Journal of Medicinal Plants Research Vol. 4(3), pp. 177-187, 4 February, 2010
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875© 2010 Academic Journals

Review

Biotechnological intervention of *Agave sisalana*: A unique fiber yielding plant with medicinal property

Mousumi Debnath[1*], Mukeshwar Pandey[1], Rohit Sharma[1], Gulab S. Thakur[2] and Pushpa Lal[1]

[1]Plant Biotechnology Laboratory, Department of Biotechnology, Jaipur Engineering College and Research Centre, Sitapura, Tonk Road, Jaipur 302022, India.
[2]Research and Development Centre, Bisen Biotech and Biopharma Pvt. Ltd., M-7, Biotechnology Park, Laxmipuram Transport Nagar, Gwalior 474010, India.

Accepted 22 December, 2009

Agave sisalana **Perr. Syn (Agavaceae) is cultivated for its fiber, ornamental and medicinal value. The plant contains saponin useful for soap making and pharmacological importance. It yields a stiff fiber traditionally used in making twine, rope and also dartboards. Despite their economic importance, the *Agave spp.* has not been genetically improved. *In vitro* propagation offers an alternative to this problem through the efficient cloning of selected high-yielding "elite" plants. The present review gives a brief account on this important fiber yielding and medicinal plant with special emphasis on the secondary metabolite and *in vitro* propagation.**

Key words: *Agave sisalana,* saponin, *in vitro* propagation, fiber.

INTRODUCTION

Medicinal plants play a key role in world health care systems. These plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people and serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. It provides also a stable economic return to local communities especially through the sale of wild-harvested material (Hamilton, 2004). However, recently many studies indicate that several medicinal plants, particularly that are wild collected, are subject to the overexploitation due to the growing demand for herbal medicine (Alves and Rosa, 2007; Botha et al., 2003; Hamilton, 2004; Sijelmassi, 1993; Stangeland et al., 2007) and also to the land conversion (Etkin, 1998). It's evident that many of these species like *Agave sisalana* (sisal hemp) that are slow-growing and slow reproducing are especially vulnerable to this situation. Consequently, many medicinal species are threatened and are in danger of extinction (Zschocke et al., 2000).

*Corresponding author. E-mail: mousumi.debnath@gmail.com.

AGAVE SISALANA: THE MIRACLE PLANT

Agave sisalana, also known as sisal*,* is an herbaceous monocotyledonous plant (Figure 1) from the Agavaceae family. Originally from Central America and Mexico, sisal grows in many tropical countries, with Tanzania and Brazil being currently the two main producers (Chand et al., 1998). *A. sisalana* can help in lowering the blood pressure. It is also used as an antiseptic and is taken to stop the growth of bacteria in the stomach and intestine. It is generally cultivated for its fibrous properties yielding a stiff fiber traditionally used in making twine, rope and also dartboards. In the first half of the twentieth century, sisal supplied about 70% of the world's long hard plant fibers (Nobel 1994). The plant contains saponins. An extract of the leaves is used as soap (Hedrick, 1972). It is likely that the root is the best source of the saponins that are used to make soap.

Sisal derives its name from a small port in the Yucatan peninsula of Mexico through which the earliest supplies of *Agave* fibers were exported, and it became known to commerce as Sisal or' sisal hemp'. The accepted botanical or Latin name of the sisal plant is *Agave sisalana* Perrine. The documented use of *Agave*s in the

Figure 1. *Agave sisalana* plant growing in natural field conditions in the Institute garden.

Peninsula of Yucatan is as a source of fiber. However, ethanobotanical exploration revealed that wild and cultivated variants have over 40 traditional uses. There are over 200 varieties of *Agave*. The most popular is Blue Weber, which is used to make tequila. Originally, blue *Agave* was used to make *Agave* syrup. During the late 1990s, there was a shortage of blue *Agave*, so wild *Agave*s were used. Currently there are *Agave* syrups made from at least half a dozen varieties, the most popular being blue *Agave*, *Agave salimiana, Agave americana* and *Agave mapisaga.* Blue *Agave*s are more expensive, wild *Agave*s are more economical. Some of the common *Agave*s are *Agave aboriginum*, *Agave abortiva*, *Agave abrupta*, *Agave acicularis, Agaveacklinicola*, *Agave affinis*, *Agave albescens*, *Agave albomarginata*, *Agave alibertii*, *Agave aloides*, *Agave amaniensis*, *Agave americana, Agave angustifolia*, *Agave angustissima*, *Agave anomala*, *Agave antillarum*, *Agave arizonica*, *Agave arubensis*, *Agave aspera*, *Agave asperrima*, *Agave atrovirens*, *Agave atrovirens var. mirabilis*, *Agave attenuata*, *Agave aurea, Agave avellanidens, Agave bovicornuta, Agave bracteosa, Agave brauniana, Agave breedlovei, Agave brevipetala, Agave breviscapa, Agave brevispina, Agave brittonia, Agave bromeliaefolia, Agave brunnea, Agave bulbifera*, *Agave cantala, Agave caymanensis, Agave chiapensis, Agave corderoyi, Agave costaricana, Agave cucullata, Agave cundinamarcensis, Agave cupreata*, *Agave deserti, Agave donnell-smithii*, *Agave durangensis*, *Agave dussiana*, *Agave eggersiana, Agave falcata, Agave filifera, Agave fourcroydes, Agave geminiflora, Agave guadalajarana, Agave havardiana, Agave impressa, Agave jaiboli, Agave lechuguilla, Agave lophantha, Agave macroculmis, Agave mckelveyana, Agave neglecta, Agave palmeri, Agave parviflora, Agave ragusae*, *Agave rasconensis, Agave regia, Agave revoluta, Agave rhodacantha, Agave rigida, Agave roezliana, Agave rudis, Agave rupicola, Agave salmiana, Agave schottii, Agave scolymus, Agave simoni, Agave sisalana, Agave stricta*, *Agave stringens*, *Agave subinermis*, *Agave subsimplex, Agave subtilis, Agave subzonata, Agave sullivani*, *Agave tequilana, Agave toumeyana, Agave tubulata, Agave utahensis, Agave victoriae-reginae, Agave vivipara, Agave weberi, Agave xylonacantha, Agave yuccaefolia, Agave zebra* (Nobel ,1988).

A. sisalana as a sexually sterile clone, probably of hybrid origin, due to its general inability to produce seed and by its chromosomes (Gentry, 1982). Nobel (1994) reports that sisal grows best on free-draining non-saline soils, and that in regions of Kenya and Tanzania with 1200 millimeters of rainfall per year (similar to Florida's range of from 1000 to 1,500 millimeters per year). *Agave*s are indigenous to tropical and sub-tropical America and they are found from South America northwards to Mexico, and beyond to the southern States of America, as well as up to the coast of California, and in the Caribbean Islands. Sisal has been introduced to different parts of the world, and it is cultivated in the West Indies, Brazil, East and West Africa, Madagascar and Indonesia, attempts are being made to establish sisal on a commercial basis in Hainan.

A. sisalana has a total above-ground dry weight productivity of about 20 tons per hectare per year. Under plantation conditions, *A. sisalana* produces about 220 leaves per plant before the emergence (referred to as "bolting") of the 5 - 6 m high inflorescence at about seven years of age (Nobel 1994). Leaves can be harvested after two years of age, which will postpone the "bolting" for 15 - 20 years. After "bolting", the plant dies. It is extremely drought resistant grows well on dry soil and can be planted as hedges or fences.

Origin and distribution

The genus *Agave* was established by Linnaeus in 1753 and contains approximately 136 species in the Agavaceae family (Nobel, 1988). *Agave*s are native to North America, with their center of origin in present-day Mexico (Gentry, 1982, Nobel, 1988). Gentry reports that the "origin of *AGAVE SISALANA* is uncertain because it was originally exported from Mexico via the port of Sisal in Yucatan, it has long been erroneously reported as of Yucatan origin. However, no botanical collections of the plant have ever been made in Yucatan, and botanists who have worked in Yucatan did not find the plant there." Fiber plantations in Yucatan are reported to be henequen, *Agave fourcroydes*. Residents in the neighboring state of Chiapas grow *Agave sisalana* as fence rows and for fiber, which is made into rope, nets, hammocks, and other functional items.

Agaves are indigenous to tropical and sub-tropical America and they are found from South America northwards to Mexico, and beyond to the southern States of America, as well as up to the coast of California, and in the Caribbean Islands. Sisal has been introduced to different parts of the world, and it is cultivated in the West Indies, Brazil, East and West Africa, Madagascar and Indonesia, attempts are being made to establish sisal on a commercial basis in Hainan. Sisal fiber, which is sometimes referred to as sisal hemp but is not related to true hemp, is fairly coarse and inflexible. Bergerws map of the distribution of the sub-genus *Agave* in Central America shows that it is confined between the latitudes of 15° N and 25°. This naturally includes *A. sisalana*, but sisal itself is essentially a tropical species. On a plantation scale it generally thrives best under high temperature, and providing that the annual rainfall does not exceed 70 inches. In the wet tropics, wherever the rainfall is in excess of this amount, its root system is adversely affected.

Cultivation

A. sisalana always requires a very well-drained soil and a sunny position (Chittendon, 1992; Huxley, 1992). The *Agave* is not very hardy in Britain tolerating temperatures down to about -3°C if conditions are not wet (Phillips and Rix, 1998). It succeeds outdoors on the south coast of England from Torbay westwards (Bean, 1981). Plants survive lower temperatures during the very cold winters. A monocarpic species, the plant lives for a number of years without flowering but dies once it does flower. However, it normally produces plenty of suckers during its life and these continue growing, taking about 10 - 15 years in a warm climate, considerably longer in colder ones, before flowering (Bean, 1981). This plant is widely used by the native people in its wild habitat; it has a wide range of uses. In a warm climate suckers take 10 - 15 years to come into flower. Members of this genus are rarely if ever troubled by browsing deer (Thomas, 1990).

Seed - surface sow in a light position, April in a warm greenhouse. The seed usually germinates in 1 - 3 months at 20°C (Rice, 1987). The seedlings are transferred into individual pots of well-drained soil when they are large enough to handle and grow them on in a sunny position in the greenhouse until they are at least 20 cm tall (Huxley, 1992). Many species are quite small and have a squat habit of growth, bearing a rosette of short fleshy leaves coated with wax. Usually the leaves are armed with formidable spines along their margins, although a minority have fine fiber-like filaments instead, or are entirely smooth-edged. Several of the largest species develop into handsome plants with exceptionally long, thick and variegated or blue, drooping leaves; these may be an at their best in the cooler and drier highlands of the East Africa. The distribution of the sub-genus *Agave* in Central America shows that it is confined between the latitudes of 15 degree N and 25 degree and this naturally includes *A. sisalana*, but sisal itself is essentially a tropical species, and on a plantation scale it generally thrives best under high temperature, and places where the annual rainfall does not exceed 70 inches. In the wet tropics, wherever the rainfall is in excess of this amount, its root system is adversely affected.

Nocturnal acid accumulation occur in *A. sisalana* .This was correlated to element levels in their chlorenchyma. Correlations was also noted between nocturnal acid accumulation and element content by fertilizer experiments with N, B, Ca, K, and P. Element levels in the chlorenchyma of the six *Agave* species were generally similar to those of previously studied cacti, including a low Na and high Ca level compared with agronomic plants (Nobel et al., 1985).

AGAVE SISALANA: A MEDICINAL PLANT

Sterols, steroidal sapogenins, steroidal alkaloids and alkaloidal amines are derived from *Agave sisalana,* and these substances derived from plant sources provide the starting materials for steroid production. All these secondary metabolites attribute to the pharmacological properties of the plant (Figure 2). Hecogenin (IV), a

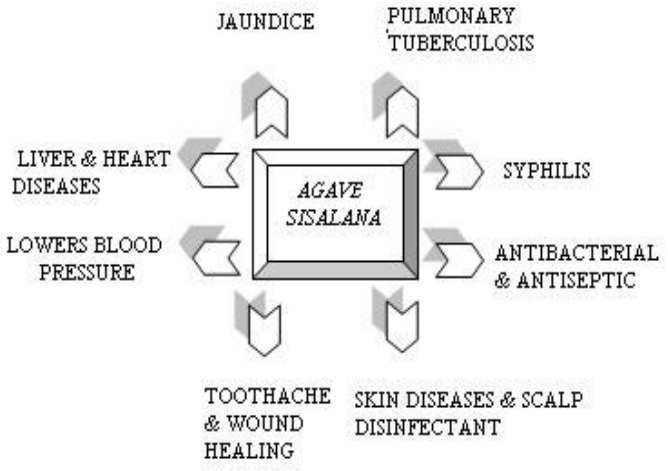

Figure 2. Medicinal properties of *Agave.*

saponin (*Agave sisalana*), was manufactured to cortisone by the process of Spensley et al. (1995) which defines the process of manufacturing the cortisone from hecogenin (IV), a saponin of *A.* sisalana (Fazli, 1968).

Pharmacological properties

The pharmacological investigation indicates that the juice obtained from *Agave sisalana* leaves stimulates the intestinal and uterine musculature, lowers the blood pressure and produces abortion in pregnant animals. The sap of *Agave* has antiseptic properties and is taken to stop the growth of bacteria in the stomach and intestine.

In Northern Morocco, the juice from the leaves of this species is used in folk medicine as a wash for skin diseases (El-Hilaly et al., 2003). It is used for syphilis and also recommended at times for pulmonary tuberculosis, diseased liver and jaundice. It can also be used as a laxative.

There are some therapeutic possibilities which may be suggested in view of the pharmacological characteristics of the juice; it is a uterine stimulant, emmenagogue and hypotensive drug (Sharaf and Zahran, 1967). The plant is also rich in saccharine and can be eaten when baked (Hedrick, 1972; Balls, 1975; Facciola, 1990).

The sap of *Agave*s has long been used in Central America as a binding agent for various powders used as poultices on wounds (Chevallier, 1996). The plant is used internally in the treatment of indigestion, flatulence, constipation, jaundice and dysentery (Bown, 1995). Water in which *Agave* fibre has been soaked for a day can be used as a scalp disinfectant and tonic in cases of falling hair (Lust, 1983). Steroid drug precursors are obtained from the leaves (Bown, 1995). A gum from the root and leaf is used in the treatment of toothache (Duke and Ayensu, 1985). The root is diaphoretic and diuretic (Chopra et al., 1986). It is used in the treatment of syphilis (Bown, 1995; Chopra et al., 1986). All parts of the plant can be harvested for use as required; they can also be dried for later use.

High prevalence of immunoglobulin E (IgE) sensitization among *Agave sisalana* processing workers has been noted in Tanzania, but its clinical implication is not obvious (Kayumba et al., 2008). All exposed workers had elevated IgE levels (>100 kU/l) and 27% of tested sera had elevated sisal specific IgE. A high prevalence of respiratory symptoms was found in both sensitized and non-sensitized sisal workers. Recently, Chen et al. (2009) reported the immunomodulatory effect of homoisoflavones and flavones from *Agave sisalana*. They reported that homoisoflavonoids have the activities of cytotoxicity (MCF-7) (Mutanyatta et al., 2003) and inhibitory activity against the enzyme COX-2 (Likhitwitayawuid et al., 2002). Due to their structural similarities, homoisoflavonoids have been reported to display activity comparable to that of flavonoids, some of which show immunopharmacological activity (Kuo et al., 2004). The activation and clonal expansion of human peripheral blood mononuclear cells (PBMC) play important roles in the generation of immune responses. Antigens or phytohemagglutinin (PHA) can stimulate resting PBMC to proliferate and differentiate. It has been studies that a series of cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) are important in the growth of PBMC induced by antigens or PHA (Chen et al., 2007).

Ethnomedicinal properties

Some species have an ethnopharmacological background, in particular *A. sisalana* which in the Bahama Islands, the central bud is boiled with salt and the decoction given as a remedy for jaundice; said to be effective within 24 h (Eldridge, 1975). *Agave attenuata* Salm-Dyck (Agavaceae) is a native species from Tropical America. In Brazil, this plant is an evergreen, perennial succulent species that lacks spines. It is non-invasive, but a widespread ornamental plant often cultivated in gardens and parks (Lorenzi and Souza, 1995). Recently, the aqueous extract of *A. attenuata* was evaluated for activity against *Bulinus africanus*, *Daphnia pulex*, *Anopheles arabiensis* and *Oreochromis mossambicus* demonstrating molluscicidal, piscicidal and larvicidal properties (Brackenbury and Appleton, 1997). Previous phytochemical study on *A. attenuata* has revealed the occurrence of sarsapogenin (Wall et al., 1954). A steroidal saponin was isolated from *A. attenuata*, along with an evaluation of its haemolytic effect and anti-inflammatory properties (Bernadete et al., 2002).

Several beverages are made from the sugar-rich juices of mature *Agave*s. The extracted juice is drunk fresh as aguamiel (honey-water) or fermented into pulque; both are popular beverages in the south of the Sonoran Desert.

Steamed heads or central stalks are mashed and allowed to ferment with added liquid. After several days, the resulting fluid is distilled into the potent liquor mescal. The most widely known local ("bootleg") variety is Bacanora, named after that Sonoran town and made from *Agave angustifolia*. Other varieties of bootleg mescal are made in nearly every Mexican village within *Agave* habitat. Tequila, the most famous legal variety of mescal, is made from the single species *Agave tequilana,* grown near the town Tequila in Jalisco.

Toothbrush sticks can be used by the vast majority of people who cannot afford buying the commercial toothbrush and toothpaste. Kassu et al. (1999) conducted an experiment on twenty different plant species and reported that the crude methanol extracts of *Agave sisalana*, Birbira and *Hypericum revolutum* test concentrations up to 500 micrograms/ml showed weak toxicity to brine shrimp. All the extracts showed antibacterial activity against *Staphylococcus aureus* and *Bacillus cereus.* Hence, the toothbrush sticks may be used for the oral and dental hygiene of the users and useful in decreasing dental caries.

AGAVE SISALANA: SECONDARY METABOLITES OF PHARMACOLOGICAL IMPORTANCE

The plant contains saponins. It is likely that the root is the best source of the saponins. The occurrence of steroidal saponins in *Agave* genus is well documented (Blunden et al., 1980; Blunden et al., 1986; Ding et al., 1989; Ding et al., 1993). The steroid sapogenin constituents of *Agave sisalana* at various phases of growth have been identified by Dawidar and Fayez (1961). It is suggested that, in the bulbils, gitogenin as first generation in sapogenin biogenesis gives tigogenin as second generation which transforms the course of the long life of the plant to hecogenin and neotigogenin as third generation. At the end of the life cycle, neotigogenin and hecogenin of the old leaves are transformed by a reverse mechanism to tigogenin in the flowering top which, as the new life approaches, changes to gitogenin. It has also been shown that hecogenin is most abundant (0.235%) in the leaves of the old plant. The major sapogenins (hecogenin, 9(11)-dehydrohecogenin and tigogenin) occurring in the *Agave* species have been analyzed by reversed-phase high-performance liquid chromatography after derivatization with benzoyl chloride (Higgins, 1976). Hecogenin and ticogin have been isolated by Cripps and Blunden (1978) from the *A.* sisalana leaf and juice samples. A gas-liquid chromatographic method has been devised for the routine estimation of the hecogenin [3-beta-hydroxy-(25R)-5-beta-spirostan-12-one] (Figure 3) and tigogenin [(25R)-5-beta-spirostan-3-beta-ol] contents of *Agave sisalana* leaf and juice samples and of the crude sapogenin concentrates known as "coffee grounds". Because of partial degradation of the sapogenins. The interesting fact is that in the third year (2000)., on in the GLC system it was found necessary to acetylate the compounds prior to their estimation. In East African samples the tigogenin proportion of the total sapogenin content is usually about 10%. At this level, the 95% inverse tolerance limits on predicted tigogenin weights are approximately +/- 7% (Cripps et al., 1974).

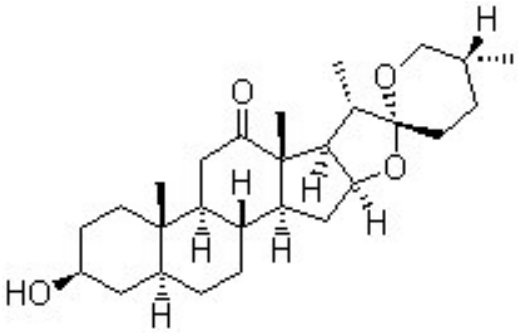

Figure 3. Hecogenin (3beta –hydroxy-5-alpha –spirotan-12-one; 5-alpha-spiostan-3 beta –ol-12-one).

Ding and co workers (1993) isolated and determined the structure of three new steroidal saponins, dongnosides C, D and E from the dried fermented residues of leaf-juices of *Agave sisalana*. In a continuing study on this plant, two additional new major steroidal saponins, named dongnosides B and A have also been reported. Their structures were characterized respectively as tigogenin-3-O-alpha-L-rhamonpyranosyl-(1,4)-beta-D-glucopyranosyl-(1,2)-[beta-D-glucopyranosyl-(1,3)]-beta-D -glucopyranosyl-(1,4)-beta-D-galactopyranoside and 3-O-alpha-L-rhamnopyranosyl-(1-->4)-beta-D-glucopyranosyl-(1,2)-[beta-D-xylopyranosyl-(1,3)-beta-D-glucopyranosyl-(1,3)]-beta-D-glucopyranosyl-(1,4)-beta-D-galactopyranoside on the basis of chemical and physicochemical evidence.

Chen et al. (2009) isolated three known flavones and seven known homoisoflavonoids (Figure 4) from the methanolic extract of the leaves of *AGAVE SISALANA*. Their structures were elucidated on the basis of spectroscopic analysis. The isolated three flavonoids are : 5,7-dihydroxyflavanone (Sirat et al., 1996), kaempferol 3- rutinoside-4′-glucoside (Geibel et al., 1994), and kaempferol 3-(2G rhamnosylrutinoside) (Kazuma et al., 2003) and seven homoisoflavonoids: 7-*O*-methyleucomol (Heller, 1976), 3′-deoxysappanone (Namikoshi and Saitho, 1987), (±)-3,9-dihydroeucomin (Mutanyatta et al., 2003), dihydro-bonducellin (Zhao et al., 2004), 7-hydroxy-3- (4-hydroxybenzyl) chromane (Meksuriyen and Cordell, 1987), 5,7- dihydroxy-3- (4′-hydroxy-benzyl)-4-chromanone and 5,7-dihydroxy-3-(3′-hydroxy-4′- methoxybenzyl)-4-chromanone (Adinolfi et al., 1986) from methanolic extraction of *AGAVE SISALANA*. Homoisoflavones (±)-3,9-dihydroeucomin, dihydrobonducellin and 5,7-dihydroxy-3-(4′-hydroxybenzyl)-4-chromanone, suppress-ed the production of IL-2 and IFN-γ. The isolated compounds

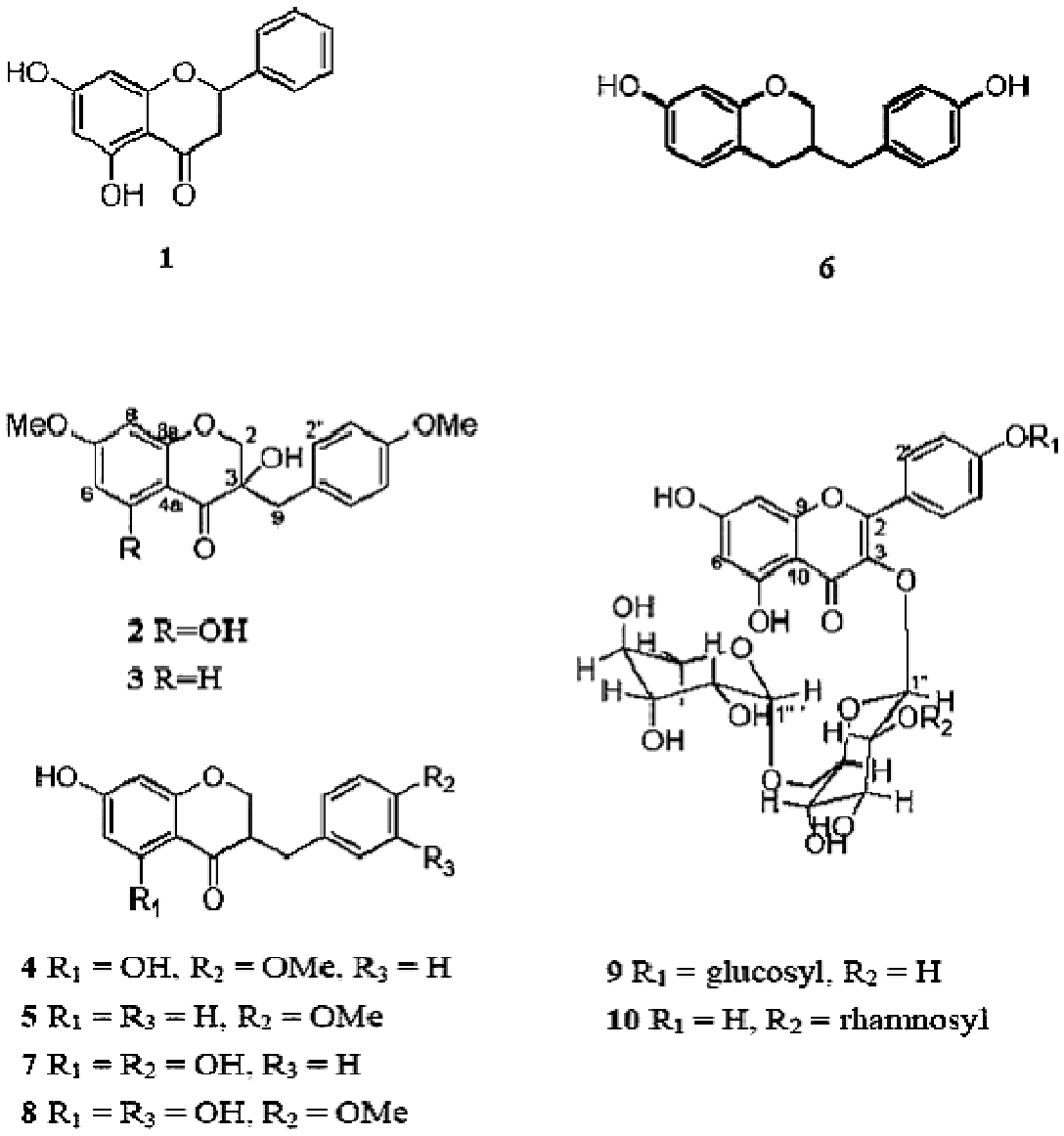

Figure 4. Chemical structures of homoisoflavones and flavones from *Agave sisalana* (Chen et al., 2009).

were also evaluated for immunopharmacological activity. (±)-3,9-Dihydroeucomin (4) showed significant immunosuppressive effects among all those compounds. The saturated groups of C-4′ methoxyl and C-5 hydroxy seem to be necessary groups for the activity.

AGAVE SISALANA: FIBRE YEILDING PROPERTIES

Sisal fiber, which is sometimes referred to as sisal hemp, is not related to true hemp and is fairly coarse and inflexible. It is valued for cordage use because of its strength, durability, ability to stretch, affinity for certain dyestuffs, and resistance to deterioration in saltwater. Sisal ropes and twines are widely employed for marine, agricultural, shipping, and general industrial use. The fibre is also made into matting, rugs, millinery, and brushes. Tanzania and Brazil are the largest producers. Traditionally used for rope and twine, sisal has many uses, including paper, cloth, wall coverings and carpets. The fibers run the entire length of the leaves, which can grow to five feet, and have been used for rope, twine, nets, upholstery padding, carpet pads, blankets, baskets, jewelry, sandals, clothing, fish stringers, musical instruments, ceremonial objects, construction material, paper pulp, and even dart boards (Nobel, 1994). A very strong fibre is obtained from the leaves is used for making rope, coarse fabrics etc (Hedrick, 1972; Balls, 1975; Usher, 1974). Although sisal is native to Mexico, commercial hard fiber from Mexico is produced primarily from henequen (*A. fourcroydes*) and lechuguilla *(A. lechuguilla).*

Pizarro et al. (1999) utilized the waste residues of sisal fiber separation from *A. sisalana* leaves to develop a larvicide for the combat of mosquito transmitting tropical diseases. They exposed larvae of *Aedes aegypti* and *Culex quinquefasciatus* to different concentrations of the *Agave* extract for 24 hours to determine lethal concentrations. The LC50 for *A. aegypti* was 322 ppm and the LC50 for *C. quinquefasciatus* was 183 ppm. To detect the active substances, saponins were investigated. It was found that the various components of the extract were effective in eliminating the larvae. Under field conditions, this formulation can probably be used at 100 ppm, which causes 100% mortality of *C. quinquefasciatus* larvae after 3-4 days. The product is not recommended for use against *A. aegypti* due to the necessity for high concentrations and to the fact that the larvae of this species live frequently on drinking water. To avoid fermentation, *Agave* extract should be used in a dehydrated form which also represents a good formulation for practical use.

The chemical composition of lipophilic extractives from *Agave sisalana* fibers, which are used for high-quality paper pulp production, was studied. The lipophilic extract, which accounted for 0.5% of total sisal fiber weight, was analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) using short- and medium-length high temperature capillary columns, respectively. For a more detailed characterization, the extract was fractionated by solid-phase extraction and the fractions obtained were analyzed by GC and GC/MS. The most predominant compounds identified were fatty acids (30% of total lipids) including α- and ω-hydroxyfatty acids, fatty alcohols (20%), free sterols (11%), alkanes (11%) and a series of ferulic acid esters of long chain alcohols and ω-hydroxy fatty acids (10%). Additionally, steroid hydrocarbons and ketones, monoglycerides, aldehydes, waxes, and sterol glycosides were also found together with minor amounts of diglycerides, and sterol esters (Gutierrez et al., 2008).

AGAVE SISALANA: IN VITRO PROPAGATION

Much of the tissue culture work in Agavaceae has been carried out on ornamental species (Madrigal et al., 1989). In *Agave*, past tissue culture work has been oriented to improve the multiplication rate of *A. atrovirens, A. fourcroydes* and *A. tequilana* (Madrigal et al., 1981). Successful regeneration and propagation was reported from seed (Groenewald et al., 1977), rhizome and stem explants of *A. foucroydes* (Robert et al., 1987) and *A. arizonica* (Powers and Backhaus, 1989).

Conventional propagation of this plant is mainly through bubils, produced in the inflorescence. Because flowers appear only once in the life span of the plant that is after 8-9 years after the time of sowing clonal production by bubils is slow. The lack of an adequate supply of planting stock is a problem common to *Agave* agroindustries (Madrigal et al., 1981). Medicinal use is the most diverse, followed by its use in construction material, and medicine. One of the most interesting uses of these species is as food; this may have been important in the domestication of the plant. Despite their economic importance, the *Agave* spp. has not been genetically improved. This is probably owing to the fact that they have very long life cycles and many of them have an inefficient sexual reproduction mechanism. The *in vitro* propagation method offers an alternative to this problem through the efficient cloning of selected high-yielding “elite” plants (Robert et al., 2005).

Organogenesis

In vitro propagation is better method for achieving large number of clonal plants for continuous plantation establishment. Successful regeneration and propagation was reported from seed (Groenewald et al., 1977), rhizome and stem explants of *A. fourcroydes* (Robert et al., 1987). Previous attempts to regenerate plants from *Agave sisalana* and an unidentified species of *Agave* (Hunault, 1979) were unsuccessful. Later on large-scale multiplication and propagation of *A. sisalana* was reported by direct shoot-bud formation (Das, 1992).

A procedure for rapid propagation of *Agave* (*A. cantala* Roxb, *A. fourcroydes* Lem. and *A. sisalana* Perrine, (Agavaceae) have been developed by Binh et al. (1960). The explants were excised from stolon plantlets, sterilized and cultivated on Murashige and Skoog (MS) basal medium containing 2% sucrose, 10% coconut water and 0.8% agar. The addition of following combination of growth substances like 0.075 mg/lit naphthalenacetic acid (NAA), 0.1 mg/lit indolyl butyric acid (IBA) and 0.5 mg/lit kinetin (KN) caused an extensive proliferation of multiple shoot primordia. Subcultures of these on the same medium were successful for the multiplication with an index of 3 - 4 times per 4 weeks subculture period. Shoots were rooted on hormone free MS medium and then transferred into a sand bed for acclimation before field planting.

In the author’s lab rhizomes and immature leaves were used as explants for *in vitro* propagation. They were harvested randomly washed thoroughly with tap water, Tween 20 and surface sterilized with 0.1% mercuric chloride for 1 min. Explants consisting of 4 - 6 mm thick segment from the rhizomes and the bulbils were used aseptically. Explants were placed in culture tubes with cotton plugs containing 15 ml freshly prepared MS (Murashige and Skoog, 1962) with 3% sucrose and solidified by about 0.8% agar supplemented with MS vitamins. Different hormones like Benzylamino purine (BAP) and kinetin (KN) were added to the nutrient medium for the morphogenesis to take place. The pH media of the media was maintained at 6.2 before auto-

claving at 120°C at 15 lbs for 15 min.

The explants showed marked morphogenesis (Figure 5) after a tenure of 40 days at different hormonal concentration. Cultures growing in MS supplemented with BAP at a concentration of 6mg/lit showed shoot elongation results ranging from 1.5 - 5 cm (Figure 5A). The shoots remain green, folded, rising straight. Also rooting was observed on the same media with length ranging from 0.5 - 2.5 cm. The roots are fine, white and tender.

The cultures in half MS media supplemented with 2 mg/lit BAP (Figure 5B, 5E) also showed remarkable growth in the length of shoot. Root induction was simultaneously followed by shooting in same cultures. Number of shoots by mean is 2. The length of shoot ranged from 1 - 5 cm with a mean value of 4 cm. In the cultures on MS media supplemented with 2 mg/lit BAP, necrosis was observed.

Cultures were also established on MS supplemented with 2 mg/lit 2, 4-D and 0.5 mg/lit Kinetin. Callogenesis as well as shoot elongation were observed (Figure 5C). This undifferentiated growth callus was brown in color, expanded and having weight of about 0.5 gm. Many cultures, under this composition, however suffered necrosis. Initiation of shoot bud were observed on MS media supplemented with 6mg/lit BAP after 40 days of culture (Figure 5D). Shoot bud elongation with rooting were observed on half MS supplemented with 2mg/lit BAP (Figure 5 F). The rooted plantlets were transferred (Figure 5G) after acclimatization and hardening to soil: vermicompost (1:1). All the plants survived and showed good growth (Figure 5H).

For shoot proliferation, growth regulators especially cytokinins (Lane, 1979; Stolz, 1979; Bhojwani, 1980; Garland and Stolz, 1981) was found to be one of the most important factors affecting the response. A range of cytokinins (Kinetin, BAP) has been used in micropropagation work (Bhojwani and Razdan, 1992). At higher levels, cytokinin tends to induce adventitious bud formation (McComb, 1978; Zimmerman and Broome, 1980). In the present study also cultures were multiplied using a range of BAP concentrations. BAP is the least expensive cytokinin source (Deora and Shekhawat, 1995) and has frequently been reported to induce better shoot multiplication than other cytokinins, particularly in many monocot species (Ahmed et al., 1990).

Callogenesis

Hazra et al. (2002) found initiation of callus from *In vitro* grown immature leaf and *ex vitro* grown mature leaf and rhizome explants of *Agave sisalana* Perr. ex. Engelm, on MS medium containing 2,4-D (9.05 M) and kinetin (4.6 M) or 2,4-D (9.05 M), kinetin (4.6 M) and CH (1000 mg l^{-1}) or mod. MS (NH_4NO_3, 1500 mg l^{-1}) containing 2,4-D (9.05 M) and kinetin (4.6 M). The author also reported similar callus induction from the base of the shoot after 30 days of culture on MS media containing 2 mg/lit of 2-4 D and 0.5mg/lit of Kinetin (Figure 5C). Light was essential for callus formation which, however, was different in three types of explants on three different media compositions. Increasing NH_4^+had a negative impact while addition of CH had a positive impact on callus formation.

Shoot regeneration from callus from CH-supplemented medium only was achieved for rhizome and immature leaf tissues. The highest rate of regeneration was obtained with BAP (26.6 M) as the sole hormone. Shoot proliferation rate increased on half-strength MS medium containing BAP (8.9 M). Microshoots developed on MS medium containing BAP (2.22 M) and GA_3 (1.44 M) and finally rooted on MS medium containing IAA (11.42 M). Acclimatized rooted plantlets showed satisfactorily growth under *ex vitro* conditions.

Callus cultures of *Agave sisalana* were also initiated from rhizome, and stem explants on MS, SH, Gamborg and White's medium supplemented with different concentrations of BA, kinetin, NAA, IAA and 2,4-D either in combination or singly. Optimum numbers of shoots were obtained from stem and rhizome explants either directly or from callus. The capacity for the shoot regeneration remained constant in the callus for more than 32 months. Regenerated shoots rooted readily within 21 - 35 days on fine sand with half the strength of inorganic salts of MS medium. 100% of the rooted plants were successfully adapted to field conditions and grown in the soil. Regenerated plants were morphologically similar to the field grown mother plants (Nikam, 1997). The author also reports 100 % successful transfer, acclimatization and adaptation of plantlets in the field (Figure 5G, 5H) .The regenerated plants showed morphologically similar and contained saponin.

Somatic embryogenesis

A protocol has been developed by Nikam et al. (2003) for somatic embryogenesis and plant regeneration of sisal (*Agave sisalana* Perr. ex. Engelm). Embryogenic callus cultures were initiated from young shoots raised *In vitro* from the stem portion of the bulbil on medium supplemented with 1 - 2 mg l^{-1} kinetin (KN) and 0.2 - 0.5 mg l^{-1} - naphthaleneacetic acid plus KN or 1 - 1.5 mg l^{-1} benzylaminopurine (BAP) or 0.25 - 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid plus BAP or 0.5 - 1.0 mg l^{-1} KN. Embryos at various developmental stages (globular-, heart- or torpedo-shaped) produced mature and germinating embryos on being transferred to a new medium containing 0 - 0.25 mg l^{-1} KN. After 28 days, a maximum of 76% germinated embryos was obtained on a medium supplemented with 0.1 mg l^{-1} KN. The capacity for embryogenesis remained constant in the callus upon subculturing on the same medium for more than 48 months. Histological observations showed a distinct multicellular origin for most of the somatic embryos as

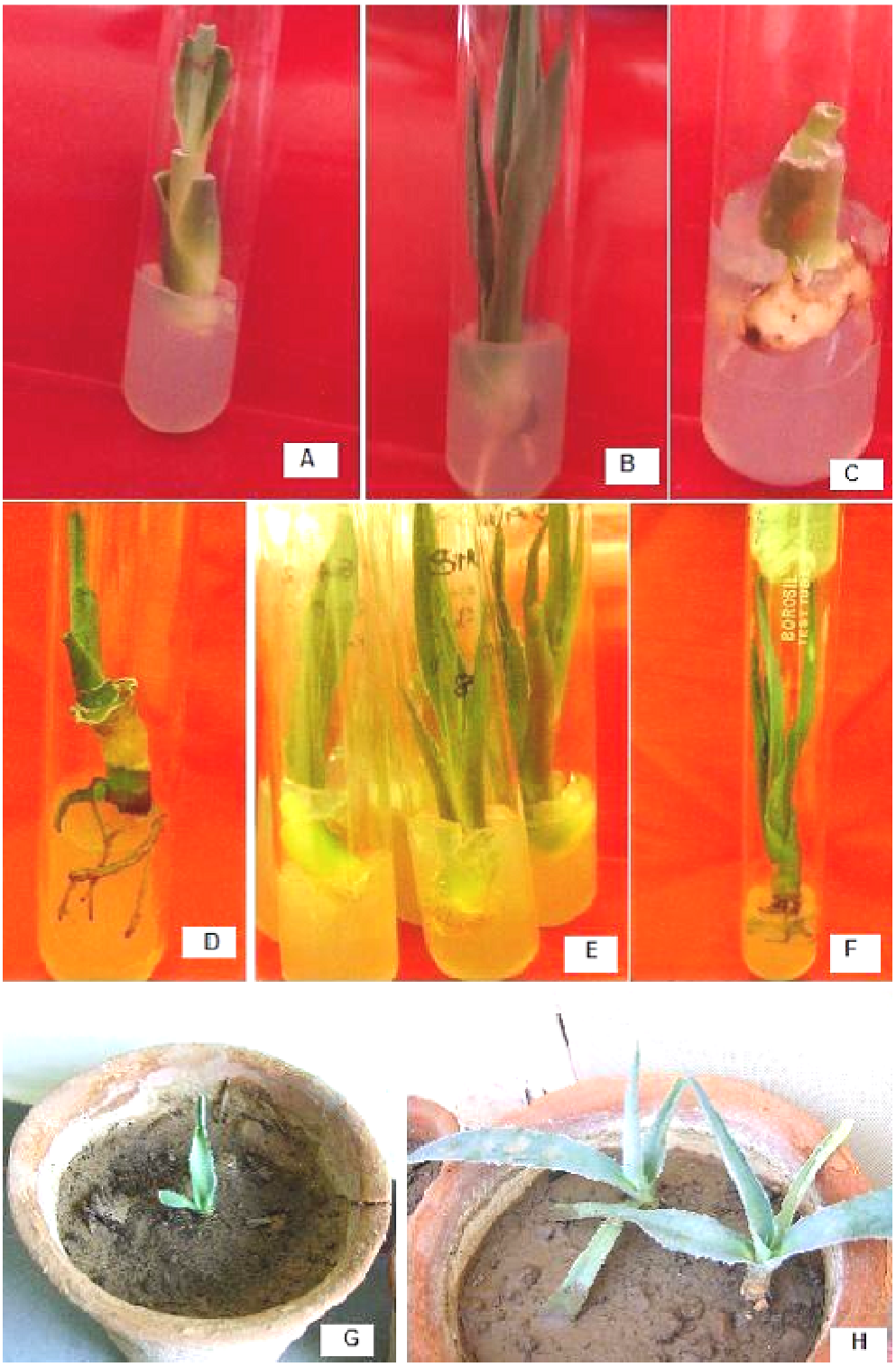

Figure 5. *In vitro* propagation of *AGAVE SISALANA* A. The explants show morphogenesis an shoot elongation on MS supplemented with BAP (6mg/lit); B. The cultures in half MS media supplemented with 2 mg/lit BAP show increase in shoot length and root induction; C: Cultures on MS supplemented with 2 mg/lit 2, 4-D and 0.5 mg/lit Kinetin showed callogenesis as well as shoot elongation. D. Initiation of shoot bud on MS media supplemented with 6mg/lit BAP after 40 days of culture E. Cultures showing different stages of shoot bud elongation and growth on different concentration of hormones F. Shoot bud elongation with rooting on half MS supplemented with 2mg/lit BAP G. Transfer of plantlets after acclimatization and hardening to soil: vermicompost (1:1). H. plants after 25 days of growth.

they developed from epidermal, sub-epidermal and inside callus cells, while a few of them originated from a superficial callus cell. Plantlets regenerated from embryos were transferred to the field where their survival rate was 100%.

Conclusion

A. sisalana is one of the major fibre yielding plants apart from jute. Development work to increases the yeild and quality has been taken up by many researchers. Another thrust area of research in this plant is to develop elite plants that can overcome and resist biotic and abiotic stresses .In future, economically feasible and sustainable production technology is under development for cropping systems for fiber crops. It is also envisaged to develop in future proper post-harvest technology for improvement of the quality of fibre and transfer of technology and human resource development in relation to fiber crops.

This plant has the potential for production of pharmaceutically important metabolites. Under the protection act of medicinal plants act, the germplasm is now be protected and conserved before it becomes endangered. In the absence of scientific system of collection and fostering regeneration of such plants, these species have been completely lost or have become endangered and / or on the verge of extinction with varying degrees. In future this serious genetic erosion can cause loss of biodiversity of resource areas. Researches on *Agave sisalana* will now concentrate on their biologically active compounds, evaluating their remedial properties, and also due attention will be paid to the sustainable management of these plants in the country in a sustainable manner.

REFERENCES

Adinolfi M, Lanzetta R, Laonigro G, Parrilli M, Breitmaier E (1986). 1H and 13C chemical shift assignments of homoisoflavanones. Magn. Reson. Chem. 24: 663-66.

Ahmed Z, Zaidi N, Shah F (1990). Micropropagation of *Melia azedarach* from mature tissue. Pak. J. Bot. 22: 172–178.

Alves R-R-N, Rosa I-L (2007). Biodiversity, traditional medicine and public health: J Ethnobiol. Ethnomed. 3(14): 1-9.

Balls E-K (1975) Early Uses of Californian Plants. University of California Press, ISBN 0-520-00072-2.

Bean W (1981). A classic with a wealth of information on the plants, Trees and Shrubs Hardy in Great Britain. pp. 1-4

da Silva BP, de Sousa AC, Graziela MS, Tatiana PM, Jose PP (2002). A New Bioactive Steroidal Saponin from *Agave attenuata*. Verlag der Zeitschrift für Naturforschung. *Tubingen* pp. 423-428.

Bhojwani S-S (1980). Micropropagation method for hybrid willow (*Salix matsudana x alba* NZ-10002). N.Z.J. Bot. 18:209-21.

Bhojwani S-S, Razdan M-K (1992). Plant tissue culture: Theory and practice, Elsevier, Amsterdam, London, New York, Tokyo.

Binh L-T, Muoi HTK, Oanh T-D, Phong D-T (1960). Rapid propagation of *Agave* by *In vitro tissue culture*. Plant Cell Tiss. Org. Cult. 23(167): 70.

Blunden G, Carabot C-A, Jewers K. (1980). Steroidal sapogenins from leaves of some species of *Agave* and *Furcraea*. Phytochem. 19(11): 2489-2490.

Blunden G, Patel A-V, Crabb T-A (1986) Barbourgenin, a new steroidal sapogenin from *AGAVE SISALANA* leaves. J Nat. Prod. 49: 687-689.

Botha J, Witkowski ETF, Shackleton C-M (2003). The impact of commercial harversting on warburgia salutaris ('pepper-bark tree') in Mpumalanga. South Afri. Bio. Con. 00: 1-24.

Bown D (1995). Encyclopaedia of Herbs and their Uses. Dorling Kindersley, London.

Brackenbury T-D, Appleton C-C (1997). A comprehensive evaluation of *Agave attenuata*, a candidate plant molluscicide in South Africa. Acta Trop. 68: 201-213.

Chand N, Tiwary RK, Rohatgi PK (1998). Bibliography resource structure properties of natural cellulosic fibres; An annotated bibliography. *J. Mater. Sci.* 23: 381–387.

Chen P-Y, Kuo YC, Chen C-H, Kuo YH, Lee CK (2009). Isolation and immunomodulatory effect of homoisoflavones and flavones from *AGAVE SISALANA* Perrine ex Engelm. *Mole.* 14:1789-1795.

Chen Y-C, Tsai W-J, Wu M-H, Lin L-C, Kuo Y-C (2007). Suberosin inhibits human peripheral blood mononuclear cells proliferation through the modulation of NF-AT and NF-B transcription factors. *Br. J. Pharmacol.*150: 298-312.

Chevallier A (1996). The Encyclopedia of Medicinal Plants. Dorling Kindersley. London ISBN 9-780751-303148.

Chittendon F (1992). Comprehensive listing of species and how to grow them. Oxford university press.

Chopra RN, Nayar SL, Chopra IC (1986). Glossary of Indian Medicinal Plants, Council of Scientific and Industrial Research, New Delhi.

Cripps AL, Blunden G (1974). A quantitative gas-liquid chromatographic method for the estimation of hecogenin and tigogenin in the leaves, juice and sapogenin concentrates of *AGAVE SISALANA*. *Steroids* 35(5): 661-66.

Das T (1992). Micropropagation of *AGAVE SISALANA*. *Plant cell Tiss. Org. cult.* 31: 253-255.

Dawidar A-A, Fayez M-B-E (1961). Steroid sapogenins, Distribution of steroid sapogenins in the sisal plant. Arch. Biochem. Biophy. 92(3): 420-423.

Deora N-S, Shekhawat N-S (1995). Micropropagation of *Capparis decidua* (Forsk.) Edgew. - A tree of arid horticulture. Plant Cell Rep. 15: 278-281.

Ding Y, Chen Y-Y, Wang D-Z, Yang CR (1989). Steroidal saponins from a cultivated form of *AGAVE SISALANA*. Phytochem. 28: 2787-2791.

Ding Y, Tian RH, Yang C-R, Chen YY, Nohara T (1993). Two new steroidal saponins from dried fermented residues of leaf-juices of *AGAVE SISALANA*. Chem Pharm Bull. (3): 557-60.

Duke JA, Ayensu ES (1985). Medicinal Plants of China, Reference Publications.

Eldridge J (1975). Bush medicine in the Exumas and Long Island, Bahamas; a field study. Econ. Bot. 29: 307-332.

El-Hilaly J, Hmammouchi M, Lyoussi B (2003). Ethno botanical studies and economic evaluation of medicinal plants in Taounate province (Northern Morocco). J. Ethnopharmacol. 86(2-3): 149-158

Etkin N-L (1998). Indigenous patterns of conserving biodiversity, Pharmacologic implications. J. Ethnopharmacol. 63: 233-245.

Facciola S (1990). Cornucopi; A Source Book of Edible Plants. Kampong Publications.

Fazli F-R (1968). Contraceptives and other steroid drugs: their production from steroidal sapogenins. Pak J. Sci. 20 (1 and 2): 64-7.

Garland P, Stoltz LP (1981). Micropropagation of Pissrdi plum, Ann. Bot. 48:n387-389.

Geibel M, Gross DC, Mo YY, Bonsall RF, Geiger H (1994). Identification of flavonol glycosides from *Prunus avium* leaves which induce the production of syringomycin by *Pseudomonas syringae*. Acta Hortic. 381: 662-666.

Groenewald EG, Wessels DCJ, Koeleman A (1977). Callus formation and subsequent plant regeneration from seed tissue of an *Agave* species (Agavaceae). J. Pflanzenphysiol. 81: 369–373

Gutierrez A, Rodriguez I-M, Jose C-R (2008). Chemical composition of lipophilic extractives from sisal. *Indus. Crops Prod.* 28(1):81-87.

Hamilton A (2004). Medicinal plants, conservation and livelihoods. Biod. Conserv 13:1477-1517.

Hazra S-K, Das S, Das A-K. (2002). Sisal plant regeneration via organogenesis. Plant Cell Tiss. Org. Cult. 70-3: 235-240(6).

Hedrick UP (1972). Sturtevant's Edible Plants of the World. Dover

Publications 0-486-20459-6.
Heller W, Andermatt P, Schaad WA, Tamm C (1976). Homoiso-flavanone. IV. neue inhaltsstoffe der eucomin-reihe von *Eucomis bicolor*. *Helv. Chim. Acta 59*: 2049-2056.
Higgins W-J. (1976). A high-performance liquid chromatographic analysis of the benzoate esters of sapogenins isolated from *Agave*s. J. Chroma. 121(2): 3290-2334.
Hunault G (1979). Recherches sur les comportements des fragments d'organs et des tissus de monocotyledones cultives *In vitro* II. Etude du cas de quelques Agavaceae. Rev. Cytol. Biol. Veget. Bot. 2: 21–66
Huxley A (1992). The New RHS Dictionary of Gardening. MacMillan Press.
Kassu A, Dagne E, Abate D, Castro A, Van Wyk B-E (1999). Ethanomedicinal aspects of the commonly used toothbrush sticks in Ethiopia. East Afr. Med. J. 76(11): 651-653.
Kayumba A-V, Van-Do T, Florvaag E, Bratveits M, Baste V, Mashalla Y, Eduard W, Moen B-E (2008). High prevalence of immunoglobulin E (IgE) sensitization among sisal (*AGAVE SISALANA*) processing workers in Tanzania. Ann. Agric. Environ. Med. 15(2): 263-270.
Kazuma K, Noda N, Suzuki M (2003). Malonylated flavonol glycosides from the petals of *Clitoria ternatea*. *Phytochem.62*: 229-237.
Kuo Y-C, Yang L-M, Lin L-C (2004). Isolation and immunomodulatory effect of flavonoids from *Syzygium samarangense*. Planta Med. *70*: 1237-1239.
Lane W-D (1979). *In vitro* propagation of *Spirea bumalda* and *Prunus cistena* from shoot apics. Can. J. Plant Sci. 59: 1025-1029.
Likhitwitayawuid K, Sawasdee K, Kirtikara K (2002). Flavonoids and stibenoids with COX-1 and COX-2 inhibitory activity from *Dracaena loureiri*. Planta Med. 68: 841-843.
Lorenzi H, Souza H-M (1995). Plantas ornamentais no Brasil arbustivas, herbaceas e trepadeiras. Editora Plantarum, Sao Paulo.
Lust J (1983). The Herb Book. Bantam books ISBN 0-553-23827-2.
Madrigal L-R, Dorantes G-M-R, Rodriguez de la O-J-L (1981). Propagacion *In vitro* de henequen (*Agave fourcroydes* Lemaire). In: Primer Simposio dal *Agave* (p 11). Cordamex SA de CV Merida, Yuc. Mexico
Madrigal L-R, Pineda-Estrada F, Rodriguez de la O-J-L (1989). *Agave* In: Ammirato, PV, Evans, DA, Sharp WR, Bajaj YPS (Eds) Handbook of Plant Cell Culture. *McGraw Hill Publ*. Co 5: 206-227
McComb J-A (1978) .Clonal propagation of woody plant species with special reference to apples, Proc. Int. Plant Prop. Soc. 28:413-426.
Meksuriyen D, Cordell G-A (1987). Traditional medicinal plants of Thailand, IX. 10-hydroxy-11-methoxydracaenone and 7,10-dihydroxy-11-methoxydracaenone from *Dracaena Loureiri*. J. Nat. Prod. 6: 1118-1125.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473–497
Mutanyatta J, Matapa B-G, Shushu DD, Abegaz B-M (2003). Homoisoflavonoids and xanthones from the tubers of wild and *In vitro* regenerated *Ledebouria graminifolia* and cytotoxic activities of some of the homoisoflavonoids. Phytochem. *62*: 797-804.
Namikoshi M, Saitoh T (1987). Homoisoflavonoids and related compounds. IV. Absolute configurations of homoisoflavonoids from *Caesalpinia sappan* L. Chem. Pharm. Bull. *35*: 3597-3602.
Nikam T-D (1997). High frequency shoots regeneration in *AGAVE SISALANA*. Plant Cell Tiss. Org. Cul. 51(3): 225-228.
Nikam T-D, Bansude G-M, Kumar A (2003). Somatic embryogenesis in sisal. Plant Cell Rep. 22(3):188-194.
Nobel PS (1988). Environmental biology of *Agave*s and cacti. Cambridge University Press.
Nobel PS (1994). Remarkable *Agave*s and cacti. Oxford University Press.
Nobel P-S, Berry W-L (1985). Elements responses of *Agave*s. Amer. J. Bot. 72(5): 686-694.
Phillips R, Rix M (1998). Conservatory and Indoor Plants, Pan Books, London.Vol. 1 and 2.
Pizarro A-P, Oliveira Filho A-M, Parente J-P, Melo M-T, dos Santos CE, Lima P-R(1999). Utilization of the waste of sisal industry in the control of mosquito larvae. Rev. Soc. Bras. Med. Trop. 32(1): 23-9.
Powers D-E, Backhaus R-A (1989). *In vitro* propagation of *Agave arizonica* Gentry & Weber. Plant Cell Tiss. Org. Cult. 16: 57–60
Rice G (1987). Growing from Seed Thompson and Morgan. Vol. 1.
Robert ML, Herrera JL, Contreras F, Scorer K-N (1987). *In vitro* propagation of *Agave fourcroydes* Lem. (Henequen). Plant Cell Tiss. Org. Cult. 8: 37–48.
Robert M-L, Herrera-Herrera J-L, Castillo E, Ojeda G, Herrera-Alamillo, M-A (2005). An Efficient Method for the Micropropagation of *Agave* Species. Plant Cell Cult. Protocol. 318:165-178.
Sharaf A, Zahran M (1967). Pharmacological investigation on *AGAVE SISALANA* with special study of its ecbolic effect. Plant Foods Hum. Nutr. 14(4): 345-351.
Sijelmassi A (1993). Les plantes médicinales du Maroc. Edition Le Fennec, Casablanca.
Sirat HM, Rahman AA, Itokawa, H, Morita H (1996). Constituents of the rhizomes of two Alpinia species of Malaysia. Planta Med. 62: 188-189.
Spensley JC, Wathes CM, Waran NK, Lines J-A (1995) Behavioral and physiological responses of piglets to naturally occurring sounds. Appl. Anim. Bahav. Sci. 44: 277.
Stangeland T, Tabuti JRS, Lye KA (2007). The influence of light and temperature on the germination of two Ugandan medicinal trees. Afr. J. Ecol. 46(4): 1-7.
Stoltz LP (1979) *In vitro* propagation of Acalypha wilkesiana. Hort Sci. 14:702-703.
Stoltz L-P (1979). *In vitro* propagation of *Acalypha wilkesiana, Hort. Sci.* 14:702-703.
Thomas G-S (1990). Plants for Ground Cover J. M. Dent & Sons.
Usher G (1974). A Dictionary of Plants Used by Man. Constable ISBN 0094579202.
Wall JM, Krider MM, Krewson CF, Eddy CR, Willaman JJ, Corell DS, Gentry H-S (1954). Steroidal sapogenins VII. Survey of plants for steroidal sapogenins and other constituents. J. Am. Pharm Ass. 63: 1-7.
Zhao P, Iwamoto Y, Kouno I, Egami Y, Yamamoto H (2004). Stimulating the production of homoisoflavonoids in cell suspension cultures of *Caesalpainia pulcherrima* using cork tissue. Phytochem. 65: 2455-2461.
Zimmerman RH, Broome OC (1981) Phloroglucinol and *In vitro* rooting of apple cultivar cuttings. J. Am. Soc. Hortic. Sci. 106: 648-652.
Zimmerman RH, Broome O-C (1981). Phloroglucinol and *In vitro* rooting of apple cultivar cuttings. J Am. Soc. Hortic. Sci.106: 648-652.
Zschocke S, Rabe T, Taylor JLS, Taylor AK, Van Staden J (2000). Plant part substitution- a way to conserve endanged medicinal plants? J. Ethnopharmacol. 71: 281-292.