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Rubia cordifolia L. and *Glycyrrhiza glabra* L. Medicinal Plants as Potential Source of COX-2 Inhibitors

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Abstract

The association of chronic inflammation with development of human cancer is well recognized. There are number of reports of involvement of inflammatory process in the initiation and progress of cancer. The search for selective inhibitors of Cyclooxygenase-2 isoenzymes and that too from natural origin is considerably important. *Rubia cordifolia* L. and *Glycyrrhiza glabra* L. find an important place in the Ayurvedic system of medicine. Several secondary plant metabolites were isolated from roots of *R. cordifolia* and rhizomes of *G. glabra* and were investigated for the COX-2 inhibitory activity using Cayman COX (ovine) inhibitory screening assay. A few molecules showed potent COX-2 inhibitory activity which may serve as lead molecules for cancer chemoprevention studies.

Keywords: Cancer chemoprevention; *Rubia cordifolia*; *Glycyrrhiza glabra*; phytochemicals; COX-2 inhibitors.

1. Introduction

Chronic inflammation induced by biological, chemical and physical factors has been associated with increased risk of human cancer at various sites [1, 2]. Autoimmune and inflammatory reactions of uncertain etiology (e.g. ulcerative colitis, pancreatitis etc.) are also associated with increased risk of cancer. There are multiple lines of evidence of an association between inflammatory tissue damage and the development of cancer [3]. In general, longer the inflammation persists, the higher the risk of cancer. Inflammation is a step by step process that includes injury, repair and resolution. All inflammatory cells (neutrophils, monocytes,

macrophages, eosinophils, dendritic cells, mast cells and lymphocytes) are recruited after damage or an infection and may contribute to the onset and progression of cancer. Key molecular players that link inflammation to genetic alterations are prostaglandins, cytokines, nuclear factor NFκB, chemokines and angiogenic factor [4]. Recent research has shown that increased amount of prostaglandin E_2 in both human and experimental tumors inhibit host immunity and may play an important role in carcinogenesis [5]. Cyclooxygenase (COX-2), an important enzyme involved in mediating the inflammatory process produces PGE_2 from endogenous arachidonic acid [6]. Several isoforms of COX have been reported. COX-1, the constitutive isoenzyme that is expressed in most tissues, controls homeostasis by maintaining the physiological level of prostaglandins. COX-2 is inducible and dramatically up-regulated by a wide variety of stimuli such as cytokines, mitogens, oncogenes, growth factor and tumor promoters and is detectable in only certain types of tissues [3, 7]. Elevated levels of PGE_2 and enhanced COX-2 activity are frequently observed in a variety of malignancies, including those of the breast, prostate, bladder, liver, pancreas, skin, lung, colon and brain [8, 9, 10]. Therefore, the suppression of prostaglandin synthesis through the selective inhibition of COX-2 is now regarded as a promising and practical approach to cancer prevention. COX inhibitors such as celecoxib, piroxicam, sulindac and aspirin have been shown to reduce the formation and growth of experimentally induced cancer in animals [11, 12, 13]. A concern relevant to the use of COX-2 inhibitors is also associated with adverse side effects that recently resulted in the withdrawl of Vioxx and Celebrax that were being investigated as potential cancer chemopreventive agents [14, 15]. The major reasons for the side effects were related to high doses of these agents and it was generally concluded that COX-2 inhibitors induce cardiovascular problems when prescribed at high doses over long durations [16].

The COX-2 inhibitors can also inhibit COX-1 as well. This is problematic because COX-1 inhibition 'turns off' some important functions such as the repair and maintenance of stomach lining, which result in varying degrees of gastric ulcerations, perforation or obstructions [17]. So, there is a need of drugs which inhibit COX-2 without affecting COX-1 (selective COX-2 inhibitors). Selective COX-2 inhibitors hold promise for cancer chemoprevention. More recently, human clinical trials with COX-2 inhibitor drugs have shown similar anti-inflammatory and analgesic efficacy to traditional NSAIDs with significantly less gastrotoxicity [18]. However, these products offer some advantage in terms of side effects but they are nine times more expensive on a daily dose comparison [19]. Fortunately, there is now some evidence that natural COX-2 inhibitors may obstruct the production of pain and inflammation and do so in a more gentle manner and for less money. Baumann and coworkers were the first to report in a study that some dietary polyphenols inhibit arachidonic acid peroxidation [20]. Since then several researches have reported that many dietary polyphenols possess COX-2 inhibitory or stimulatory effects [21, 22, 23]. COX inhibition by polyphenols may account for anti-inflammatory effects, which reduce prostaglandin synthesis. Therefore, it should be noted that the concurrent use of polyphenols and NSAIDs could be beneficial or deleterious and thus necessitates constant attention by healthcare providers.

We have been testing different classes of synthetic compounds and their derivatives for COX-2 inhibitory activities [24, 25, 26] besides evaluating antimutagenic/antigenotoxic activity of polyphenolic extract/fractions isolated from Ayurvedic medicinal plants [27-34]. In the present study, it was planned to evaluate the COX-2 inhibitory potential of various polyphenols and related compounds (natural plant products) isolated from medicinal plants viz. *R. cordifolia* and *G. glabra*.

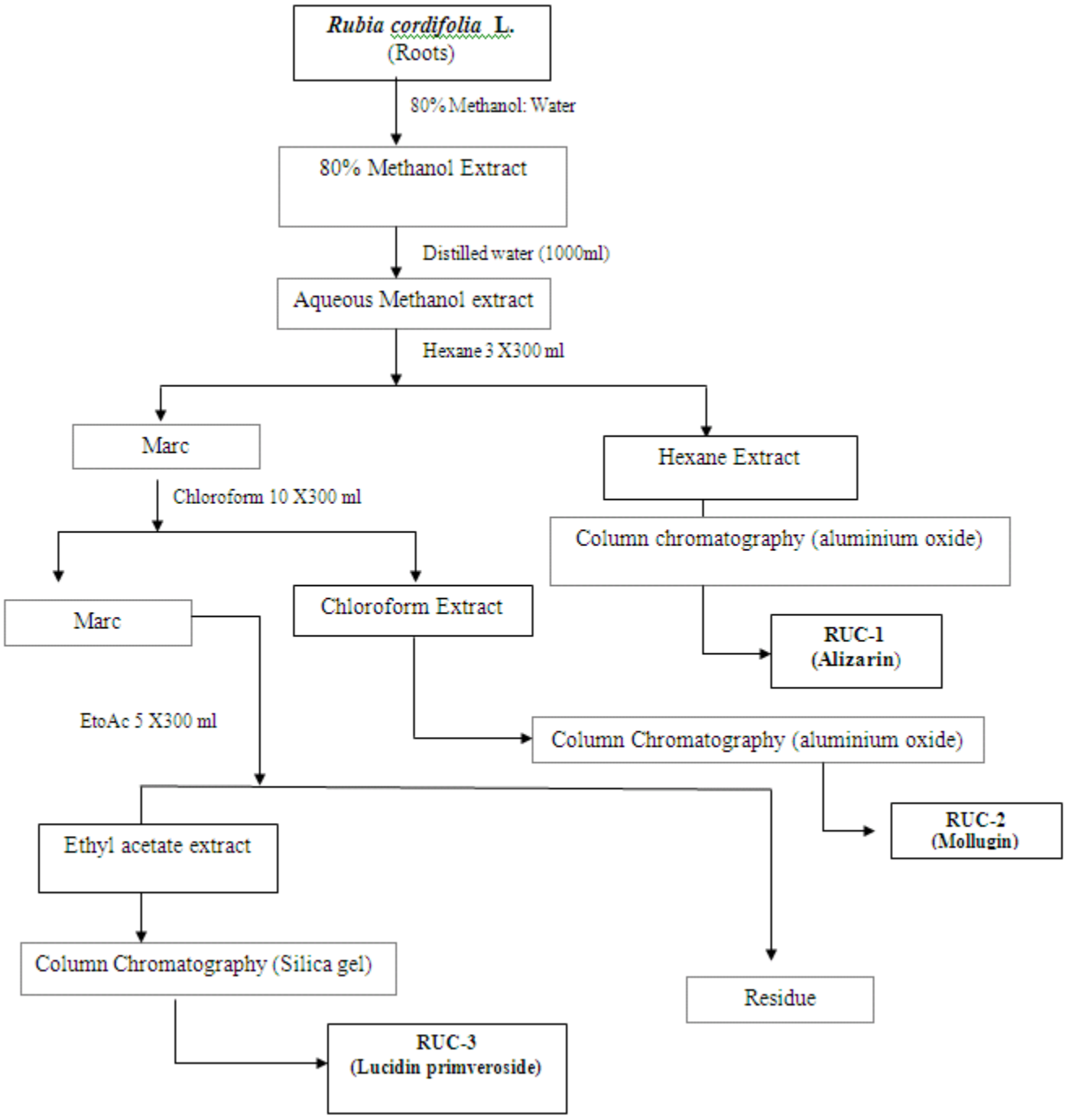
2. Materials and methods

The roots of *R. cordifolia* and *G. glabra* were purchased from a local market at Amritsar, India. Voucher specimens No. 0342-B-03/2006 (*R. cordifolia*) and 0342-A-03/2006 (*G. glabra*) have been kept in the herbarium of the Department of Botanical and Environmental Sciences, Guru

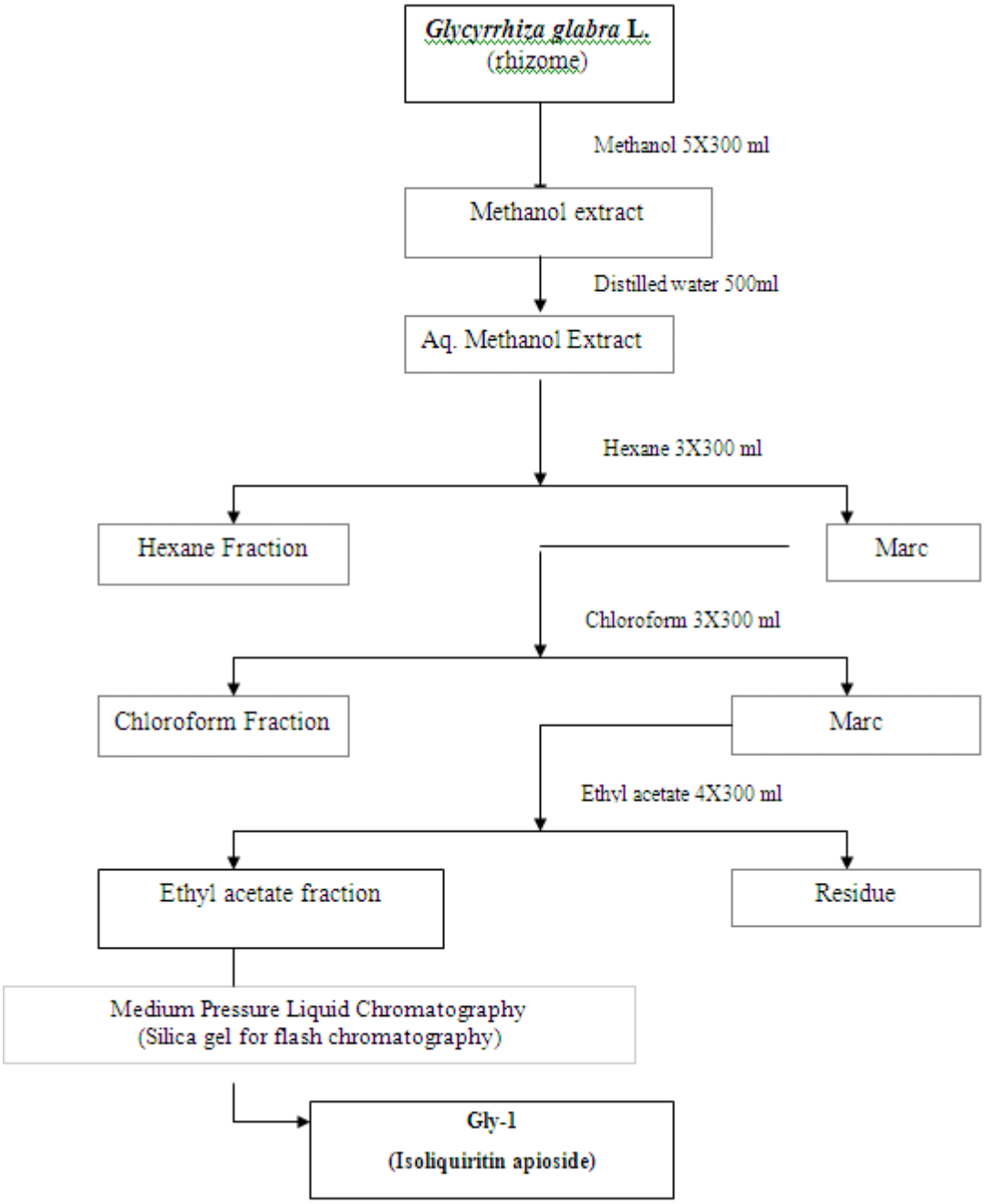
Nanak Dev University, Amritsar, Punjab, India. Plant material were washed with tap water, dried at 40^0C and crushed to make powder. The bioassay kit was purchased from Cayman Chemicals.

2.1 Isolation of Phytochemicals

The powdered roots of *R. cordifolia* and rhizomes of *G.glabra* were percolated with 80% methanol to obtain the methanol extract. The methanol extract was further fractionated with a series of organic solvents to obtain respective fractions. The various molecules were isolated as per Flow chart I, IIa and IIb. Structure elucidation of the isolated molecules was done using Nuclear Magnetic Resonance and Mass spectroscopic techniques.



Flow Chart I: Isolation of phytochemicals from *Rubia cordifolia* L.

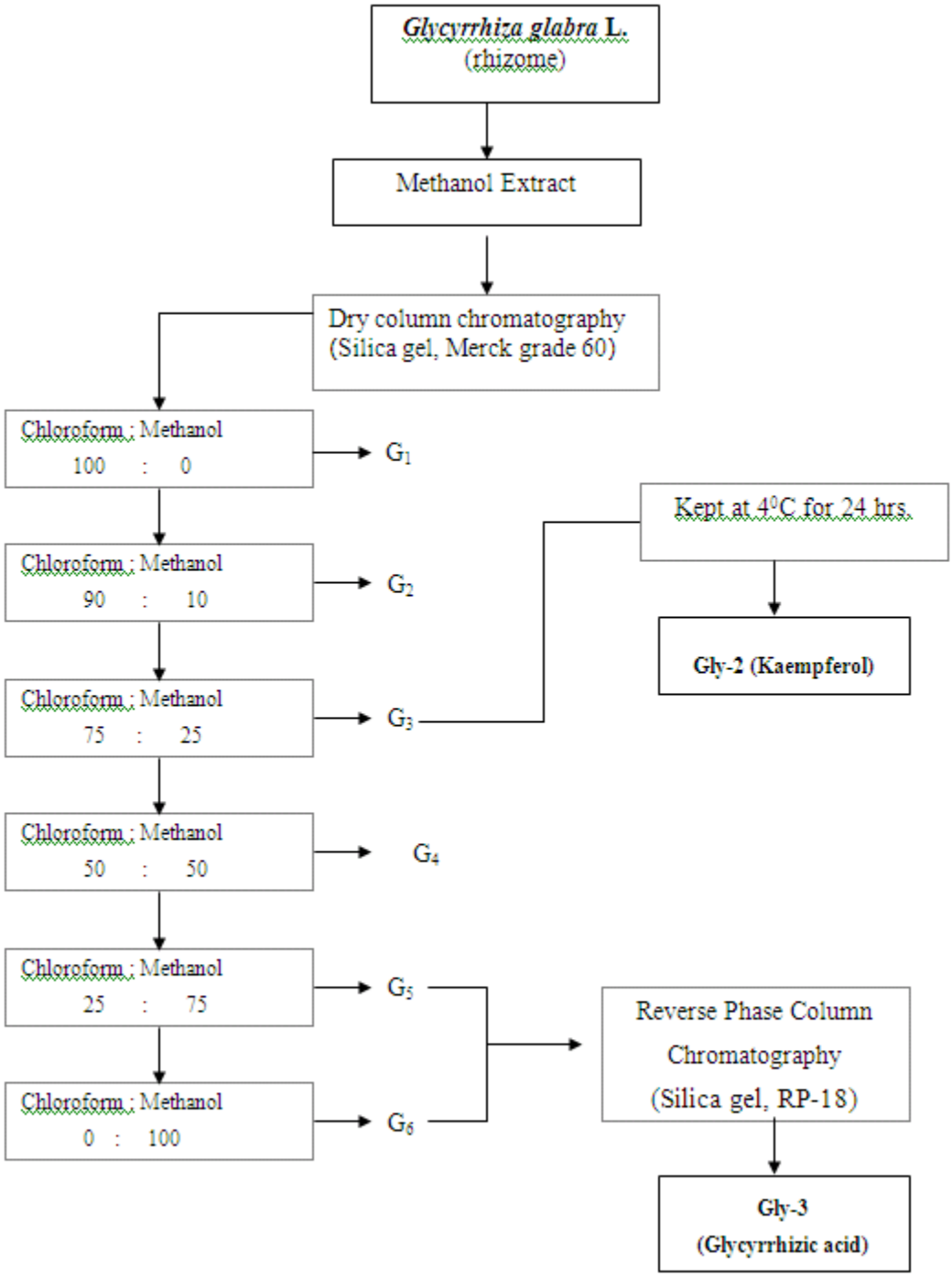


Flow Chart IIa: *Isolation of phytochemicals from* Glycyrrhiza glabra *L.*

2.2 COX-2 inhibitory activity

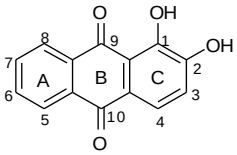
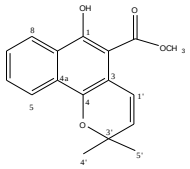
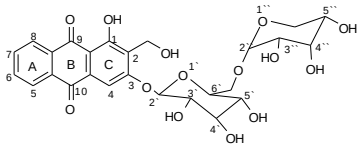
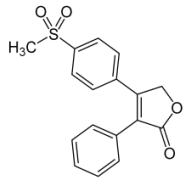
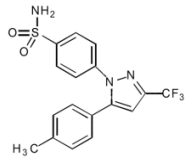
In vitro COX-2 inhibiting activities of the compounds have been evaluated using 'COX (ovine) inhibitor screening assay' kit with 96-well plates. Both ovine COX-1 and COX-2 enzymes were included. This screening assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. COX-1, COX-2, initial activity tubes were prepared taking 950µl of reaction buffer, 10µl of heme and 10µl of COX-1 and COX-2 enzymes in respective tubes. Similarly, COX-1, COX-2 inhibitor tubes were prepared by adding 20µl of inhibitor (compound under test) in each tube in addition to the above ingredients. The background tubes correspond to inactivated COX-1 and COX-2 enzymes obtained after keeping the tubes containing enzymes in boiling water for 3 min. along with vehicle

control. Reactions were initiated by adding 10µl of arachidonic acid in each tube and quenched with 50µl of IM HCl. PGH_2 thus formed was reduced to $PGF_{2\alpha}$ by adding 100µl $SnCl_2$. The prostaglandin produced in each well was quantified using broadly specific prostaglandin antiserum that binds with major prostaglandins and reading the 96-well plate at 405 nm. The wells of the 96-well plate showing low absorption at 405 nm indicated the low level of prostaglandins in these wells and hence the less activity of the enzyme. Therefore, the COX inhibitory activities of the compounds could be quantified from the absorption values of different wells of the 96-well plate.



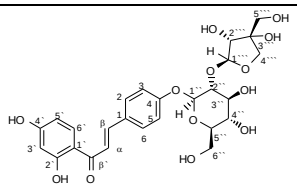
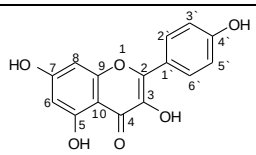
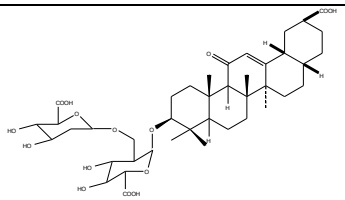
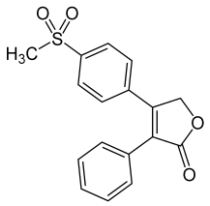
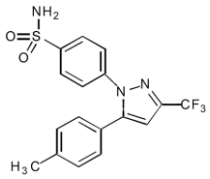
Flow chart IIb: *Isolation of phytochemicals from* Glycyrrhiza glabra *L.*

Table 1: *In vitro* percentage inhibition and IC_{50} values for COX-1 and COX-2 enzymes by phytochemicals isolated from *R. cordifolia* L.

Compound	% Inhibition			IC_{50} (µM)		COX-2 Selectivity*
	COX-2		COX-1	COX-2	COX-1	
	1 µM	**10** µM	**10** µM			
 Alizarin (RUC-1)	58.60	92.63	40.20	<1.0	>10	>10
 Mollugin (RUC-2)	49.05	88.53	26.08	1.21	>10	> 8.26
 Lucidin primveroside(RUC-3)	47.63	91.22	42.34	1.49	>10	> 6.71
 Rofecoxib**	75	100	75	0.3	40	~133
 Celecoxib **	50	100	65	1.2	14	~10

**Reported in literature [63].
*COX-2 selectivity = IC_{50} (COX-1)/ IC_{50} (COX-2)

Table 2: *In vitro* percentage inhibition and IC_{50} value for COX-1 and COX-2 enzymes by phytochemicals of *G. glabra* L.

Compound	% Inhibition			IC_{50} (µM)		COX-2 Selectivity*
	COX-2		COX-1	COX-2	COX-1	
	1 µM	**10** µM	**10** µM			
 Isoliquiritin apioside(Gly-1)	57.2	89.03	62.74	<1.0	<10	<10
 Kaempferol(Gly-2)	51.94	97.08	64.24	<1.0	<10	<10
 Glycyrrhizic acid(Gly-3)	72.60	95.08	18.66	<1.0	>10	>10
 Rofecoxib**	75	100	75	0.3	40	~133
 Celecoxib **	50	100	65	1.2	14	~10

**Reported in literature [63].
*COX-2 selectivity = IC_{50} (COX-1)/ IC_{50} (COX-2)

3. Results

Some phytoconstituents isolated from *R. cordifolia* and *G. glabra* showed promising results as selective COX-2 inhibitors.

3.1 *Rubia cordifolia* L.

As seen from Table 1 **Alizarin** (RUC-1) was found to be the most selective inhibitor of COX-2 (COX-2 selectivity >10) among the compounds isolated from *R.cordifolia*. At a concentration of 10 μM, it inhibited the COX-1 by 40.20% whereas COX-2 was inhibited by 92.63%. M**ollugin (RUC-2)** inhibited the COX-2 by 88.53% at concentration of 10 μM in comparison to inhibition of COX-1 by 26.08%. It showed COX-2 selectivity >8.26. **Lucidin primveroside (RUC-3)** also inhibited COX-2 (COX-2 selectivity >6.71) It showed 42.34% inhibition of COX-1 at concentration of 10 μM and inhibited the COX-2 by 91.22% at the same concentration.

3.2 *Glycyrrhiza glabra* L.

Isoliquiritin apioside (Gly-1) showed 89.03% inhibition of COX-2 at 10 μM and 62.74% of COX-1. The molecule showed COX-2 selectivity less than 10. **Kaempferol** (Gly-2) inhibited COX-2 by 97.08% at a concentration of 10 μM in comparison to COX-1 by 64.24%. It showed COX-2 selectivity less than 10 (Table 2). **Glycyrrhizic acid (Gly-3)** was found to be most selective inhibitor of COX-2 amongst the phytochemicals isolated from *G.glabra*. At a concentration of 10 μM, it inhibited the activity of COX-2 by 95.08% as compared to COX-1 which was inhibited by18.66%. It showed COX-2 selectivity more than 10 (Table 2).

4. Discussion

Carcinogenesis is a long and multistep process that include initiation, promotion and progression [35]. Initiation is a result of rapid and irreparable assault to the cell. Causes of cancer initiation include oxidative stress, chronic inflammation [36] and genotoxic damage by carcinogen [37]. DNA damage can result in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all processes associated with carcinogenesis [38]. COX-2, the inducible form of cyclooxygenase that catalyse the rate-limiting steps in prostaglandin synthesis from arachidonic acid, plays an important role in cancer. Several lines of evidence indicate the initial role of COX-2 in carcinogenesis as a well-established tumor promoter [39]. Over expression of COX-2 leads to malignant cell proliferation and invasion and this effect is reversed by non-steroidal anti-inflammatory agents elucidating the importance of COX-2 inhibitors in cancer chemoprevention [40].

Although there are various drugs as COX-2 inhibitors are available in the market but due to their adverse side effects, continuous withdrawal of these drugs takes place from time to time. There is utmost need to search for the natural selective COX-2 inhibitors (mainly of dietary origin) as these are regarded as safe.

Polyphenols are one such class of molecules which vary from simple structures to complex ones and may act as NSAIDs like compounds [41] and this activity appeared to be related to their phenol function [42]. Mollugin (napthaquinone), Alizarin (anthraquinone) and Lucidin primveroside (anthraquinone glycoside) were isolated from *R. cordifolia*. These molecules are of much interest as cancer chemopreventive agents. However, they have been very less explored for COX-2 inhibitory activity. The present investigation showed that mollugin (RUC-2), selectively inhibited COX-2. This is in consistence with the report of Oku and Ishiguro that 1, 4-napthaquinones (impatienolate and balsaminolate) isolated from *Impatiens balsamina* L. showed selective COX-2 inhibitory activities [43]. Napthaquinone derivatives, furonapthaquinone and β-Lapachone were also shown to exhibit selective inhibition of COX-2 [44, 45]. Shikonin, another napthaquinone derivative has been reported as a potent inhibitor of prostaglandin E_2 [46]. The cyclooxygenase inhibitory activity of anthraquinone molecules Alizarin (RUC-1) and Lucidin primveroside (RUC-3) is in concordance with the various reports that anthraquinone derivatives can inhibit cyclooxygenases. Anthraquinone rich extracts of *Aloe vera* gel possess anti-inflammatory activity by inhibiting the arachidonic acid pathway via

inhibition of cyclooxygenases [47]. Recently, Gan and coworkers reported that 3-alkylaminopropoxy-9,10-anthraquinone derivatives interfere with the conversion of arachidonic acid to prostaglandin (PGH_2) [48].

Among the molecules isolated from *G.glabra*, Glycyrrhizic acid (Gly-3) (triterpene glycoside) was the most potent in inhibiting the COX-2 activity. It showed strong inhibition of COX-2 as compared with COX-1. Triterpenes isolated from aerial parts of *Aralia cordata* possess COX-2 inhibitory activity [49]. Asiatic acid, a triterpene isolated from leaves of *Centella asiatica* inhibited the nitric oxide and prostaglandin E_2 production in RAW 264.7 macrophage cells [50]. Triterpenes isolated from *Eriobotrya japonica* prevent pulmonary inflammatory diseases by inhibiting iNOS, COX-2 and cytokines (TNF-alpha, IL-Ibeta and IL-8) production in human lung epithelial cells (A-549) [51]. Kaempferol (Gly-2) isolated from *G.glabra* also showed moderate selectivity for COX-2. Flavonoids are the well studied class of polyphenols as COX-2 inhibitors. Kaempferol and its derivatives isolated from seeds of *Prunus tomentosa* exhibited inhibitory activities on nitric oxide (NO) and prostaglandin E_2 (COX-2) production [52]. Kaempferol and quercetin exhibited anti-inflammatory activities by inhibiting iNOS and COX-2 protein levels in cultured human umbilical vein endothelial cells [53]. Luteolin and galangin, well known flavonoid molecules were studied as first dietary polyphenols as inhibitors of arachidonic acid peroxidation [20]. After this Chrysin and luteolin were considered as potent anti-inflammatory agents as they effectively suppressed COX-2 activity [54]. In 2008, Li and coworkers [55] reported that a new molecule Malsudone along with known flavonoids luteolin, isoquercetin, 7-methoxyflavone and luteolin-7-O--glycoside possess potent inhibitory effect on COX-2 with moderate inhibition of COX-1. Quercetin was demonstrated to protect against colon cancer by suppressing the expression of proinflammatory mediators (COX-1, COX-2, iNOS) [56]. Kolaviron, isolated from seeds of *Garcinia kola* has been reported to possess anti-inflammatory activities by inhibiting COX-2 and iNOS expression through down regulation of NF-Kappa B and AP-1 DNA binding activity [57]. Isoliquiritin apioside (Gly-1) a chalcone glycoside also showed good activity as COX-2 inhibitor. Certain reports show that chalcones and its derivatives also possess the potential to inhibit COX-2 [58]. Some chalcone derivatives were shown to be potent and selective COX-2 inhibitors [59]. Isoliquiritigenin isolated from roots of *Glycyrrhiza uralensis* [60] and Cardamnin, isolated from the fruits of *Alpinia rafflesiana* [61] inhibited COX-2 and iNOS expression in RAW 264.7 macrophage cells.

Among the phytochemicals tested from the medicinal plants, the percentage inhibition and IC_{50} values of 'RUC-1' 'RUC-2' from *R. cordifolia* and 'Gly-3' from *G. glabra* are in between that of corresponding reported values of Rofecoxib and Celecoxib. The natural origin and moderate selectivity of these compounds for COX-2 in comparison to rofecoxib may also make them better substitutes of Rofecoxib and Celecoxib as their too much selectivity for COX-2 leads to the cardiac toxicity. Studies carried out in our laboratory have also shown these molecules to possess antigenotoxic activity against H_2O_2 and 4NQO in SOS chromotest using *E. coli* PQ37 and in Comet assay using human blood lymphocytes [32, 62].

The chemopreventive effects of various phytochemicals have often been associated with their anti-inflammatory activities especially due to the inhibition of COX-2. Since the isolated phytochemicals showed potent COX-2 inhibitory activity these may serve as potential candidates for chemopreventive/chemotherapeutic studies.

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