureus* 29213, *E. coli* ATCC 25922, *C. albicans* ATCC 90028 and *A. fumigatus* showed the extracts to be inactive.

PLANT PART: LEAVES

Comparative antitumor activity of extracts of leaves of *Iris* ensata and *Iris hookeriana*

Sulpharhodamine B assay (SRB) was performed which revealed increase in growth inhibition during 48h incubation against a panel of human cancer cell lines. The increase in growth inhibition depicted that the incubation of the cells for 48h with the test material containing petroleum ether and dichloromethane extracts of leaves of *Iris hookeriana* at 10 and 50µM concentration resulted in growth inhibition of breast (MCF-7), pancreatic Miapaca, prostate (PC-3) and lung (A549) cancer cell lines. Dichloromethane extract and Ethyl acetate extract of *Iris ensata* exhibited promising inhibition for breast (MCF-7) and lung (A-549) cell lines at lower concentration. Whereas methanol water extract showed promising results for prostate (PC-3) cell line. Taken together, the results depicted that the incubation of different human cancer cell lines of varied tissue origin even with 50µM of methanol water and ethyl acetate extract of *Iris ensata* and petroleum ether and dichloromethane extract of *Iris hookeriana* for 48h imparted cytotoxic effects on cellular cytotoxicity to a great extent.

Comparative antioxidant Activity of extracts of leaves of *Iris ensata* and *Iris hookeriana*

DPPH radical scavenging activity showed *Iris ensata* to be more potent than *Iris hookeriana*. The IC_{50} of methanol extract of *Iris ensata* equal to 2.34 which was almost comparable with the IC_{50} of the quercetin used as a standard. The ethyl acetate and dichloromethane extracts of *Iris ensata* possessed IC_{50} equal to 3.24 and 5.65 respectively whereas extracts of *Iris hookeriana* showed higher values of IC_{50} compared to the extracts of *Iris ensata* thereby concluding *Iris ensata* to be more potent antioxidant than *Iris hookeriana*.

Comparative antimicrobial activity of extracts of leaves of *Iris ensata* and *Iris hookeriana*

All the extracts of the two species showed no significant activity towards inhibition of microbial growth. Preliminary screening of the extracts of the two species for the microbial strains *S. aureus* 29213, *E. coli* ATCC 25922, *C. albicans* ATCC 90028 and *A. fumigatus* showed the extracts to be inactive.

Appendices

Appendix I : List of Symbols/Abbreviations

ATCC	American Type Culture Collection
CC	Column chromatography
$CDCl_3$	Deuterated Chloroform
CFU	Colony forming units
cm^{-1}	Centimeter inverse
°C	Degree Celsius
d	Doublet
DMSO (d_6)	Deuterated dimethyl sulphoxide
ESI-MS	Electrospray Ionization Mass Spectrometry
Fig.	Figure
g/gm	Gram
Hz	Hertz
IR	Infrared
J	Coupling constant
m	Multiplet
M	Molecular mass
MeOD	Deuterated Methanol
mg	Milligram
µg	Microgram
MHz	Mega Hertz
MIC	Minimum inhibitory concentration
mM	Millimolar
M.p.	Melting point
m/z	Mass to Charge ratio

Na	Sodium
NAM	Nutrient agar medium
NBGP	Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose
NMR	Nuclear Magnetic Resonance
OD	Optical density
ppm	Parts Per Million
RA	Reference antibiotic
Rf	Retention factor
s	Singlet
TLC	Thin Layer Chromatography
UV	Ultra Violet
v/v	Volume by Volume
α	Alpha
β	Beta
δ	Delta
λ_{max}	Lamda maximum

Appendix II : List of Figures

No.	Title
1.1	Structures of some representative natural product drugs.
1.2	Structures of some representative traditional drugs derived from plants.
1.3	Some representative drugs derived from natural products.
1.4	Hypothesis of the links between the working mechanisms of flavonoids and their effects on disease.
1.5	Structures of new isoflavones (1-5) isolated from *Iris*.
1.6	Structures of new flavones (25-29) isolated from *Iris*.
1.7	Structures of new monosaccharides (6-10) isolated from *Iris*.
1.8	Structures of isoflavone disaccharides and trisaccharides isolated from *Iris*.
1.9	Alkali degradation of isoflavones.
1.10	MS fragmentation of 2'-methoxyisoflavones.

6.1. ^{1}HNMR spectra of Ih/DC.

6.2. ^{13}CNMR spectra of Ih/DC.

6.3. ^{1}HNMR spectra of Ih/DC2.

6.4. ^{13}CNMR spectra of Ih/DC 2.

Appendix III : List of Tables

No.	Title
1.	Enzyme modulator activity of flavonoids.
2.	Phytoconstituents of various species of *Iris*.
3.	Antitumor activity of rhizomes of *Iris ensata*.
4.	IC 50 value of rhizomes of *Iris ensata*
5.	Antimicrobial activity of rhizomes of *Iris ensata*.
6.	Antitumor activity of leaves of *Iris ensata*.
7.	IC 50 value of leves of *Iris ensata*
8.	Antimicrobial activity of leaves of *Iris ensata*.
9.	Antitumor activity of rhizomes of *Iris hookeriana*.
10.	IC 50 value of rhizomes of *Iris hookeriana.*
11.	Antimicrobial activity of rhizomes of *Iris hookeriana*.
12.	Antitumor activity of leaves of *Iris hookeriana*.
13.	IC 50 value of leaves of *Iris hookeriana.*
14.	Antimicrobial activity of leaves of *Iris hookeriana*.
15.	Yields of extracts**.**
16.	Qualitative chemical analysis.
17.	Yields of extracts.
18.	Qualitative chemical analysis.
19.	Antitumor activity of Irigenin and its acetylated and methylated derivative.

Appendix IV: PHYTOCHEMICAL TESTS

1. **Test for Steroids**: In a small quantity of petroleum ether extract, 2ml of acetic anhydride solution was added in $CHCl_3$. This was followed by addition of conc.H_2SO_4. A greenish colour was produced which turns to blue indicates the presence of steroids.

2. **Test for Flavonoids**: Dilute ammonia (5ml) was added to a portion of an aqueous filtrate of the extract, concentrated sulphuric acid (1ml) was added. A yellow colouration that disappears on standing indicates the presence of flavonoids.

3. **Test for terpenoids**: To 0.5gm of the methanolic extract was added 2ml of chloroform. Concentrated sulphuric acid (3ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

4. **Test for Glycosides**: A small quantity of the extract was dissolved in 1ml of water. Sodium hydroxide solution was added, yellow colour appeared that indicates the presence of glycosides.

5**. Test for Alkaloids**: Few mg of methanolic extract was dissolved in 5ml of distilled water. 2ml hydrochloric acid was added until an acid reaction occurs then 1ml of Dragendroffs reagent was added, orange red precipitate was formed immediately indicated the presence of alkaloids.

Appendix V: SPRAY REAGENTS

1. Anisaldehyde-sulphuric acid reagent: 0.5 ml of anisaldeyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml conc. H_2SO_4

2. Cerric ammonium sulphate reagent: To 6gms of cerric ammonium sulphate 3ml of sulphuric acid was added and the final volume of the solution was made to 100 ml with distilled water.

3. Ferric chloride reagent: To 5 % solution of $FeCl_3$, NH_4OH solution is added with shaking drop by drop till the permanent precipitate is obtained (or) 1% Ferric (III) Chloride in Methanol/water (1:1).

4. Dragendroffs reagent:

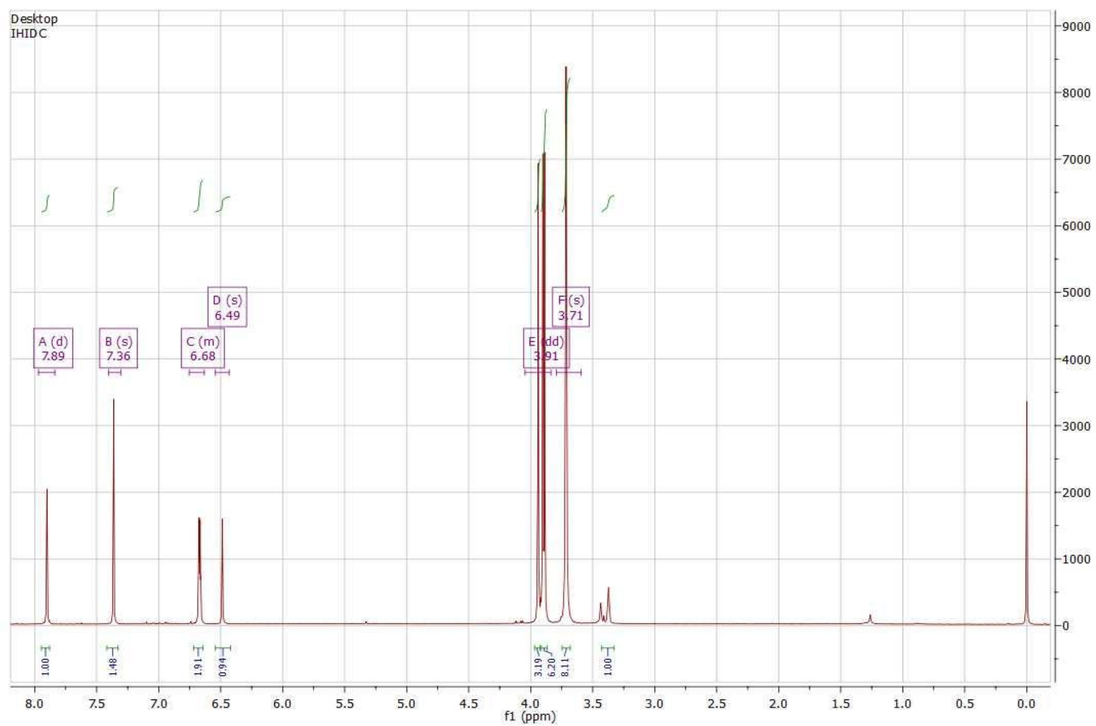
Solution a: 0.85 gm of basis bismuth nitrate was dissolved in a mixture of 10 ml acetic acid and 40 ml water.

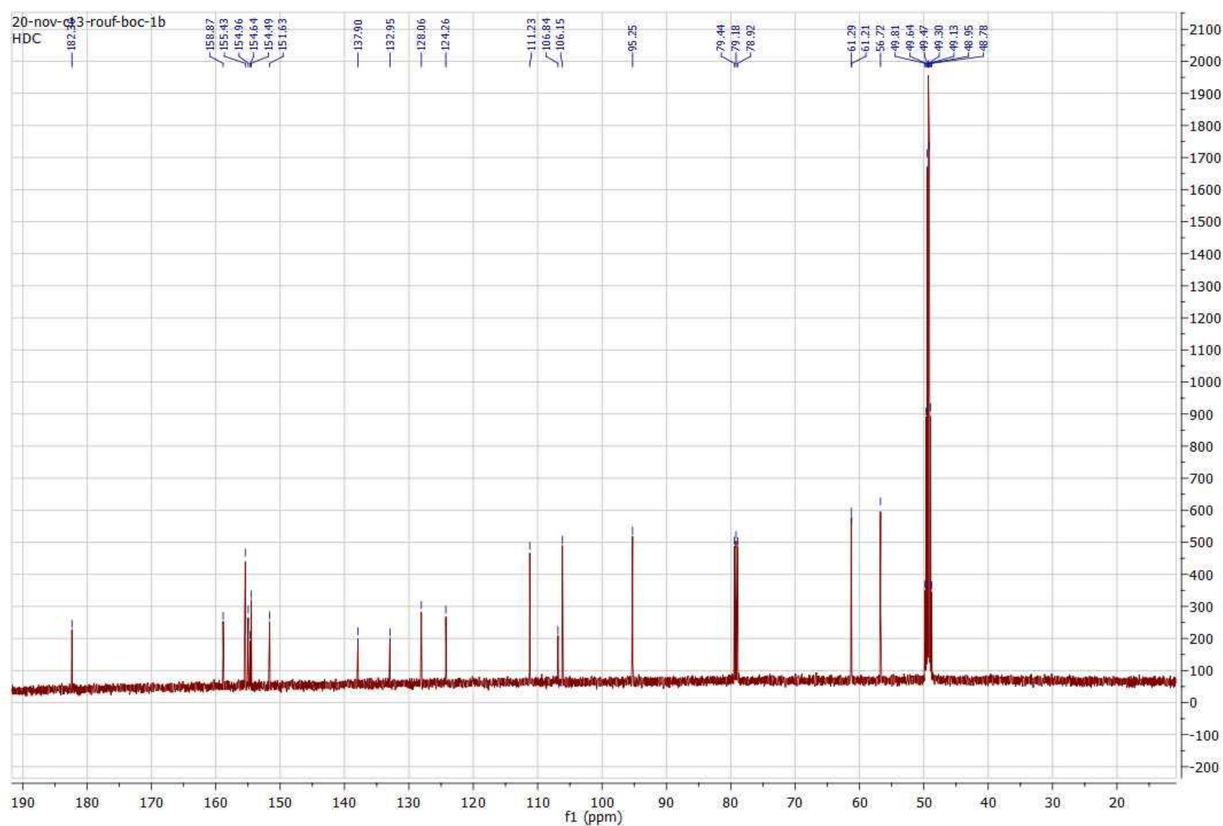
Solution b: A solution was made by dissolving 8 gms of potassium iodide in 20 ml water.

Stock solution: Equal volumes of a and b were mixed.

Spray reagent: 1 ml stock solution was mixed with 2 ml acetic acid and 10 ml water before use.

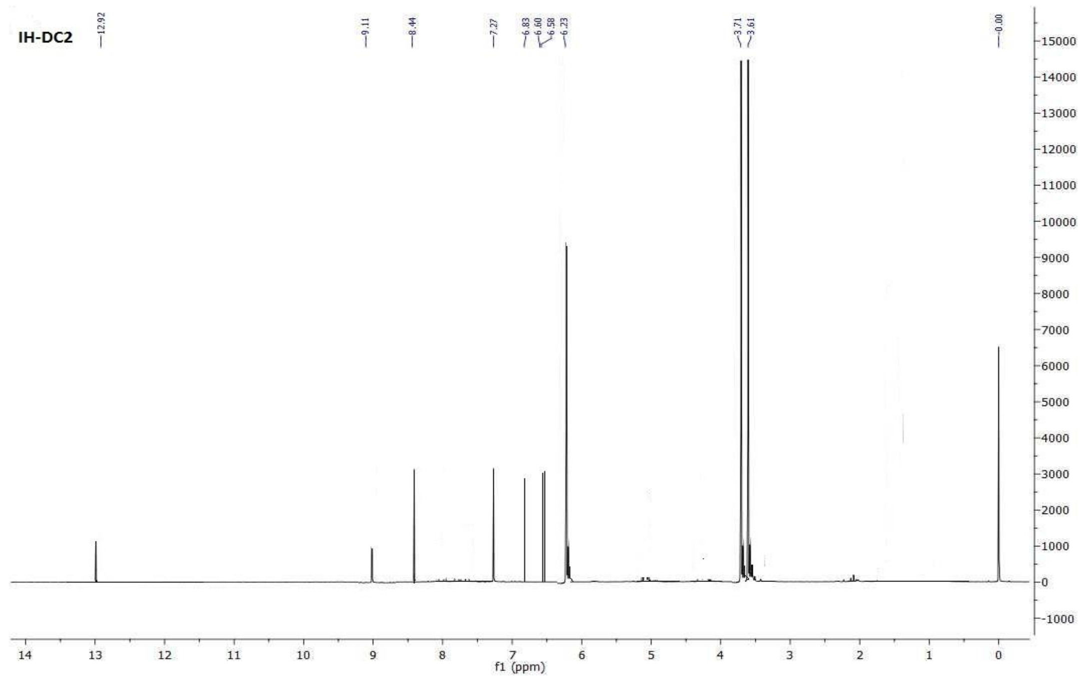
6.1. ^{1}HNMR spectra of Ih/DC

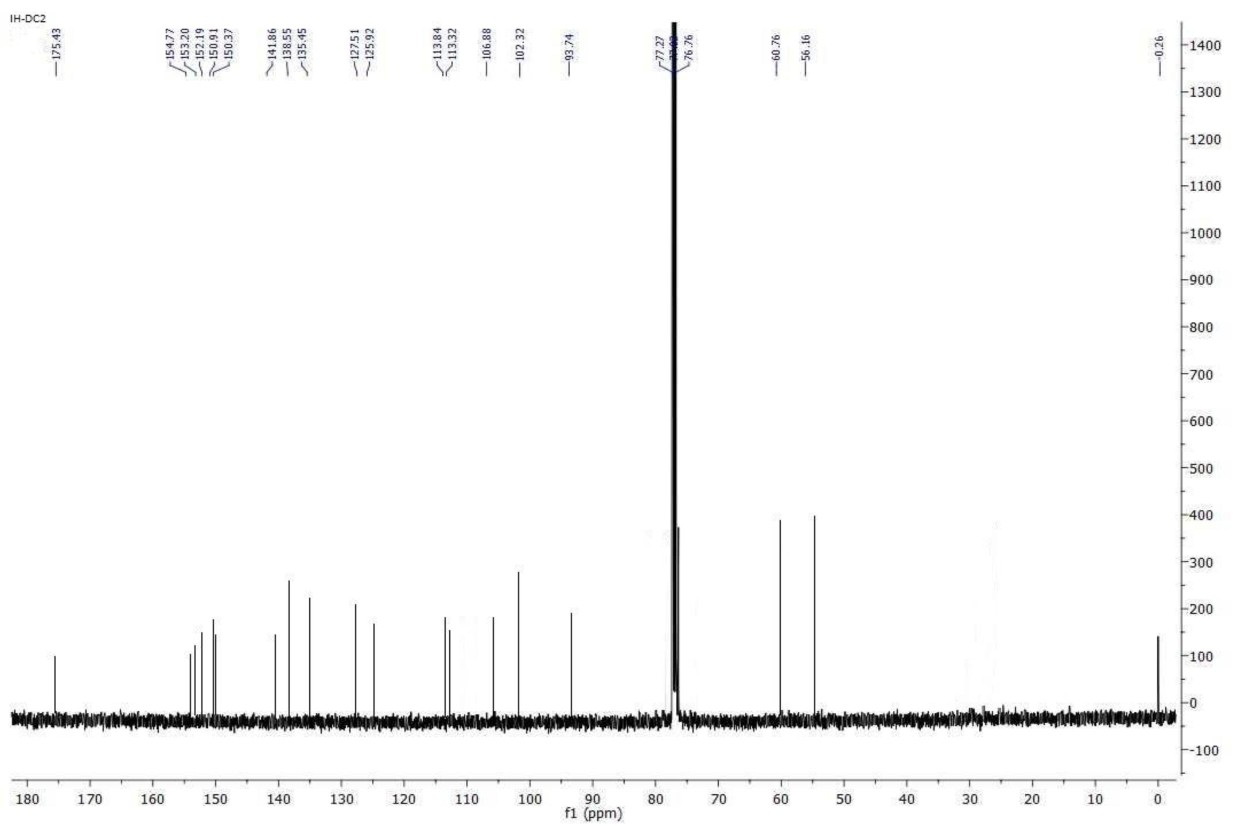




6.2. ^{13}CNMR spectra of Ih/DC

6.3. ^{1}HNMR spectra of Ih/DC2





6.4. ^{13}CNMR spectra of Ih/DC2

Publication and Congress attended:

S. M. Buchh, F.A. Mir, S.u. Rehman, M.. A. Qurishi and J. A. Banday. Isoflavone: a brief study on structural and optical properties, Eur. Phy. J. App. Phy., 62, 3201, (2013) 1-4

Presented an oral presentation in 8th JK Science Congress: organized by University of Kashmir in collaboration with DST Govt. of India and S&T council J&K Govt, 2012.